VNIVERSITAT DE VALÈNCIA

FACULTAD DE FARMACIA

DEPARTAMENTO DE FISIOLOGÍA

Programa de Doctorado en Fisiología

ESTRÉS POR DISULFURO EN PANCREATITIS AGUDA EXPERIMENTAL. IMPLICACIONES FISIOPATOLÓGICAS

TESIS DOCTORAL presentada por

Mª LUZ MORENO SANCHO

Dirigida por:

Dr. Juan Sastre Belloch

Dr. Javier Escobar Cubiella

Valencia, 2014

D. Juan Sastre Belloch, Doctor en Farmacia y Catedrático del Departamento de Fisiología de la Universidad de Valencia.

D. Javier Escobar Cubiella, Doctor en Biología e Investigador del Instituto de Investigación Sanitaria La Fe.

CERTIFICAN:

Que Doña Mª Luz Moreno Sancho, Licenciada en Farmacia por la Universidad de Valencia, ha realizado bajo su dirección la presente tesis titulada:

ESTRÉS POR DISULFURO EN PANCREATITIS AGUDA EXPERIMENTAL. IMPLICACIONES FISIOPATOLÓGICAS

Para la obtención del título de Doctora

Y para que conste a los efectos oportunos, firman la presente certificación.

Valencia, 2014

Fdo. D. Juan Sastre Belloch

Fdo. D. Javier Escobar Cubiella

La consecución de la presente Tesis Doctoral ha sido posible gracias a la financiación obtenida de los siguientes proyectos y beca:

- **SAF2009-09500.** "Identificación de dianas de estrés oxidativo y nitrosativo claves en la pancreatitis aguda en obesidad experimental". Ministerio de Economía y Competitividad.

- **CONSOLIDER-Ingenio 2010: CSD2007-00020.** "Papel funcional del estrés oxidativo y nitrosativo en grandes sistemas biológicos". Ministerio de Educación y Ciencia.

- **SAF2012-39694.** "Papel de la señalización redox en la respuesta inflamatoria local y sistémica de la pancreatitis aguda en animales obesos". Ministerio de Economía y Competitividad.

- Beca de **Formación de Personal Investigador (BES-2010-034718).** Ministerio de Economía y Competitividad 2010-2014

A MIS PADRES,

MI HERMANA

Y VÍCTOR

AGRADECIMIENTOS

Primero de todo quiero dedicar esta Tesis a mis padres, porque os lo debo todo. Me habéis dado todo lo que soy hoy por hoy, no sólo a nivel profesional sino también a nivel personal. Gracias por todo lo que habéis hecho por mí. Por enseñarme entre muchos otros valores, el trabajo, la constancia y el esfuerzo diarios, sin ellos, no hubiera llegado hasta aquí. Gracias por apoyarme durante toda la Tesis. También agradecer a mi hermana, Eva, por escucharme siempre y por darme tan buenos consejos. Para mí, además de una hermana eres mi gran amiga.

También quiero dedicar esta Tesis a Víctor, mi gran compañero de viaje. Gracias por tu cariño y paciencia y por tu apoyo incondicional durante todo este período, gracias a los cuales las cosas han sido mucho más fáciles. Sin ti, nada hubiera sido lo mismo.

Gracias a mis directores de Tesis Juan y Javi por toda su ayuda y consejos durante estos años. Gracias Juan por haberme dado la oportunidad de hacer la Tesis. Gracias Javi por todo lo que me has enseñado.

La colaboración de los grupos de investigación dirigidos por Antonio Martínez-Ruíz y Joaquim Ros también han sido claves por su aportación y ayuda en la realización de algunos experimentos.

Agradecer al personal del SCSIE: Mª Jesús y Ana (cultivos celulares), Oreto y Luz (protéomica) e Inma (animalario) porque vuestro trabajo también ha contribuido a la realización de esta Tesis.

También me gustaría dar las gracias a todos mis compañeros del departamento de Fisiología de la Facultad de Farmacia: José E., Ángel, Julián, María R., Inés, Toni, Juanlu, Mariam, Dan, Javier, Tere, Xelo, José C., María C., Joan, Germán, Chabeli, Pilar, Salva B., y a los del grupo de Juan: Anabel, Alessandro, Ana, Raquel y Salva.

And last but not least, I want to thank everybody from Phil's laboratory. It was a pleasure for me to have the opportunity to work with all of you. You have an incredible group: Sasha, Olena, Jenna, Becky, Kathryn, Ewald, Alisa, Joe, Yemi and Daniel. Also, I would like to thank my little Spanish team in London, Mariana and Abel.

A TODOS, MUCHAS GRACIAS

AA:	Arachidonic acid/Ácido araquidónico
ADH:	Alcohol dehydrogenase/Alcohol deshidrogenasa
AP:	Acute pancreatitis/Pancreatitis aguda
AP:	Activator protein/Proteína activadora
APE:	Apurinic/apyrimidinic endonuclease/Endonucleasa apurínica/apirimidínica
APS:	Amonium persulfate/Persulfato amónico
ARE:	Antioxidant response element/Elemento de respuesta antioxidante
ASK:	Apoptosis-inducing kinase/Kinasa inducida por apoptosis
ATP:	Adenosine triphosphate/Adenosina trifosfato
BCA:	Bicinchoninic acid/Ácido bicinconínico
BSA:	Fetal serum albumin/Albúmina de suero fetal
CARS:	Compensatory anti-inflammatory syndrome/Síndrome antiinflamatorio compensatorio
CAT:	Catalase/Catalasa
CBS:	Cystathionine β-synthase/Cistationina β-sintasa
CCK:	Cholecystokinin/Colecistoquinina
3Cl-tyr:	3-Chlorotyrosine/3-Clorotirosina
CoQ:	Coenzyme Q/Coenzima Q
COX:	Cyclooxygenase/Ciclooxigenasa
Ct:	Threshold cycle/Ciclo umbral
Cu^{+}:	Cuprous ion/Ion cuproso
Cu/ZnSOD:	Cooper and zinc superoxide dismutase/Cobre y zinc superóxido dismutasa
Cul3:	Cullin-3 E3-ubiquitin ligase/Cullin-3 E3-ubiquitín ligasa
Cys:	Cysteine/Cisteína
DEPC:	Diethylpyrocarbonate/Dietilpirocarbonato

DNA:	Deoxyribonucleic acid/Ácido desoxirribonucleico
DNP:	2,4-Dinitrophenyl/2,4-Dinitrofenilo
DSPs:	Dual specificity phosphatases/Fosfatasas de especificidad dual
DTT:	Dithiothreitol/Ditiotreitol
EDTA:	Ethylenediaminetetraacetic acid/Ácido etilendiaminotetraacético
EGTA:	Ethyleneglycol tetraacetic acid/Etilenglicol del ácido tetraacético
ERK:	Extracellular signal-regulated kinases/Quinasas reguladas por señales extracelulares
$FADH_2$:	Flavin adenine dinucleotide/Flavín adenín dinucleótido
Fe^{2+}:	Ferrous ion/Ion ferroso
Fe^{3+}:	Ferric ion/Ion férrico
GCL:	Glutamate cysteine ligase/Glutamato cisteína ligasa
GDP:	Guanosine diphosphate/Guanosín difosfato
GFP:	Green fluorescent protein/Proteína verde fluorescente
GPx:	Glutathione peroxidase/Glutatión peroxidasa
GR:	Glutathione reductase/Glutatión reductasa
Grx:	Glutaredoxin/Glutarredoxina
GSH:	Reduced glutathione/Glutatión reducido
GSNO:	S-nitrosoglutathione/S-nitrosoglutatión
GSOH:	Glutathione sulphenic acid/Ácido sulfénico del glutatión
GSSG:	Oxidized glutathione
GTP:	Guanosine triphosphate/Guanosín trifosfato
γ-C:	γ-cystathionase/ γ-cistationasa
γ-GT:	γ-glutamyltranspeptidase/ γ-glutamiltranspeptidasa
H^+:	Proton/Protón

HEPES:	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid/Ácido 4-(2-hidroxi-etil)-1-piperazin-etan-sulfónico
HETE:	Hydroxyeicosatetraenoic acid /Ácido hidroxieicosatetraenoico
HIF:	Hypoxia inducible factor/Factor inducible por hipoxia
HNE:	Hydroxynonenal/Hidroxinonenal
HNO_2:	Nitrous acid/Ácido nitroso
HO^-:	Hydroxyl anion/Anión hidroxilo
HO•:	Hydroxyl radical/Radical hidroxilo
H_2O_2:	Hydrogen peroxide/Peróxido de hidrógeno
HOCl:	Hypochlorous acid/Ácido hipocloroso
HOBr:	Hypobromous acid/Ácido hipobromoso
HPDP:	N-[6-(biotinamide)hexyl]-3'-(2'-pyridyldithio)propionamide/N-[6-(biotinamida)hexil]-3'-(-2'-piridilditio)propionamida
HPLC-MS/MS:	High-performance liquid cromatography coupled with mass spectrometry/Cromatografía líquida de alta resolución acoplada a espectrometría de masas
HRP:	Horseradish peroxidase/Peroxidasa de rábano
IAA:	Iodoacetic acid/Ácido iodoacético
IAM-B:	Biotinylated iodoacetamide/Iodoacetamida biotinilada
IGF:	Insulin-like growth factor/Factor de crecimiento de tipo insulínico
IκB:	Inhibitor of κB/Inhibidor de κB
IL-1:	Interleukin/Interleuquina
JAK:	Janus kinase/Quinasa janus
JNK:	c-Jun N-terminal kinases/Quinasa N-terminal de Jun
Keap:	Kelch-like ECH-associated protein/Proteína asociada a ECH semejante a Kelch
LP:	Lipoxigenase/Lipooxigenasa

L-phe:	L-phenylalanine/L-fenilalanina
LT:	Leukotriene/Leucotrienos
L-tyr:	L-para-tyrosine/L-para-tirosina
MAPK:	Mitogen-activated protein kinase/Proteín quinasa activada por mitógenos
MAPKK:	MAPK kinase/ MAPK quinasa
MAPKKK:	MAPKK kinase/MAPKK quinasa
MBrB:	Monobromobimane/Monobromobimano
MDA:	Malondialdehyde/Malondialdehído
MeO:	Methionine sulfoxide/Sulfóxido de metionina
MKP:	MAPK phosphatase/MAPK fosfatasa
MLK:	Mixed-lineage kinase/Quinasa de linaje mixto
MMTS:	Methylmethanesulphonate/Metilmetanotiosulfonato
MnSOD:	Manganese superoxide dismutase/Manganeso superóxido dismutasa
MODS:	Multiple organ dysfunction syndrome/Síndrome de disfunción multiorgánica
MPO:	Mielopreroxidase/Mieloperoxidasa
mRNA:	Messenger RNA/RNA mensajero
mTOR:	Mammalian target of rapamycin/Diana de rapamicina en mamíferos
m-tyr:	L-meta-tyrosine/L-meta-tirosina
m/z:	Mass/charge/masa/carga
NAC:	N-acetylcysteine/N-acetilcisteína
NADH:	Nicotinamide adenine dinucleotide/Nicotín adenina dinucleótido
NADPH:	Nicotinamide adenine dinucleotide phosphate/Fosfato de nicotín adenina dinucleótido

NEM:	N-ethylmaleimide/N-etilmaleimida
NF-ĸB:	Nuclear factor kappa B/Factor nuclear kappa B
3nitro-tyr:	3-Nitrotyrosine/3-Nitrotirosina
NO:	Nitric oxide/Óxido nítrico
NO•:	Nitric oxide radical/Radical del óxido nítrico
NO_2•:	Nitrogen dioxide radical/Radical del dióxido de nitrógeno
NP:	Normal phase/Fase normal
Nrf2:	NF-E2-related factor 2/Factor relacionado con NF-E2 2
O_2:	Oxygen/Óxigeno
$O_2^{\bullet-}$:	Superoxide anion/Anión superóxido
O_3:	Ozone/Ozono
ONOO•:	Peroxynitrite radical/Radical peroxinitrito
ONOOH:	Peroxynitrite/Peroxinitrito
o-tyr:	L-ortho-tyrosine/L-orto-tirosina
p38:	Stress activated protein kinase (SAPK)/Proteín quinasa activada por strés
PAGE:	Electroforesis en gel de poliacrilamida/Electroforesis en gel de poliacrilamida
PBS:	Phosphate Buffer Solution/Solución de tampón fosfato
PCA:	Perchloric acid/Ácido perclórico
PCR:	Polymerase chain reaction/Reacción en cadena de la polimerasa
PDI:	Protein disulfide isomerase/Proteína disulfuro isomerasa
PG:	Prostaglandin/Prostaglandina
PheH:	Phenylalanine hydroxylase/Fenilalanina hidroxilasa
PI3K:	Phosphatidylinositide 3-kinase/Fosfoinositol 3-quinasa
PP1:	Protein phosphatase 1/Proteín fosfatasa 1
PP2:	Protein phosphatase 2/Proteín fosfatasa 2

PP2A:	Protein phosphatase 2A/Proteín fosfatasa 2A
PP2Ac:	Protein phosphatase 2A catalytic subunit/Subunidad catalítica de la proteín fosfatasa 2A
PP2B:	Protein phosphatase 2B/Proteín fosfatasa 2B
PP2C:	Protein phosphatase 2C/Proteín fosfatasa 2C
PPP:	Serine/threonine phosphatase/Serín/treonín fosfatasa
Prdx:	Peroxiredoxin/Peroxirredoxina
PSOH:	Sulphenic acid/Ácido sulfénico
PSO_2H:	Sulphinic acid/Ácido sulfínico
PSO_3H:	Sulphonic acid/Ácido sulfónico
PTP:	Protein tyrosine phosphatase/Proteín tirosín fosfatasa
PUFA:	Polyunsaturated fatty acid/Ácido graso poliinsaturado
Raf:	Rapidly accelerated fibrosarcoma/Fibrosarcoma rápidamente acelerado
Ref:	Redox effector factor/Factor efector redox
RNA:	Ribonucleic acid/Ácido ribonucleico
RNS:	Reactive nitrogen species/Especies reactivas del nitrógeno
ROS:	Reactive oxygen species/Especies reactivas del oxígeno
RP:	Reverse phase/Fase reversa
RPTP:	Receptor-like PTP/Receptor semejante a PTP
R-SNO:	S-nitroso derivative/Derivado S-nitroso
RT-PCR:	Real time PCR/PCR a tiempo real
SAH:	S-adenosylhomocysteine/S-adenosilhomocisteína
SAM:	S-adenosylmethionine/S-adenosilmetionina
SDS:	Sodium dodecylsulfate/Dodecilsulfato sódico
Ser:	Serine/Serina
SHP:	Protein tyrosine phosphatase with a homologous domain to Src/Proteín tirosín fosfatasa con un dominio homólogo al Src

SIRS:	Systemic inflammatory response syndrome/Síndrome de respuesta inflamatoria sistémica
SOD:	Superoxide dismutase/Superóxido dismutasa
STAT:	Signal transducer and activator of transcription/Señal de transducción y activadora de la transcripción
TAK:	TGFβ-activated kinase/Quinasa activada por TGFβ
TBS-T:	Tris buffer solution-Tween/Solución de tampón Tris-Tween
TCA:	Trichloroacetic acid/Ácido tricloroacético
TEMED:	Ethane 1,2-Bis(dimethylamino)/Etano 1,2-Bis(dimetilamino)
TFA:	Trifluoroacetic acid/Ácido trifluoroacético
Thr:	Threonine/Treonina
TNF-α:	Tumor necrosis factor alpha/Factor de necrosis tumoral alfa
Tris:	Tris(hydroxymethyl)aminomethane/ Tris(hidroximetil)aminometano
tRNA:	Transfer RNA/RNA de transferencia
Trx:	Thioredoxin/Tiorredoxina
TrxR:	Thioredoxin reductase/Tiorredoxina reductasa
TX:	Thromboxane/Tromboxano
Tyr:	Tyrosine/Tirosina

I. ABSTRACT/RESUMEN

• ABSTRACT

Oxidative stress is defined as the disturbance in the balance between oxidants and antioxidants species in favor of the formers. While there are many types of oxidative damage, increasing evidence suggests an important role for protein oxidation in multiple diseases.

Disulfides are formed in the cytosol during oxidative stress functioning as redox switches. The tri-peptide glutathione is a major thiol antioxidant that plays a key role in numerous cell functions. So far the glutathione redox status has been considered a reliable indicator of oxidative stress in cells because it reflects the balance between antioxidant status and pro-oxidant reactions.

Nowadays, acute pancreatitis is a relatively common disease. However, the main questions that raise both doctors and researchers remain unanswered. How we could diagnose acute pancreatitis earlier? Which treatment we should give to a patient to attenuate the inflammatory process? What is the period of time that we can permit between the data of the patient is taken and the diagnosis of acute pancreatitis? In order to answer these questions, it is necessary to find out the molecular mechanisms that occur during the development of the pancreatic inflammatory response. For these reasons, one of the aims of this Thesis was to analyze the redox status of thiols and to identify mixed disulfides and targets of redox signaling in pancreas in experimental acute pancreatitis as a model of acute inflammation associated with glutathione depletion.

There are different experimental models to induce and study acute pancreatitis in animals, all characterized and well established to induce mild and severe acute pancreatitis. For the development of this Thesis, it has been used a model of severe acute necrotic pancreatitis induced by retrograde infusion of sodium taurocholate in rats. This model has a high reproducibility and has been handled by our research group for several years. In addition, it was used *in vitro* cell line model of pancreatic acinar cells (266-6) that come from mice to investigate and further demonstrate the mechanisms involved. Therefore, for the study of acute pancreatitis it was used both *in vitro* and *in vivo* models.

It is known that there is an association between acute inflammation and depletion of reduced glutathione (GSH). In addition, GSH depletion in pancreas is a hallmark of the early course of acute pancreatitis and contributes to the severity of the disease. One of the aims of this Thesis was to characterize the consequences of GSH depletion in acute pancreatitis at the molecular level. Glutathione depletion in pancreas in acute pancreatitis is not associated with any increase in oxidized glutathione levels or protein glutathionylation. Cystine and homocystine levels as well as protein cysteinylation and γ-glutamyl cysteinylation markedly rose in pancreas after induction of pancreatitis. Protein cysteinylation was undetectable in pancreas under basal conditions. The quantification of these low molecular weight thiols, its reduced and oxidized forms, as well as the mixed disulfide bridges (bound to proteins) was carried out after processing of the corresponding fresh samples from rat pancreatic tissue and subsequent analysis by mass spectrometry coupled to high resolution liquid chromatography (HPLC-MS/MS).

To assess the formation of disulfide bridges during the course of acute pancreatitis, it was performed the “redox monobromobimane switch”. The increase in fluorescence after pancreatitis onset showed that reversible thiol oxidation is formed in the course of the disease.

After confirming disulfide formation during acute pancreatitis, it was investigated whether disulfides were formed with low molecular free thiols, such as GSH, cysteine, etc., or between proteins. The majority of protein disulfides seem to be ascribed to low molecular weight thiols since the diagonal electrophoresis performed after the redox switch revealed a marked increase in chemiluminescence along the diagonal at 6h after the induction of acute pancreatitis.

When the formation of disulfide bridges in acute pancreatitis was studied, it was evaluated another oxidative modifications of proteins, S-nitrosylation and carbonylation. S-nitrosylation was assessed by “biotin switch” and carbonylation by bidimensional electrophoresis using an antibody against carbonylated residues. There were no significant changes in protein S-nitrosylation and carbonylation during the course of acute pancreatitis. We show in the present Thesis that acute pancreatitis is an inflammatory pathology associated with oxidative stress without carbonylation and S-nitrosylation protein oxidation but with a remarkable formation of disulfide bridges. Thiol-disulfide exchanges in proteins may cause profound effects on protein activity and cell signaling. In this Thesis it is described disulfide stress as a type of oxidative stress in acute inflammation in mammals associated with oxidation of the pair cysteine/cystine and protein cysteinylation, but without glutathione oxidation or changes in protein glutathionylation.

The next step in this Thesis was to identify targets of disulfide stress that were oxidized in the course of acute pancreatitis. Targets of disulfide stress were identified by different techniques: Western blotting under reducing and non-reducing conditions, diagonal electrophoresis, proteomic methods and phosphatase activity in the presence and absence of N-acetylcysteine. All the potential targets of disulfide stress that were studied in the present Thesis were chosen by their importance and particular relevance because of their implication in redox stability, redox signaling and the inflammatory cascade that occurs in acute pancreatitis. The study of protein phosphatases in this Thesis enabled a further elucidation of the mechanisms involved in the inflammatory process during acute pancreatitis and allowed the identification of protein phospathases as targets of disulfide stress.

All the identified targets of disulfide stress could be classified into two groups: Redox buffers and redox signaling thiols. Redox buffers would include ribonuclease inhibitor and albumin. Redox-signaling thiols are thioredoxin 1, APE1/REF1, KEAP1, tyrosine phosphatases and serine/threonine phosphatases, and protein disulfide isomerase. These targets of disulfide stress exhibit great relevance in biological processes involving enzyme activity, DNA repair, cell proliferation, apoptosis, endoplasmic reticulum stress, and the inflammatory response. Therefore, disulfide stress would be a specific type of oxidative stress involved in redox signaling in mammals.

Because of its relationship with oxidative stress, redox homeostasis, and its action on MAPKs, PP2A could play a key role in early phases of acute pancreatitis, particularly in the severe form of the disease, participating critically in the inflammatory cascade control. Based on this hypothesis as well as on the oxidation observed in the catalytic subunit of PP2A and its loss of

activity in acute pancreatitis, we decided to silence PP2Ac in pancreatic acinar 266-6 cells in order to study the role of PP2A inactivation in the activation of the MAPK cascade and in the up-regulation of inflammatory cytokines. What is more, non-silenced and PP2Ac-silenced pancreatic acinar 266-6 cells were incubated with 10 ng/mL of TNF-α for 1h. Additionally, in order to simulate *in vitro* the conditions of the *in vivo* experimental model induced by sodium taurocholate, non-silenced and PP2Ac-silenced 266-6 cells were treated with 0,5% sodium taurocholate for 2h. Then, the mRNA expression of the genes *il-6, cxcl1* and *tnf-α* was assessed by real time PCR.

PP2A silencing in pancreatic acinar cells, *in vitro* leads to activation of p38 and JNK MAPKs and up-regulation of cytokines CXCL-1, IL-6 and TNF-α.

All these findings would help to better understand the molecular basis of the pancreatic disease and the main targets of oxidative stress that are affected during acute pancreatitis. Knowledge of the molecular biology of a disease is important because, ultimately, has its expression in a macromolecular level. The targets identified and the study of the antioxidant and inflammatory response also may contribute to improve the therapeutic treatment of acute pancreatitis. On the other hand, the new mechanism that is involved in acute inflammation in mammals would provide a new tool for the study of other diseases in which inflammation is present.

• RESUMEN

El estrés oxidativo se define como la alteración en el equilibrio entre las especies oxidantes y antioxidantes, a favor de las primeras. Mientras que hay muchos tipos de daño oxidativo, cada vez más pruebas sugieren un papel importante en la oxidación de proteínas en múltiples enfermedades.

Los puentes disulfuro se forman en el citosol durante el estrés oxidativo y funcionan como intercambiadores redox. El glutatión es un tripéptido y es uno de los principales antioxidantes, lo que le confiere un papel clave en numerosas funciones celulares. El estado redox del glutatión se considera el mejor indicador de estrés oxidativo en las células, ya que refleja el equilibrio entre el estado antioxidante y las reacciones pro-oxidantes.

Hoy en día, la pancreatitis aguda es una enfermedad relativamente común. Sin embargo, las principales cuestiones que plantean tanto los médicos y los investigadores siguen sin respuesta. ¿Cómo podríamos diagnosticar la pancreatitis aguda antes? ¿Qué tratamiento hay que dar a un paciente para atenuar el proceso inflamatorio? ¿Cuál es el período de tiempo que nos podemos permitir entre que los datos del paciente son tomados y el diagnóstico de la pancreatitis aguda? Con el fin de responder a estas preguntas, es necesario conocer los mecanismos moleculares que ocurren durante el desarrollo de la respuesta inflamatoria del páncreas. Por estas razones, uno de los objetivos de esta Tesis ha sido analizar el estado redox de los tioles y los disulfuros mixtos y de identificar dianas de señalización redox en páncreas en la pancreatitis aguda experimental como modelo de inflamación aguda asociada con la depleción de glutatión.

Existen diferentes modelos experimentales para inducir y estudiar la pancreatitis aguda en los animales, todos caracterizados y bien establecidos para inducir la pancreatitis aguda leve y grave. Para el desarrollo de esta Tesis, se ha utilizado un modelo de pancreatitis aguda necrótica inducida por la infusión retrógrada de taurocolato sódico en ratas. Este modelo tiene una alta reproducibilidad y ha sido utilizado por nuestro grupo de investigación durante varios años. Además, se ha utilizado un modelo *in vitro* a través de una línea celular de células acinares pancreáticas (266-6) procedentes de ratones para investigar en mayor profundidad los mecanismos implicados. Por lo tanto, para el estudio de la pancreatitis aguda se utilizaron tanto modelos *in vitro* como *in vivo*.

Se sabe que existe una asociación entre la inflamación aguda y la depleción de glutatión reducido (GSH). Además, la depleción de GSH en el páncreas es una característica distintiva de las primeras fases de la pancreatitis aguda y contribuye a la severidad de la enfermedad. Uno de los objetivos de esta Tesis ha sido caracterizar las consecuencias de la depleción de GSH en la pancreatitis aguda a nivel molecular. La depleción de glutatión en el páncreas en la pancreatitis aguda no está asociada a ningún aumento en los niveles de glutatión oxidado o glutationilación de proteínas. Los niveles de cistina y homocisteína, así como la cisteinilación y γ-glutamilcisteinilación de proteínas aumentaron notablemente en el páncreas después de la inducción de la pancreatitis. La cisteinilación de proteínas fue indetectable en el páncreas en condiciones basales. La cuantificación de estos tioles de bajo peso molecular, sus formas reducidas y oxidadas, así como los puentes disulfuro mixtos (formas unidas a proteínas) se llevó a cabo en muestras de tejido pancreático fresco de rata y el posterior análisis por espectrometría de masas acoplada a cromatografía líquida de alta resolución (HPLC-MS/MS).

Para evaluar la formación de puentes disulfuro durante el curso de la pancreatitis aguda, se llevó a cabo un intercambio redox con monobromobimano. El aumento de la fluorescencia después de la aparición de la pancreatitis mostró que la oxidación reversible de tioles se forma en el curso de la enfermedad.

Tras confirmar la formación de puentes disulfuro durante la pancreatitis aguda, se investigó si los puentes disulfuros se formaban con tioles libres de bajo peso molecular, tales como GSH, cisteína, etc, o entre las proteínas. La mayoría de los disulfuros que sufren las proteínas parece ser debido a la unión de tioles de bajo peso molecular ya que la electroforesis diagonal llevada a cabo después del intercambio redox con biotina reveló un marcado aumento de quimioluminiscencia a lo largo de la diagonal a las 6h tras la inducción de la pancreatitis aguda.

Después de estudiar la formación de puentes disulfuro en la pancreatitis aguda, se evaluaron otras modificaciones oxidativas de las proteínas: La S-nitrosilación y la carbonilación. La S-nitrosilación se evaluó mediante el intercambio con biotina y la carbonilación por electroforesis bidimensional utilizando un anticuerpo contra residuos carbonilados. No hubo cambios significativos en la S-nitrosilación y carbonilación de proteínas durante el curso de la pancreatitis aguda.

Por tanto, en la presente Tesis mostramos que la pancreatitis aguda es una patología inflamatoria asociada con el estrés oxidativo, pero en la que no aparece carbonilación ni S-nitrosilación de proteínas y sin embargo, se acompaña de una considerable formación de puentes disulfuro. Los intercambios tiol-disulfuro en las proteínas pueden causar grandes efectos

sobre la actividad de las mismas y sobre la señalización celular. En esta Tesis, se describe el estrés por disulfuro como un tipo de estrés oxidativo que ocurre en la inflamación aguda en mamíferos y que está asociado con la oxidación del par cisteína/cistina y la cisteinilación de proteínas, pero sin cambios en la oxidación del glutatión o en la glutationilación de proteínas.

El siguiente paso en esta Tesis fue identificar dianas de estrés por disulfuro y que por tanto se oxidan en el curso de la pancreatitis aguda. Las dianas de estrés por disulfuro se identificaron mediante diferentes técnicas: *Western blotting* en condiciones reductoras y no reductoras, electroforesis diagonal, métodos proteómicos y el estudio de la actividad fosfatasa en presencia y ausencia de N-acetilcisteína. Todas las dianas potenciales de estrés por disulfuro que se estudiaron en la presente Tesis fueron escogidas por su importancia y relevancia particular en su implicación en la estabilidad redox, la señalización redox y la cascada inflamatoria que se produce en la pancreatitis aguda. El estudio de las proteín fosfatasas en esta Tesis permitió una elucidación adicional de los mecanismos implicados en el proceso inflamatorio durante la pancreatitis aguda así como la identificación de proteín fosfatasas como dianas de estrés por disulfuro.

Todas las dianas identificadas de estrés por disulfuro se pueden clasificar en dos grupos: Los tampones redox y los tioles de señalización redox. Los tampones redox incluyen el inhibidor de la ribonucleasa y la albúmina. Los tioles de señalización redox son la tiorredoxina 1, APE1/REF1, KEAP1, las tirosín fosfatasas, las serín/treonín fosfatasas y la proteína disulfuro isomerasa. Estas dianas de estrés por disulfuro presentan gran relevancia en los procesos biológicos que implican actividad enzimática, la reparación del ADN, la proliferación celular, la apoptosis, el estrés del retículo endoplasmático y la

respuesta inflamatoria. El estrés por disulfuro sería por tanto un tipo específico de estrés oxidativo implicado en la señalización redox en mamíferos.

Debido a su relación con el estrés oxidativo, la homeostasis redox, y su acción sobre las MAPKs, la PP2A podría desempeñar un papel clave en las fases tempranas de la pancreatitis aguda, particularmente en la forma grave de la enfermedad, participando críticamente en el control en cascada inflamatoria. Sobre la base de esta hipótesis, así como en la oxidación observada en la subunidad catalítica de la PP2A y su pérdida de actividad en la pancreatitis aguda, decidimos silenciar la PP2Ac en células acinares pancreáticas 266-6 con el fin de estudiar el papel de la inactivación de la PP2A en la activación de la cascada de MAPK y en la regulación de las citoquinas inflamatorias. Además, las células acinares pancreáticas 266-6 silenciadas y no silenciadas la PP2Ac, se incubaron con 10 ng/mL de TNF-α durante 1 h. Y, con el fin de simular *in vitro* las condiciones del modelo experimental *in vivo* inducido por taurocolato sódico, las células acinares pancreáticas 266-6 silenciadas y no silenciadas la PP2Ac fueron tratadas con taurocolato sódico al 0,5% durante 2 h. A continuación, la expresión del ARNm de los genes *il-6, cxcl1* y *tnf-α* se evaluó mediante PCR en tiempo real.

El silenciamiento *in vitro* de la PP2Ac en células acinares pancreáticas conduce a la activación de las MAPKs p38 y JNK y a la regulación de las citoquinas CXCL-1, IL-6 y TNF-α.

Todos estos hallazgos podrían ayudar a entender mejor la base molecular de la enfermedad pancreática y a las principales dianas de estrés oxidativo que se ven afectadas durante la pancreatitis aguda. El conocimiento de la biología molecular de una enfermedad es importante porque, en última

instancia, tiene su expresión a nivel macromolecular. Las dianas identificadas y el estudio de la respuesta antioxidante e inflamatoria también pueden contribuir a mejorar el tratamiento terapéutico de la pancreatitis aguda. Por otra parte, el nuevo mecanismo que está implicado en la inflamación aguda en mamíferos proporcionaría una nueva herramienta para el estudio de otras enfermedades en las que la inflamación está presente.

II. INTRODUCTION

1. OXYGEN AND LIFE

Oxygen is essential for aerobic life, but also may trigger toxicity through the formation of reactive oxygen species (ROS) (Halliwell 1996). These species are fundamental in all biological processes (activation of transcription factors, modulation of the oxidation-reduction state, cell signaling, etc.) but they have also been linked with enzyme abnormalities, damage to cell membranes, impairment of mitochondrial function, etc... They also induce mutations when reacting with nitrogenous bases and this in turn leads to nitrosative stress in higher organisms. During the evolutionary process, aerobic beings have developed different strategies (antioxidant enzymes, redox processes, etc.) to avoid toxicity that ROS may produce in the structure of proteins, lipids and DNA as a consequence of oxidative stress.

1.1 OXIDANTS

There are many biochemical oxidizing agents in cellular systems, both derived from oxygen and nitrogen (table 1), or sulfur. Reactive oxygen species are symbolized as ROS, while nitrogen derived species are called reactive nitrogen species (RNS). ROS can cause oxidative stress while RNS can generate nitrosative stress.

The oxidizing agent can have free radical or non-radical chemical structure.

A free radical is an atom or molecule, which contains one or more unpaired electrons in their outer orbital. The presence of unpaired electrons makes these species highly reactive. The average lifespan of these chemical

species is reduced, about milliseconds, although this varies depending on the type of free radical.

OXIDANTS	
FREE RADICALS	NON-RADICALS
Superoxide anion ($O_2^{\bullet -}$)	Hydrogen peroxide (H_2O_2)
Hydroxyl ($HO\bullet$)	Ozone (O_3)
Nitric oxide ($NO\bullet$)	Nitrous acid (HNO_2)

Table 1. Classification of some ROS and RNS

Because of the topic of this Thesis is related to reactive oxygen species, since now, it will be discussed only about ROS and oxidative stress.

1.1.1 REACTIVE OXYGEN SPECIES

Reactive oxygen species are generally small molecules highly reactive due to the presence of a layer of unpaired valence electrons. They include oxygen ions, free radicals and peroxides, both inorganic and organic. These species are formed naturally as products of the normal oxygen metabolism and, as it has been mentioned before, play an important role in biological functions. However, under oxidative stress ROS levels can greatly increase, which can result in significant damage to cell structures. Indeed, ROS are very powerful oxidizing agents.

1.1.2 TYPES OF REACTIVE OXYGEN SPECIES

The main ROS of interest from the biological point of view are superoxide anion, hydrogen peroxide and hydroxyl radical. These three molecules exhibit different properties (Rinalducci et al. 2008)

1.1.2.1 Superoxide anion, $O_2^{\bullet-}$

$O_2^{\bullet-}$ is formed from an oxygen molecule in the presence of an amount of energy enough to enable it to acquire a supplemental electron. This specie is produced by a large number of enzymes, autoxidation reactions and non-enzymatic electron transfer from the reduced molecular oxygen. The superoxide anion formed *in vivo* has a half-life of the order of milliseconds, it is dismutated by superoxide dismutases (SODs) with very fast reaction constant, or via non-enzymatic mechanism to H_2O_2.

Even though $O_2^{\bullet-}$ is not a strong oxidant, it is a precursor of many other reactive oxygen species, and it also becomes involved in the propagation of oxidative chain reactions (Turrens 2003).

In addition, $O_2^{\bullet-}$ can react with thiol groups (-SH), but the rate of this reaction is usually quite low (Aruoma et al. 1989). The reaction of $O_2^{\bullet-}$ with glutathione also produces sulfur radicals which are more damaging to the cell than the anion peroxide (Schoneich et al. 1989).

Under conditions of overproduction of $O_2^{\bullet-}$ and depletion of antioxidants, $O_2^{\bullet-}$ can interact with -SH from vicinal proteins and enzymes and

inactivate them, deplete glutathione and initiate a cascade of oxidative events. This will be discussed in later sections of this thesis.

1.1.2.2 Hydrogen peroxide, H_2O_2

H_2O_2 is formed *in vivo* by spontaneous or enzymatic (through SODs and various oxidases in oxidation-reduction reactions) dismutation of $O_2^{\bullet-}$ during cellular metabolism (Rojkind et al. 2002).

At low concentrations, H_2O_2 is relatively stable, however, at high concentrations it can interact with energy generation systems of cells and inactivate them. Furthermore, H_2O_2 can oxidize thiol groups from proteins and cause breaking of DNA strands. Its most harmful effect is the formation of HO• catalyzed by transition metals. Physiologically, H_2O_2 is removed by the action of glutathione peroxidase (GPx), catalase (CAT) and peroxirredoxins (Prdx).

1.1.2.3 Hydroxyl radical, HO•

HO• is the most reactive radical found in biological systems (Aruoma et al. 1989). It has the ability to quickly react with all kinds of cellular components. Generally it is produced from hydroperoxides through the Fenton's reaction (Goldstein et al. 1993) requiring transition metal traces as catalysts. In this regard iron plays the main role. There are no specific antioxidants *in vivo* for this radical specie.

1.1.3 ROS GENESIS

All free radicals mentioned above are formed endogenously (inside the organism), but the body is also exposed to free radicals from external sources, for instance the diet, smoke tobacco, pollution, ozone, etc. (Ames 1983; Pryor et al. 1995; Rock et al. 1996). Thus, reactive oxygen species can have an endogenous or exogenous origin (Freeman and Crapo 1982; Frei 1994).

1.1.3.1 Exogenous sources

The most important exogenous sources in the generation of reactive oxygen species are:

- **Antineoplastic agents**, such as adriamycin, bleomycin, daunorubicin and some antibiotics (Doroshow and Davies 1983). Some of the effects of these drugs have been attributed to their ability to reduce oxygen to superoxide, hydrogen peroxide and hydroxyl radical.

- **Radiations**, such as electromagnetic radiations and particle radiations (electrons, protons, neutrons, deuterons, α and β particles) (von Sonntag 1994).

- **Environmental factors**, such as photochemical air pollutants, hyperoxia, pesticides, solvents, anesthetics and aromatic hydrocarbons. These agents either have free radicals or become radicals by cellular metabolism and detoxification processes (Trush et al. 1982).

1.1.3.2 Endogenous sources

1.1.3.2.1 Mitochondrial electronic transport chain

The mitochondrial electronic transport chain is one of the main sources of free radicals in the cell. Although ROS are produced under physiological conditions (Boveris et al. 1978),there are many diseases in which it is reported that free radicals are generated in mitochondria, this being the cause of oxidative stress experienced by the cell (Vina et al. 2003).

Classically, it has been considered the mitochondrial chain as the source of ROS (figure 1). In 1966 Jensen described that mitochondria produces hydrogen peroxide. The electronic transport chain is composed of redox proteins capable of reducing oxygen to H_2O, during oxidative phosphorylation producing ATP (energy source).However, between 2 and 5% of O_2 molecules are activated due to the incorporation of an electron in the form of $O_2^{\bullet-}$. This activation occurs in proportion to the activity of the electron transport chain (Tiidus et al. 1996). Complexes I and III (redox centers) add electrons to oxygen molecules becoming main sources of $O_2^{\bullet-}$ (figure 1).

Once the $O_2^{\bullet-}$ is produced it can take two ways: either it reduces cytochrome C in the intermembrane space or is converted into H_2O_2 and O_2 in the same intermembrane space.

In the case of continuous increase in the activity of mitochondria, there would be a continuous generation of $O_2^{\bullet-}$ that can reduce transition metals and generate HO• (Turrens 2003).

The respiratory control maintenance depends on the integrity of the mitochondrial membrane, if it receives any damage, uncouples electron transport and ATP synthesis.

The mitochondrial ROS production increases when the supply of substrate to the mitochondrial electron transport chain exceeds energy demand. Under these conditions, increases the partial oxygen pressure that can increase ROS production (Skulachev 1996). Under oxidative stress, simultaneous collapse of the mitochondrial membrane potential and a transient increase in ROS generation by the electron transfer chain can result in mitochondrial release of ROS to cytosol (Go and Jones 2008).

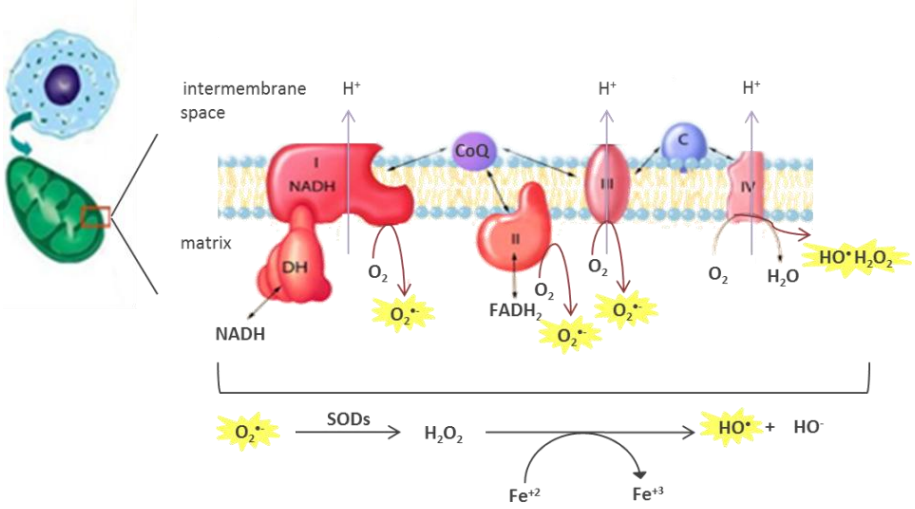


Figure 1. Endogenous sources of ROS

In addition to the electron transport chain, mitochondrial ROS may also be generated by pyruvate, α-ketoglutarate dehydrogenase, glycerol-3-phosphate dehydrogenase and monoamine oxidase (Sandri et al. 1990; Starkov et al. 2004; Tretter and Adam-Vizi 2004).

Other endogenous sources of ROS are xanthine oxidase, cytochrome P450, cyclooxygenase and lipooxigenase among others.

1.1.3.2.2 Fenton-Haber-Weiss Reaction

This reaction consists of the reduction of H_2O_2 by ions from transition metals to yield the hydroxyl radical. The H_2O_2 is a relatively stable molecule in the absence of catalysts that promote its decomposition. However, in the presence of ions from transition metals (specially ferrous ion Fe^{2+} and, in lesser extent, cuprous ion Cu^{+}) Fenton discovered at the end of nineteenth century (Fenton 1894) that organic molecules could be oxidized by mixing hydrogen peroxide and Fe^{2+} (Fenton's reagent). Later on, Haber and Weiss gave an explanation of the reaction mechanism: the hydrogen peroxide suffers reduction resulting in the formation of hydroxyl radical and hydroxyl ion, and the corresponding metal suffers oxidation by transfer of an electron to the specie that is reduced (Haber and Weiss 1934). The representation of the reaction is as follows:

$$H_2O_2 + Fe^{2+} \longrightarrow HO\bullet + HO^{-} + Fe^{3+}$$

Despite all this, it is not completely clear how metal ions are able to act as redox catalysts in biological systems by Fenton's reaction and Haber-Weiss. Processes of iron uptake and distribution, and metal ions, are generally very

strictly regulated in mammals. A large number of metal binding proteins (ferritin, transferrin, ceruloplasmin,...) act as a reservoir and prevent these ions been involved in redox reactions (Halliwell 1991; Rouault and Klausner 1996).

1.2 ANTIOXIDANTS

As the cellular machinery is continuously producing free radicals, biological systems must neutralize these oxidants. Halliwell, in 1995 defined antioxidant as *"any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate"* (Halliwell et al. 1995). Hence, an antioxidant is a substance capable of neutralizing the oxidizing action of free radicals. Antioxidants can act in the following ways:

- Preventing the formation of ROS
- Intercepting the attack of ROS
- Kidnapping reactive metabolites and converting them into less reactive molecules
- Amplifying the resistance of the sensitive biological targets against ROS
- Maintaining a favorable environment for the action of other antioxidants

From the biochemical point of view, antioxidants can be divided into non-enzymatic and enzymatic antioxidants.

1.2.1 NON-ENZYMATIC ANTIOXIDANTS

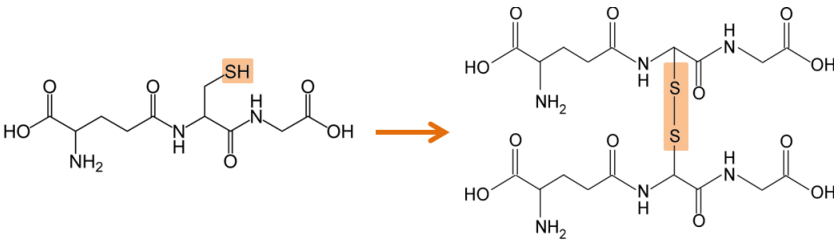
The non-enzymatic antioxidants are the first line of defense against both extracellular and intracellular free radicals.

These antioxidants can be lipophilic molecules such as vitamin E, estrogens, bilirubin, flavonoids and carotenoids, or hydrophilic as glutathione (GSH), vitamin C (ascorbic acid) and uric acid (Halliwell and Gutteridge 1990; Niki and Traber 2012).

1.2.1.1 Glutathione

Glutathione is the most abundant non-protein thiol in mammalian cells. It was discovered by Hopkins in 1921 and consists of three amino acids: glutamic acid, cysteine and glycine. Its structure gives certain characteristics which give broad and important functionality in the cell. The thiol group (-SH) of the cysteine is involved in redox reactions, while the γ-glutamyl bond between the cysteine and glutamic residues makes it resistant to degradation by most cellular peptidases.

This tripeptide can be in two forms depending on its redox state: as reduced (GSH) or as oxidized (GSSG) glutathione (figure 2). GSSG is a combination of two molecules of GSH bound by a disulfide bridge (S-S).



GSH **GSSG**

Figure 2. Structure of reduced (GSH) and oxidized (GSSG) glutathione

GSH plays many important metabolic functions: detoxifies ROS in cells and protects against harmful effects of radiation (Sies 1991). GSH is also involved in DNA synthesis (Estrela et al. 2006) and in amino acid uptake in some tissues (Vina et al. 1989), modulates enzyme activities (Pajares et al. 1992), regulates calcium homeostasis (Bellomo et al. 1982), cell proliferation (Terradez et al. 1993) and is a reservoir of cysteine (Tateishi et al. 1974). Many of these functions are due to its chemical structure.

1.2.1.1.1 Glutathione detoxification mechanism

GSH can reduce peroxides by glutathione peroxidase, or react directly with ROS without any enzymatic intervention, exchanging electrons through the sulfur of cysteine of its structure:

$$GSH + R^{\bullet} \longrightarrow RH + GS^{\bullet}$$

$$2GS^{\bullet} \longrightarrow GSSG$$

1.2.1.1.2 GSH synthesis

In physiological conditions, the limiting factors for the synthesis of GSH are the activity of the enzyme glutamate cysteine ligase (GCL) (Diaz et al. 2002) and the availability of cysteine (Tateishi et al. 1974; Tateishi et al. 1977).

- **Glutamate cysteine ligase**

GCL produces γ-glutamylcysteine from L-glutamic acid and L-cysteine. Its activity is regulated by negative feedback of GSH synthesis because GSH inhibits GCL (Richman and Meister 1975).

The second step in GSH synthesis is catalyzed by the enzyme GSH synthase that bounds glycine to γ-glutamylcysteine. The new GSH intracellularly synthetized may be transported to cell exterior.

- **Availability of cysteine: Transsulfuration pathway**

The transsulfuration pathway constitutes the major route of cysteine biosynthesis. The initial substrate entering the transsulfuration pathway is methionine. The metabolism of homocysteine, one of the intermediates and a non-protein-forming sulfur amino acid, is at the junction of two metabolic pathways: remethylation to methionine and transsulfuration to cysteine. In remethylation (figure 3), homocysteine acquires a methyl group from N-5-methyltetrahydrofolate or from betaine to form methionine. A considerable proportion of methionine is then activated by ATP to form S-adenosylmethionine (SAM). SAM serves primarily as a universal methyl donor to a variety of acceptors. S-adenosylhomocysteine (SAH), the by-product of

these methylation reactions, is subsequently hydrolyzed, thus regenerating homocysteine, which then becomes available to start a new cycle of methyl-group transfer (Selhub 1999).

In the transsulfuration pathway (figure 3), homocysteine condenses with serine to form cystathionine in an irreversible reaction catalyzed by cystathionine β-synthase (CBS). Cystathionine is then hydrolyzed by γ-cystathionase (γ-C), to form cysteine and α-ketobutyrate. Excess cysteine is oxidized to taurine or inorganic sulfates or is excreted in the urine (Selhub 1999).

Since cystathionine β-synthase acts at the intersection of the transsulfuration and remethylation pathways, changes in the activity or concentration of this enzyme would be expected to lead to a shift in the direction of the homocysteine flux. CBS is therefore a reasonable target for redox regulation (Deplancke and Gaskins 2002). Given the role of cysteine as a precursor for GSH synthesis, it seems logical that the transsulfuration pathway, the major cysteine biosynthesis pathway, would be redox sensitive. Thus, oxidizing conditions enhance cystathionine β-synthase activity and likely also γ-cystathionase and glutamate cysteine ligase activities through post-translational modification, inhibit the remethylation pathway, and thereby significantly increase the flux of sulfur through the transsulfuration and GSH biosynthesis pathways (Deplancke and Gaskins 2002; McBean 2012).

1.2.1.1.3 GSH degradation: Meister cycle

After its synthesis, GSH can be used or degraded. GSH degradation is performed one part inside and another part outside the cell. GSH is exported

out of the cell via a specific transporter. In this moment the enzyme γ-glutamyltranspeptidase (γ-GT) hydrolyzes the γ-glutamyl bond. The glutamic acid formed, enters into the cell.

A dipeptidase hydrolyzes cysteinilglycine formed by γ-GT (Meister and Anderson 1983). Therefore the activity of γ-GT is a source of GSH amino acids precursors and favors its synthesis (Leung and Chan 2009).

The set of reactions involved in the synthesis and degradation of GSH is called γ-glutamyl cycle and was postulated by Meister and Anderson (Meister y Anderson, 1983).

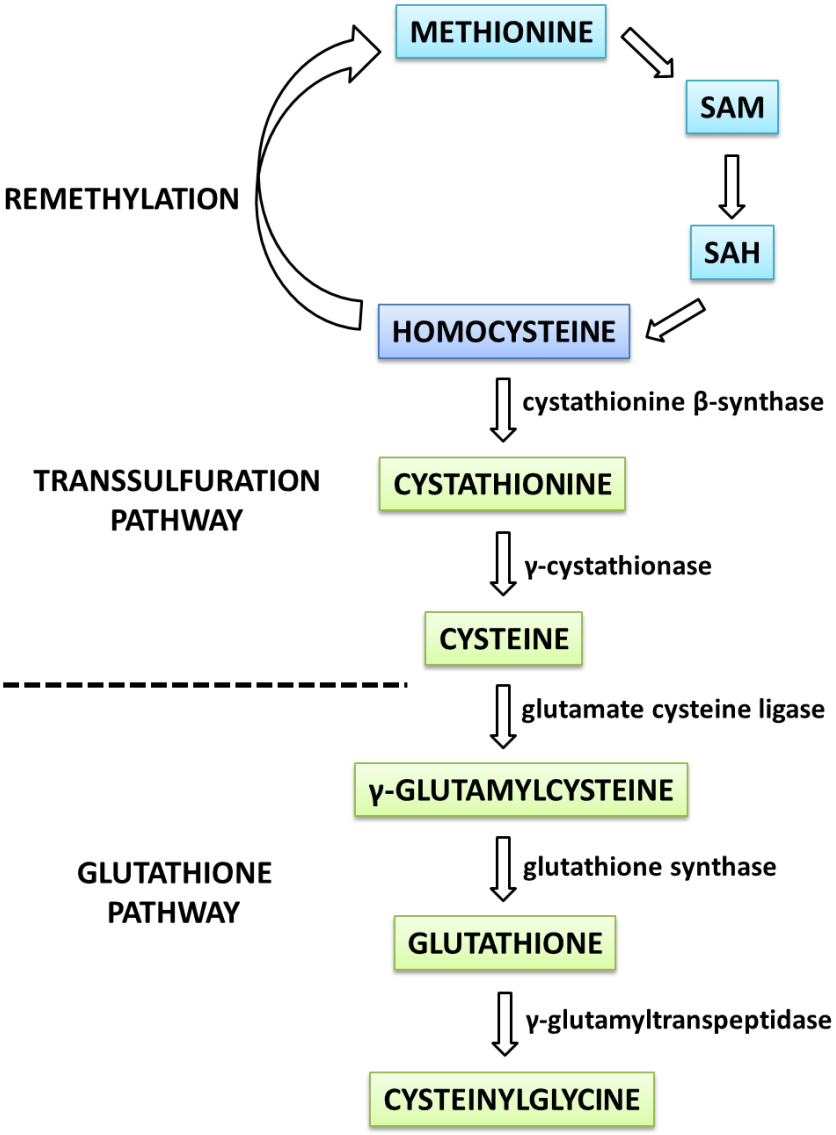


Figure 3. Relationship between remethylation, transsulfuration and glutathione pathways

1.2.1.2 N-Acetylcysteine

N-Acetylcysteine (NAC) is a cysteine amino acid derivative with a -SH group that confers it a high antioxidant capacity and an acetyl group that prevents cysteine oxidation. Thus, NAC can reach tissues where it undergoes deacetylation serving as precursor for GSH synthesis (Cotgreave 1997).

Besides its mucolytic effect (Sheffner 1963), NAC has been shown to reduce ROS toxicity associated with acute inflammatory processes (Feddersen et al. 1993).

NAC is able to inhibit redox signaling pathways and those involved in the synthesis of inflammatory mediators, interfering in the phosphorylation of MAPKs (Ramudo et al. 2005) as well as activation of NF-kB transcription factors (Yubero et al. 2009). Thus, NAC administration is able to reduce the levels of cytokines and chemokines, an effect observed *in vivo* (Kim et al. 2000) and *in vitro* (Li and Karin 1999; Yu et al. 2002; Ramudo et al. 2009) studies.

Thanks to all this, NAC has been shown to have an important protective role in tissue damage in acute pancreatitis, as evidenced by analysis conducted in pancreas and extrapancreatic organs such as lung and liver (Demols et al. 2000; Sevillano et al. 2003).

1.2.2 ENZYMATIC ANTIOXIDANTS

The main defense against free radicals is carried out by the following systems of enzymes: superoxide dismutase family (Cu/ZnSOD, MnSOD and EC-SOD), catalase (CAT), glutathione-dependent system and thioredoxin-

dependent system (figure 4). In the absence of oxidative stress, generated ROS are held to very low levels due to the coordinated action of these enzyme systems. If there is a reduction in the antioxidant capacity or an unilateral deficiency of one of these enzyme systems, it will increase cell vulnerability against ROS (Arthur 2000).

1.2.2.1 Superoxide dismutase

Superoxide dismutase (SOD) is present in all cells that use oxygen in their metabolism (Meister 1988). Its activity was first described by McCord and Fridovich in 1969 (Berndt et al. 2007). The superoxide dismutase activity plays a key role in antioxidant defense because it is one of the first lines of defense of the organism against oxidants. SOD catalyzes the reaction that converts radical superoxide into hydrogen peroxide (Yoshitake et al. 1994; Holmgren 2000).

$$O_2^{\bullet -} + O_2^{\bullet -} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

Several common forms of SOD: Cu/ZnSOD or SOD1 (which binds both copper and zinc), MnSOD or SOD2 (which binds manganese) and EC-SOD.

The mitochondrial matrix contains MnSOD (Dubuisson et al. 2004). The expression of MnSOD is further induced by agents that cause oxidative stress (Engstrom et al. 1974; Watabe et al. 1994). Cu/ZnSOD is present in the intermembrane space of the mitochondria, nuclear compartments and also in the cytoplasm of eukaryotic cells (Rhee et al. 2005). EC-SOD is extracellular. Its expression pattern is highly restricted to the specific cell type and tissues where its activity can exceed that of MnSOD and Cu/ZnSOD (Zelko et al. 2002).

1.2.2.2 Catalase

Catalase (CAT) participates in the detoxification of hydrogen peroxide through a reaction that leads to water and oxygen (Chance et al. 1979). It is a very important enzyme in protecting the cell from oxidative damage by ROS. Likewise, catalase has one of the highest turnover numbers of all enzymes; one catalase molecule can convert millions of molecules of hydrogen peroxide to water and oxygen each second. It is mainly located in peroxisomes (Tolbert and Essner 1981) although in 2000 it was shown some activity of this enzyme in mitochondria and cytosol (Edman et al. 1985).

$$2\ H_2O_2 \xrightarrow{\text{Catalase}} 2\ H_2O + O_2$$

1.2.2.3 Glutathione-dependent enzyme system

It is an antioxidant system dependent upon GSH. The enzymes included in this system are glutathione peroxidases (GPx), glutathione reductase (GR) and glutaredoxins (Grx) (figure 4).

Glutathione peroxidase reduces hydrogen peroxide to water by utilizing electrons provided by GSH. The GSSG formed is reduced back by glutathione reductase that utilizes NADPH as a reducing equivalent generated mainly by glucose-6-phosphate dehydrogenase. In parallel, glutaredoxin catalyzes the glutathionylation of proteins (Brigelius-Flohe and Maiorino 2013).

1.2.2.3.1 Glutathione peroxidase

Is the general name of an enzyme family with peroxidase activity whose main biological role is to protect the organism from oxidative damage. The biochemical function of glutathione peroxidases is to reduce alkyl hydroperoxides and hydrogen peroxide (Arthur 2000). The presence of selenocysteine in the catalytic moiety guarantees a fast reaction with the hydroperoxides and a fast reducibility by GSH (Brigelius-Flohe and Maiorino 2013). Several isoenzymes are encoded by different genes, which vary in subcellular location and substrate specificity. Glutathione peroxidase 1 (GPx1) is the most abundant isoenzyme, found in the cytoplasm and mitochondria of nearly all mammalian tissues, whose preferred substrate is hydrogen peroxide. Glutathione peroxidase 2 (GPx2) is an intestinal enzyme, while glutathione peroxidase 3 (GPx3) is extracellular, especially abundant in plasma. Glutathione peroxidase 4 (GPx4) has a high preference for lipid hydroperoxides; it is expressed in nearly every mammalian cell, though at much lower levels. Seven proteins exhibiting glutathione peroxidase activity have been described; four of them are now known as classical GPx1–GPx4. Three other enzymes, GPx5–GPx7, still require better identification and characterization (Kulinskii and Kolesnichenko 2009).

1.2.2.3.2 Glutathione reductase

Glutathione reductase reduces GSSG to form GSH. In this process, NADPH is required. The reaction catalyzed by this flavoprotein provides reducing power for various coupled thiol transferases and peroxidases. The binding sites for NADPH and GSSG are at opposite sides of each subunit of the dimer; FAD at the center of the subunit and an adjacent cystine moiety

mediate transfer of reduction equivalents. This enzyme and several others that contain active site dithiol moieties that undergo reversible oxidation (e.g. thioredoxin reductase and lipoamide dehydrogenase) exhibit similarities in their active site amino acid sequences (Meister 1988).

1.2.2.3.3 Glutaredoxins

Glutaredoxins can catalyze both the formation and the reduction of mixed disulfides between protein thiols and GSH (Yoshitake et al. 1994). S-glutathionylation will be discussed in depth in section 2.1.3.2.3.

There are two prominent and well-characterized isoforms of mammalian glutaredoxins, namely Grx1 and Grx2. Grx1 is primarly localized to the cytosol and possibly in the nucleus of certain cells. Grx2 is in the mitochondrial matrix and catalyzes the reversible oxidation and glutathionylation of mitochondrial membrane proteins (Holmgren 2000); There is a second mitochondrial Grx, named Grx5, due to its homology to yeast Grx5 (Berndt et al. 2007).

In general, there is upcoming evidence that the formation of mixed disulfides of protein thiols with glutathione is a key event in the regulation of the cellular response to oxidative stress. Molecular biological studies show that Grx2 has a central role in GSH-dependent redox regulation in mitochondria (Beer et al. 2004).

1.2.2.4 Thioredoxin-dependent enzyme system

It is an antioxidant system dependent upon thioredoxin. The enzymes included in this system are peroxiredoxins (Prdx), thioredoxin reductase (TrxR) and thioredoxins (Trx) (figure 4).

Peroxiredoxins reduce hydrogen peroxide to water by utilizing electrons provided by thioredoxin. The oxidized form of peroxiredoxin is reduced back to its functional form by thioredoxin. The regeneration of reduced Trx by thioredoxin reductase utilizes NADPH as a reducing equivalent generated by glucose-6-phosphate dehydrogenase (Holmgren 1989).

1.2.2.4.1 Peroxiredoxins

Peroxiredoxins are members of a super-family of selenium-free and heme-free peroxidases which function as thioredoxin peroxidases to catalyze the reduction of hydrogen peroxide, alkyl hydroperoxide and peroxynitrite (Dubuisson et al. 2004). Peroxiredoxin uses thioredoxin to recharge its reduced form after reducing H_2O_2 in the following reactions:

$$\text{Prdx (reduced)} + H_2O_2 \longrightarrow \text{Prdx (oxidized)} + 2\ H_2O$$

$$\text{Prdx (oxidized)} + \text{Trx (reduced)} \longrightarrow \text{Prdx (reduced)} + \text{Trx (oxidized)}$$

Mammals express six peroxiredoxins. Prdx1 is located in the cytosol; Prdx2 is one of the most abundant proteins in erythrocytes after hemoglobin; Prdx3 is exclusively localized in mitochondria; Prdx4 is in the endoplasmic reticulum; Prdx5 has been found in mitochondria and other compartments (peroxisomes, cytosol and nucleus) and Prdx6 is cytosolic. (Watabe et al. 1994;

Knoops et al. 1999; Banmeyer et al. 2004). Peroxiredoxins are proposed to play a role in cell signaling by regulating H_2O_2 levels. In addition, Prdx3 and Prdx5 play an important role in mitochondrial redox regulation by functioning as antioxidants (Zhang et al. 2007).

1.2.2.4.2 Thioredoxins

Thioredoxins are enzymes that act as antioxidants by facilitating the reduction of other proteins by cysteine thiol-disulfide exchange. Thioredoxin is an oxidoreductase enzyme containing a dithiol-disulfide active site. Thioredoxins are characterized at the level of their amino acid sequence by the presence of two vicinal cysteines in a CXXC motif. These two cysteines are the key to the ability of thioredoxin to reduce other proteins. Thioredoxin proteins also have a characteristic tertiary structure termed the thioredoxin fold (Martin 1995).

Thioredoxins are kept in the reduced state by thioredoxin reductase, in a NADPH-dependent reaction.

Mammalian cells contain two thioredoxins, the cytosolic thioredoxin (Trx1) and the mitochondrial thioredoxin (Trx2). Mammalian Trx1 was first described as an electron donor for enzymes that form a disulfide during their catalytic cycle, like ribonucleotide reductase (Engstrom et al. 1974), methionine sulfoxide reductases (Brot and Weissbach 1983), and peroxiredoxins (Rhee et al. 2005). Trx1 is normally a cytosolic protein, but certain stimuli like mild oxidative stress can induce its translocation to the nucleus or its export from the cell (Hirota et al. 1999).

The thioredoxin active site has also been found in thioredoxin-like domains in several proteins of higher molecular weight. It was shown that protein disulfide isomerase contains two presumed active-site sequences with strong homology to *E. coli* thioredoxin (Edman et al. 1985). Protein disulfide isomerase is implicated in the formation of native disulfide bonds in proteins (Bulleid and Freedman 1988) and is localized in the endoplasmic reticulum. Recently, protein disulfide isomerase was shown to be a substrate for both thioredoxin reductase and thioredoxin, and thus a member of the thioredoxin system.

1.2.2.4.3 Thioredoxin reductases

Thioredoxin reductases (TrxR) are NADPH-dependent enzymes of the nucleotide-disulfide oxidoreductase family which function to reduce thioredoxins. TrxRs are ubiquitously found in mammalian tissues, are homodimeric and contain a C-terminal selenocysteine necessary for catalytic activity. Two isoforms have been identified; the cytosolic TrxR1, and the mitochondrial TrxR2. Cytosolic TrxR1 and mitochondrial TrxR2 function independently. TrxR shares some features with glutathione reductase (Mustacich and Powis 2000).

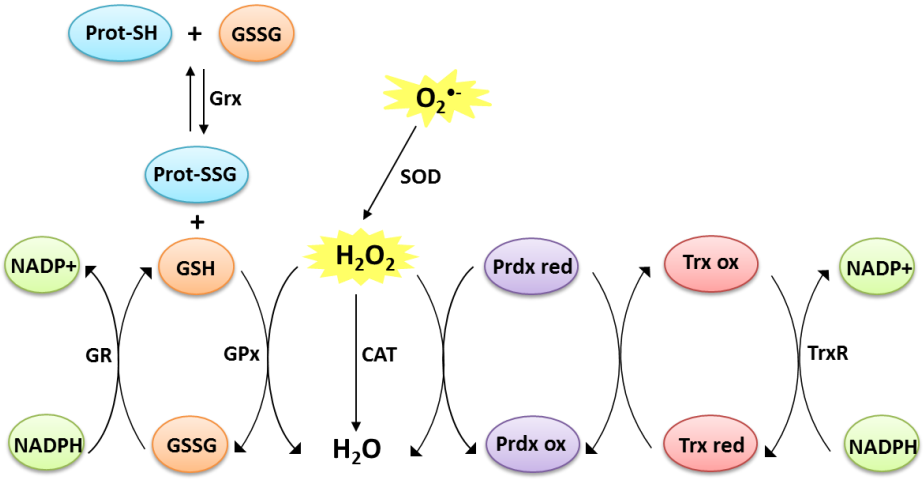


Figure 4. Enzymatic antioxidants

2. OXIDATIVE STRESS

Under normal physiological conditions, the balance between ROS generation and removal maintains properly the intracellular redox-sensitive signaling. However, certain factors can modify this balance causing a rapid increase of intracellular ROS levels and hence oxidative stress occurs because the production of ROS exceeds the cellular antioxidant defense.

Oxidative stress is defined as the disturbance in the balance between oxidants and antioxidants species (Sies 1986). It can be caused by an excess of oxidants, antioxidant deficiency, or both factors simultaneously.

Oxidative stress can lead to cell death and contribute to the development of pathology (figure 5) (Serero et al. 2008). Many diseases are associated with oxidative stress: cancer, diabetes mellitus type 2,

atherosclerosis, myocardial infarction, and acute pancreatitis. In the case of acute pancreatitis, oxidative stress seems to contribute to the progression of mild to severe disease.

Oxidative stress can cause damage to the major cellular macromolecules (lipids, proteins and DNA) (figure 5).

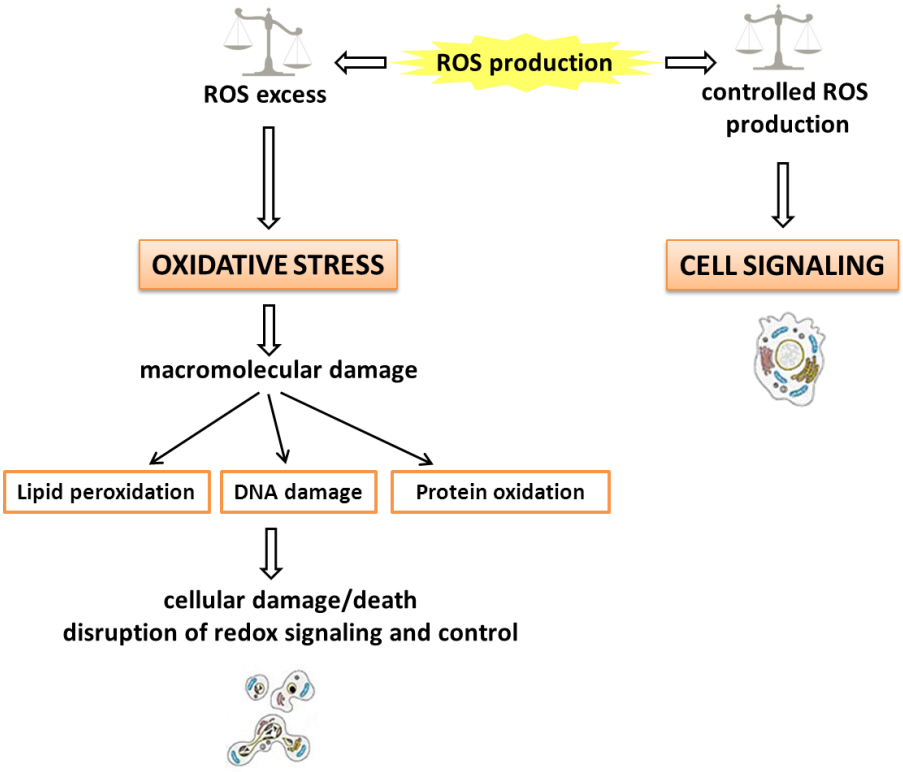


Figure 5. Balance and imbalance in oxidant production and antioxidants

2.1 OXIDATIVE STRESS AND DAMAGE TO BIOMOLECULES

2.1.1 OXIDATIVE DAMAGE TO LIPIDS

The oxidative damage to lipids induced by ROS is known as lipid peroxidation. The main targets for lipid peroxidation are cholesterol (which is related to atherosclerosis) and polyunsaturated fatty acids (PUFAs) affecting the membrane lipid bilayer integrity (Gueraud et al. 2010; Spickett et al. 2010). Lipid peroxidation is commonly observed in many diseases, especially those involving inflammatory processes. Complexity of lipid peroxidation originates from the large number of products that can be produced. Among PUFAs, peroxidation of the omega-6 (ω-6) fatty acid arachidonic acid (AA) is known to play a crucial role in inflammation. AA peroxidation mediated by the enzymes lipoxygenases (LPs) leads to the formation of leukotrienes (LTs) and hydroxyeicosatetraenoic acid derivatives (HETEs), whereas peroxidation by cyclooxygenases (COX) yields prostaglandins (PGs) and thromboxanes (TXs); all of them are known to be inflammatory mediators and referred to as eicosanoids (Ecker 2012).

Lipid peroxidation can be originated from different sources and it may be classified as enzymatic, non-enzymatic non-radical peroxidation, and non-enzymatic free-radical mediated peroxidation.

Lipid peroxidation mediated by free radicals generates a great amount of products more stable than peroxyl radicals, which may be separated into primary lipid hydroperoxides and secondary products such as malondialdehyde, hydroxynonenal, isoprostanes, neuroprostanes, isofuranes and neurofuranes (Therond et al. 2000). These secondary products are used as

lipid peroxidation biomarkers. Malondialdehyde (MDA) is mainly produced by cleavage of oxidized PUFAs containing at least three double bonds (Gueraud et al. 2010). Hydroxynonenal (HNE) is a major product formed by β-cleavage of oxidized ω-6-PUFAs (Therond et al. 2000; Dalle-Donne et al. 2006). Isoprostanes and isofuranes are oxidative derivatives of arachidonic acid whereas neuroprostanes and neurofuranes derive from docosahexaenoic acid.

Products of oxidized lipids may themselves initiate further oxidative damage. They may attack amino acid side chains in proteins (Requena et al. 2003) and cause fragmentation of DNA (Wang et al. 2006).

2.1.2 OXIDATIVE DAMAGE TO DNA

Oxidation may also affect other biomolecules, such as DNA. The HO• may attack deoxyribose phosphate backbone as well as the nitrogenous bases (nucleobases) of DNA nucleotides, generating a broad variety of base and sugar modification products (Dalle-Donne et al. 2006). Physiologically, oxidative DNA modifications are being produced continuously and enzymatic DNA repair mechanisms like base excision repair or nucleotide excision repair are crucial to maintain a low steady state of oxidative DNA damage preventing the organism from mutagenesis and carcinogenesis processes (Valavanidis et al. 2009). Determination of oxidative DNA derivatives may reflect the balance between DNA damage and repair. Among the oxidative DNA derivatives, interest is focused on nucleobase modifications such as cytosine glycol, thymine glycol, 8-hydroxy- and 2-hydroxyadenine and specially 8-oxo-2'-deoxyguanosine because it is an abundant lesion formed *in vivo*, which can be quantitatively measured (Valavanidis et al. 2009).

DNA is not the primary target of oxidative stress (Thorpe et al. 2004). In fact, elevated DNA damage is a secondary outcome of direct protein inactivation (Jin et al. 2003; Youn et al. 2005; Serero et al. 2008). Damage to protein is probably more important factor in ROS-induced lethality than DNA damage (Thorpe et al. 2004).

2.1.3 OXIDATIVE DAMAGE TO PROTEINS

Proteins are the most abundant cellular targets of oxidative species, more than DNA and lipids, making up to around 68% of the oxidized molecules in the cell (Rinalducci et al. 2008). The effect of oxidative stress in proteins occurs mainly by amino acid oxidation and loss or modification of biological function.

While there are many types of oxidative damage, increasing evidence suggests an important role for protein oxidation in aging and multiple diseases (Sohal 2002; Stadtman and Levine 2003; Youn et al. 2005). The importance of protein oxidation towards cellular homeostasis derives from the fact that proteins serve vital roles in regulating cell structure, cell signaling, and the various enzymatic processes of the cell. Protein oxidation can therefore rapidly contribute to oxidative stress by directly affecting cell signaling, cell structure, and enzymatic processes involved in metabolism. There are many different modes of inducing protein oxidation including metal catalyzed oxidation, oxidation induced cleavage, amino acid oxidation, and the conjugation of lipid peroxidation products.

Several amino acids can be directly modified via side chain reactions with ROS. The most sensitive amino acids are those with aromatic side chain groups and those containing sulfhydryl groups.

Certain proteins are more susceptible to oxidative targeting than others. The factors determining such selectivity include the relative content of oxidation-sensitive amino acid residues, the presence of metal-binding sites, protein localization in the cell, molecular conformation and rate of degradation. Mounting evidence also shows that newly synthesized proteins are the most prone to oxidative damage, indicating that complete folding and incorporation into protein complexes confers protection from oxidation-driven degradation (Holland et al. 2007; Medicherla and Goldberg 2008).

Disturbances that can suffer proteins in response to oxidative stress are mainly classified into those that do not modify or alter sulfur-containing amino acids and those that do it (figure 6).

2.1.3.1 Oxidative modifications to proteins not affecting sulfur-containing amino acids

2.1.3.1.1 Carbonylation

Carbonylation involves the oxidation of side chains of amino acids to aldehydes or ketones. Much knowledge on the redox state of the proteins has been acquired through identification of carbonyl groups in proteins.

Among the reactions involving sulphur-containing amino acids, carbonylation is the most commonly occurring oxidative protein modification.

Lysine, arginine, proline, and threonine side-chains can be oxidatively converted to reactive aldehyde or ketone groups (carbonylation) causing inactivation, crosslinking or breakdown of proteins (Levine and Stadtman 2001; Dalle-Donne et al. 2003; Eaton 2006).

Carbonyl formation is irreversible (Avery 2011; Cai and Yan 2013). The pattern of proteins that become carbonylated during oxidative stress appears to be quite conserved across distantly related organisms. Protein carbonylation has a biochemical consequence, as the modification provides an irreversible marker for damaged proteins to be inactivated by proteasomal degradation. However, there is a limit to the cell capacity to process carbonylated proteins, particularly as the proteasome itself is a target for oxidative inactivation (Wu et al. 2009). Carbonylated proteins that are not degraded may form potentially toxic aggregated species. Thus elevated levels of carbonylated proteins can be linked to loss of cell viability (Desnues et al. 2003).

2.1.3.1.2 Oxidation of aromatic residues in proteins

Amino acids with aromatic side chain groups are targets of oxidative stress. These amino acids are phenylalanine, tyrosine and tryptophan.

Under physiological conditions, oxidation of the aromatic amino acid L-phenylalanine (L-phe) to L-para-tyrosine (L-tyr) is carried out enzymatically by the action of phenylalanine hydroxylase (PheH) (Zhang et al. 2011). However, in pathophysiological situations the spontaneous oxidation of L-phe to L-tyr isomers may be given in the presence of HO•, leading to the production of L-ortho-tyrosine (o-tyr) (Lubec et al. 1997; Matayatsuk et al. 2007) and L-meta-tyrosine (m-tyr) (Matayatsuk et al. 2007). In addition, the attack of ROS such as

peroxynitrite (ONOO-) or hipochlorous acid (HClO) may directly attack L-tyr producing oxidized L-tyr derivatives such as 3-nitrotyrosine (3nitro-tyr) or 3-chlorotyrosine (3Cl-tyr), respectively (Saravanabhavan et al. 2010). Indeed, L-phe and L-tyr derivatives are reliable biomarkers of oxidative damage to proteins. Furthermore, 3nitro-tyr is also used as an index of nitrosative stress and 3Cl-tyr as a marker of inflammation because it indicates myelopreroxydase (MPO) activation (MPO converts H_2O_2 to HClO) (Davies et al. 1999; Orhan et al. 2004; Saravanabhavan et al. 2010). Tyrosine oxidation can alter residue hydrophobicity, as any change in aromatic residues, with subsequent effects on protein structure. Tryptophan oxidation is another irreversible protein modification that results in the formation of N-formylkynurenine (Shacter 2000).

2.1.3.2 Oxidative modifications to proteins affecting sulfur-containing amino acids

Methionine and cysteine are the two sulfur-containing amino acids that are present in peptides and proteins (Kim et al. 2014). They are extremely vulnerable to oxidation and they play an important role during oxidative stress, redox regulation and cell signaling (Drazic and Winter 2014). The most important common characteristic of cysteine and methionine residues in proteins is that both are subject to reversible oxidation and reduction, mediated either enzymatically or non-enzymatically (Kim et al. 2014). While cysteine forms cysteine through a disulfide linkage, methionine forms methionine sulfoxide (MeO) by addition of oxygen to its sulfur atom. Disulfides may be reduced back to the thiol form by various reductases, often utilizing thioredoxin (Arner and Holmgren 2000). MeO is reduced back to methionine by the methionine sulfoxide reductases, thioredoxin-dependent enzymes that are

universal among organisms (Brot and Weissbach 1983; Zhang and Weissbach 2008). High concentrations of ROS can further oxidize MeO to the irreversible methionine sulfone (Drazic and Winter 2014).

Due to the aims of this Thesis, this section will be will be focus on cysteine amino acid.

Cysteinyl thiols are particularly susceptible to oxidative modification and can undergo a diverse array of redox reactions (figure 6), which are largely dependent on the species and concentration of oxidants they contact. Two major determinants of the susceptibility of thiols to redox regulation are the accessibility of the thiol within the three-dimensional structure of the protein and surrounding amino acids.

Thiol oxidation is of interest both as a consequence of oxidative stress and also in increasingly recognized redox signaling. Thiol modifications include direct oxidation, formation of mixed disulfides (with GSH, cysteine and homocysteine), and formation of intra/intermolecular protein disulfides (figure 6).

2.1.3.2.1 Direct oxidation

The thiol of cysteine can be oxidized to sulfenic acid (PSOH), and further to sulfinic acid (PSO_2H) and sulfonic acid (PSO_3H). The first two of these modifications are readily reversible, while the latter one is often described as irreversible (figure 6).

Not only ROS react with thiols to form sulfenic acids (Kettenhofen and Wood 2010), but also peroxynitrite (ONOOH), hypochlorous acid (HOCl), and hypobromous acid (HOBr) react with thiols, leading to the formation of sulfenic acid.

Sulfenic acid has important roles as a catalytic center in enzymes and as a sensor of oxidative and nitrosative stress in enzymes and transcription factors. Depending on the environment, sometimes sulfenic acid may be present as a metastable oxidized form, and other times it acts as an intermediate leading to more stable disulfides, sulfinic acid, or sulfenyl-amide forms (Poole et al. 2004). Sometimes sulfenic acid is required as an essential intermediate prior to disulfide bond formation in the catalytic cycle (Roos and Messens 2011).

Oxidant exposure, in addition to resulting in irreversible oxidation of cysteine residues to sulfonic acids, can lead to disulfide bonding, protein misfolding, and aggregation (Sitia and Molteni 2004; Cumming and Schubert 2005). Excessive disulfide bonding may lead to covalent aggregates that are difficult to be reduced even when intracellular redox conditions are restored to normal.

2.1.3.2.2 Protein disulfides

Under normal physiological conditions, disulfides of proteins are formed by the action of protein disulfide isomerase (PDI) in the endoplasmic reticulum (Xiao et al. 2004; Benham 2012; Narayan 2012). Under oxidative conditions, inappropriate disulfides can be formed between two proteins (interprotein) or within a protein (intraprotein), causing changes in protein

aggregation and conformation. Cysteine oxidation by formation of intra/intermolecular disulfide bridges is a major contributor to protein stability. Disulfide bonds formed between free cysteine residues upon oxidative stress may play a beneficial role in cellular defense systems against a variety of stress challenges (Shimizu and Hendershot 2009). For example, it is well established that formation of disulfide linkage within KEAP1 in response to cellular stimuli by electrophiles or oxidants (Wakabayashi et al. 2004; Kensler et al. 2007) is essential for activation of the NF-E2-related factor 2 (NRF2) that then upregulates the expression of phase II antioxidant enzymes under a variety of physiological and pathophysiological conditions (Baird and Dinkova-Kostova 2011). This will be further discussed in section 4.2.

2.1.3.2.3 Mixed disulfides

Protein-SH groups can form mixed disulfides with low-molecular-weight thiols such as homocysteine, γ-glutamylcysteine, cysteinylglycine, free cysteine and glutathione (Eaton 2006). The formation of protein disulfides can protect the protein from further potentially more damaging oxidation. Glutathionylation is the most common mixed disulfide intracellularly, but cysteinylation of extracellular proteins may be prevalent. These modifications can be reversed by specific reducing disulfide enzymes such as thioredoxin reductase and glutaredoxin (Holmgren 1989).

Because GSH and free cysteine are the most abundant low-molecular-mass thiols *in vivo*, S-glutathionylated and S-cysteinylated proteins are the main mixed disulfides.

• **S-glutathionylation**

The glutathione redox couple dynamically regulates protein function by the reversible formation of mixed disulfides between protein cysteines and GSH (Gilbert 1984; Ziegler 1985; Thomas et al. 1995; Cotgreave and Gerdes 1998). Most of the protein-bound thiols (>85%) are accounted for by GSH (Chai et al. 1994; Ravichandran et al. 1994; Schuppe-Koistinen et al. 1994).

Protein S-glutathionylation can occur by several reactions: (i) direct interaction between partially oxidized (activated) protein sulfhydryls and GSH; (ii) thiol/disulfide exchange reactions between protein thiols and GSSG or PSSG; (iii) reaction between protein thiols and intermediate S-nitrosothiols such as S-nitrosoglutathione (GSNO) (Giustarini et al. 2005; Martinez-Ruiz and Lamas 2007); (iv) direct interaction between a free protein cysteinyl residue and GSH.

Furthermore, glutathione sulfenic acid (GSOH) is considered alternative mediator of S-glutathionylation (Konorev et al. 2000; Tao and English 2004; Giustarini et al. 2005).

Although the disulfide bond linking the protein and glutathione is readily reversible under reducing conditions, under oxidizing conditions the S-glutathionylation can be maintained indefinitely in persistently glutathionylated proteins (Gilbert 1995; Beer et al. 2004). However, in many situations protein S-glutathionylation is only transient, as an adjacent protein thiol displaces the glutathione moiety to form an intraprotein disulfide (Gilbert 1995). Thus, there are two major classes of S-glutathionylated proteins: those that are transiently glutathionylated before protein disulfide formation, and those that are persistently glutathionylated (Beer et al. 2004).

S-glutathionylation has been implicated in buffering of oxidative stress, stabilization of extracellular proteins, protection of proteins against irreversible oxidation of critical cysteine residues, and regulation of enzyme activity (Gilbert 1984; Ziegler 1985; Thomas et al. 1995; Cotgreave and Gerdes 1998).

- **S-cysteinylation**

The intracellular concentrations of free cysteine and cystine are much lower than those of GSH, whereas extracellular free cysteine is more abundant than GSH. Actually, the cysteine/cystine redox couple quantitatively represents the largest pool of low-molecular-mass thiols and disulfides in plasma and in the extracellular compartment on the whole. The major low-molecular-mass sulfhydryl/disulfide system in cells, GSH/GSSG, is principally in the reduced form, whereas the major low-molecular-mass system in the extracellular compartment, cysteine/cystine, is principally in the disulfide form, cystine (Giustarini et al. 2009). Thus, extracellular proteins may be prevalently S-cysteinylated, whereas intracellular proteins may be prevalently S-glutathionylated. For example, whereas the fraction of S-thiolated hemoglobin in red blood cells is only S-glutathionylated (Sampathkumar et al. 2005), plasma proteins such as albumin are mainly S-cysteinylated, possibly after formation of an intermediate sulfenic acid (Carballal et al. 2003; Carballal et al. 2007).

Although much is known about S-glutathionylation and there are numerous studies on it, there is still much to learn about the role of S-cysteinylation. In fact, most of S-cysteinylation studies have been performed *in vitro* (Hochgrafe et al. 2007).

2.1.3.2.4 Other thiol modification: S-nitrosylation

S-nitrosylation is a post-translational modification of protein that has emerged as an important signaling pathway related to nitric oxide (NO) production, with some particularities in terms of specificity (Hernansanz-Agustin et al. 2013). It is a reversible modification that consists in the formation of a nitrosothiol (R-SNO) at a protein cysteine thiol (R-SH) by several chemical mechanisms (Hernansanz-Agustin et al. 2013).

NO itself is rarely a direct S-nitrosylating agent, most of these mechanisms proceed through formation of reactive nitrogen species (RNS), including higher nitrogen oxides formed by the reaction of NO and O_2 (Martinez-Ruiz and Lamas 2004; Hernansanz-Agustin et al. 2013). Among them, N_2O_3 is considered to be the main nitrosylating agent (Martinez-Ruiz and Lamas 2004).

Not all cysteines in a protein can be S-nitrosylated. S-nitrosylation depends on the chemical reactivity between the nitrosylating agent and the target (Martinez-Ruiz and Lamas 2004). Cysteines that are surrounded by a particular amino acid motif are likely candidates for this modification (Hess et al. 2005). Thus, the cysteine environment is what determines its reactivity (Martinez-Ruiz and Lamas 2004). A specific modification by S-nitrosylation typically affects protein activity, thus mediating NO signaling pathways (Choi et al. 2000; Uehara et al. 2006). Proteins also can be denitrosylated by redox-sensitive enzymes such as the thioredoxin and S-nitroso-glutathione reductase systems, PDI, or alcohol dehydrogenase (ADH) class III (Benhar et al. 2009).

S-nitrosylation has been regarded as functionally equivalent to protein phosphorylation or dephosphorylation as it might represent a post-translational modification highly relevant for intracellular signaling (Wang et al. 2006; Akhtar et al. 2012). But S-nitrosylation is also potentially different from phosphorylation in that nitrosylation may not involve a delicate network consisting of kinases or enzymes that catalyze, respectively, nitrosylation and denitrosylation. In addition, some authors propose S-nitrosylation as a pathway to transport NO (Pawloski et al. 2001) and potentially as a reservoir mechanism. Nonetheless, S-nitrosylation has been demonstrated to be a key modification of cysteine residues under a variety of physiological and pathophysiological conditions (Foster et al. 2009; Yan et al. 2012; Haldar and Stamler 2013). In particular, in connection with nitric oxide-based redox regulation of protein function, S-nitrosylation has been found to be involved in protective mechanisms in many disorders (Sun et al. 2007; Foster et al. 2009; Murphy et al. 2012).

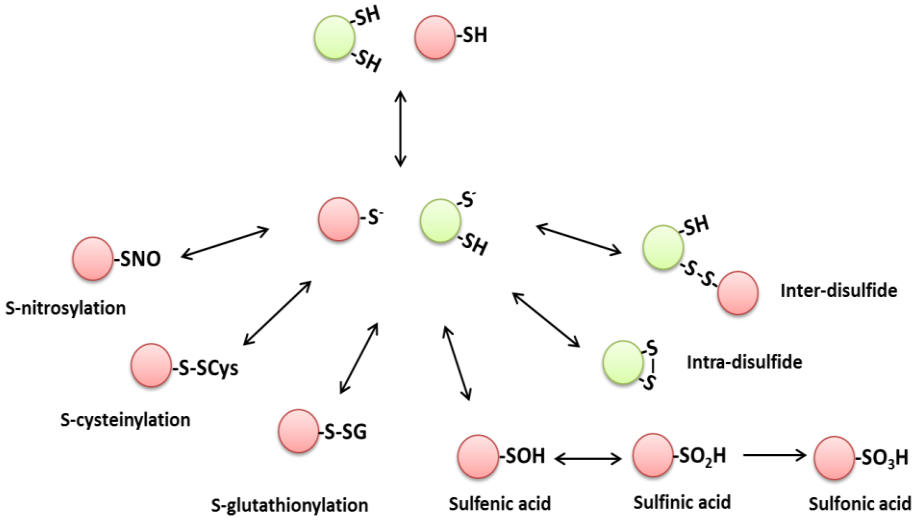


Figure 6. Oxidative modifications of protein cysteines

3. REDOX SIGNALING

Oxidant species are known to contribute to disease and dysfunction in biological systems. However, evidence has been progressively accumulating that demonstrates a more fundamental role for many oxidant species in the regulation of everyday function of healthy cells. How oxidants are viewed in health and disease has changed over the past decade. Oxidants were once almost exclusively considered harmful entities that cause damage and dysfunction. However, it is now understood that oxidants are produced in healthy tissue, with regulated control of their production (see in figure 5). In addition, there is now considerable evidence that oxidants themselves can in fact serve as signaling molecules (Hess et al. 2005).

The reaction of oxidants with biomolecules is the molecular basis for sensing alterations in the cellular redox state. The transduction of an oxidant signal into a biological response can be mediated in several ways, but one principal mechanism involves the oxidation of protein cysteine residues. For specific and transient signal transduction, cysteine modifications have to be reversible and target specific (Spadaro et al. 2010). The thiol (-SH) moiety on the side chain of the amino acid cysteine is particularly sensitive to redox reactions and is an established redox sensor. Thiols can interact with a variety of oxidants to form in many cases a reversible covalent modification. These reversible oxidative modifications, which include disulfide bond formation, are a major mechanism by which protein function can be controlled (Eaton 2006). Redox dependent signaling events involving the post-translational oxidative modification of proteins have now been accepted as an important regulatory process. Modification of redox-sensitive amino acids as means to regulate protein function may be comparable to other post-translational modifications

such as phosphorylation (Finkel 1998). The thiol functional group of cysteine residues of proteins can act as a ROS sensor (Spadaro et al. 2010; Antelmann and Helmann 2011).

Not all cysteinyl thiols are important as redox sensors, as most protein thiols do not react with oxidants under the conditions and at the concentrations they are found in cells. However, some thiols ionise to the thiolate anion state more readily (i.e., those with a low pKa) because their surrounding environment promotes it. These thiols have enhanced reactivity for many oxidants, and this provides a basis for specificity in thiol-mediated redox signaling (Eaton 2006). A common feature of redox-reactive cysteines is that they are often adjacent to or in proximity of a polar amino acid (Salsbury et al. 2008). Polar amino acids have been suggested to lower the pKa of the cysteine thiol group, resulting in stabilization of the thiolate anion form, which is more reactive than the thiol form. The structural microenvironment may be more important for cysteine reactivity than a specific consensus sequence (Ascenzi et al. 2000). The amino acids that are relevant for influencing cysteine reactivity may not necessarily be directly adjacent to cysteine in the amino acid sequence (Ascenzi et al. 2000).

Reactive thiols often form transient catalytic intermediates in the reaction cycle of many enzymes (Eaton et al. 2002). This often means that the most important protein thiols in terms of function are those susceptible to redox-dependent modification. This allows oxidants to alter the activity of some proteins by modifying the redox state of functionally essential thiols, and serves as a simple signal transduction mechanism, which couples protein redox state directly to functional activity. Redox alterations can integrate more widely into cell signaling by changing the activity of kinases and phosphatases. This

may be achieved in the simplest form by direct modification of kinases or phosphatases by oxidants (Groen et al. 2005; Salmeen and Barford 2005), or redox alterations of upstream effector proteins such as receptors (Nishida et al. 2000).

For precise signaling control, regulated generation of ROS would be required possibly in specific locations within the cytoplasm (Stone and Yang 2006; Genereux et al. 2010). Specific local redox environments may also be achieved through compartmentalization of ROS in distinct organelles and through localized ROS production in specific subcellular compartments (Pani et al. 2001; Go and Jones 2008).

Important cellular processes that involve regulation by redox mechanisms include apoptosis (Circu and Aw 2010), angiogenesis (Maulik 2002) and cell cycle control. Cysteine modification represents a direct mechanism for sensing of changes in the redox potential.

3.1 ROS AS SECOND MESSENGERS

A crucial feature of a second messenger is having specificity in its interaction with effectors in signaling pathways. The specificity of signaling by ROS is defined by both kinetics and spatial relationships.

Superoxide oxidizes thiol to thiyl radical, which can initiate a chain reaction, but the rate constant for this reaction is quite slow. Inside the cell, $O_2^{\bullet-}$ would rapidly suffers dismutation to H_2O_2 and O_2. Therefore, although a role for $O_2^{\bullet-}$ in signaling has been proven, targets that react with $O_2^{\bullet-}$ have not

been demonstrated *in vivo*. Thus, the signaling role of $O_2^{\bullet-}$ is more likely as a precursor of H_2O_2.

Hydroxyl radical has also been thought to be involved in signaling (Bauer 2002). However, HO• has no specificity as it reacts with almost any organic molecule with rate constants near the limit of diffusion. Some of the products of lipid peroxidation (generated by HO•) can signal through receptors (Mohler et al. 1996), while others can participate in signaling by modifying signaling proteins, such as Keap1 (Levonen et al. 2004) or SHP-1, a protein tyrosine phosphatase (Rinna and Forman 2008). However, this is not evidence of a direct role of HO• as a second messenger (Forman et al. 2010).

H_2O_2 has all the properties that are required for a second messenger: it is enzymatically produced and degraded, and it specifically reacts with thiols that are prone to oxidation (Forman et al. 2010).

4. ANTIOXIDANT RESPONSES TO OXIDATIVE STRESS

In order to prevent oxidative stress, cells must respond to ROS by mounting an antioxidant defense system. Antioxidant enzymes play a major role in reducing ROS levels; thus, redox regulation of transcription factors via redox effector factor 1 (REF-1) and NRF2 pathways are significant in determining gene expression profile and cellular response to oxidative stress.

4.1 REF-1 PATHWAY

Redox effector factor-1 (REF-1) was shown to be identical to the apurinic/apyrimidinic (AP)-endonuclease 1 (APE-1) (Demple et al. 1991). Thus,

REF-1/APE-1 is a multifunctional protein that not only regulates transcription factor activity, but also mediates base excision repair. The N-terminal region of REF-1/APE-1 is redox sensitive while the AP-endonuclease activity domain is located at the C-terminal region (Martinez-Ruiz and Lamas 2007). Cys-65 of REF-1 appears to be a major redox active site (along with Cys-93) that is required for reduction and increased DNA binding of the associated transcription factors (Walker et al. 1993).

The redox status of reactive Cys residue, located within the DNA-binding domain of some transcription factors, may control their transcriptional activity. REF-1 (henceforth we will use this term to name it) has been identified as a protein with nuclear redox activity capable of inducing the DNA binding activity of several transcription factors, such as activator protein 1 (AP-1), p53, nuclear factor kappa B (NF-ĸB), and hypoxia inducible factor 1 (HIF-1α) (Tell et al. 2009). In every case, REF-1 maintains the cysteine residues of the transcription factors in the reduced state (the active form).

Under oxidative stress, REF-1 translocates into the nucleus by nuclear importins (figure 7). There, REF-1 upregulates key transcription factors involved in cellular defense. Oxidized REF-1 is then subjected to redox regulation by nuclear translocated thioredoxin through the Trx catalytic center (Cys-32 and Cys-35). Thioredoxin was shown to translocate into the nucleus and interact with Ref-1 (figure 7). The interaction of thioredoxin with REF-1 and the subsequent activation of REF-1 target proteins appear to be regulated by the redox active Cys-32 and Cys-35 residues of thioredoxin (Kensler et al. 2007; Shimizu and Hendershot 2009; Baird and Dinkova-Kostova 2011) which are responsible for its reducing activity (Xiao et al. 2004).

REF-1 was shown to be upregulated by genotoxic agents and oxidants, such as H_2O_2, and so protects cells from DNA oxidative damage (Foster et al. 2003).

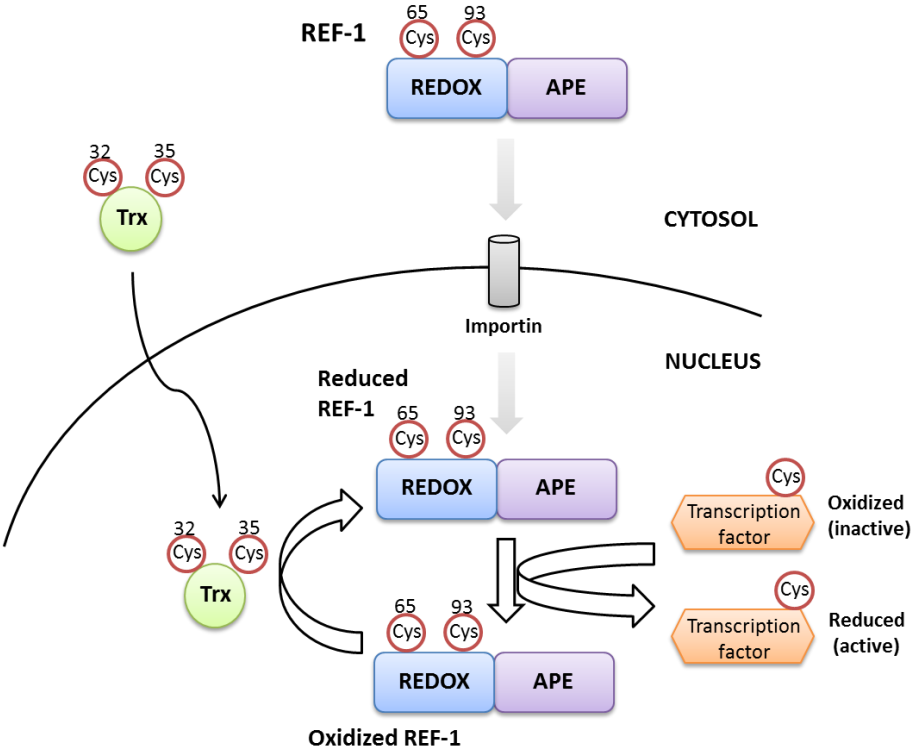


Figure 7. REF-1-mediated redox cell signaling

4.2 NRF2 PATHWAY

Under oxidative stress conditions, a specific group of antioxidant detoxification genes is transcriptionally activated in an AP-1/NF-kB-independent manner. These antioxidant genes are regulated by a highly

homologous enhancer termed the antioxidant/electrophile response element (ARE/EpRE).

The primary transcription factor involved in ARE activation under oxidative stress conditions is NRF2 (Itoh et al. 1999). Under non-stressed conditions, the majority of NRF2 resides in the cytoplasm and associates with an inhibitory protein, Kelch-like ECH-associated protein-1 (KEAP1) (Itoh et al. 1999) KEAP1 interacts with the cullin-3 E3-ubiquitin ligase (Cul3) and serves as a platform for the ubiquitination and resultant proteasomal degradation of NRF2 (figure 8) (Furukawa and Xiong 2005; Villeneuve et al. 2010). Reactive KEAP1 cysteines (Dinkova-Kostova et al. 2002; Zhang and Hannink 2003) are redox sensors, and upon oxidation by ROS, results in the dissociation of NRF2 from KEAP1/Cul3, which allows NRF2 stabilization and translocation into the nucleus (figure 8). In the nucleus NRF2 dimerizes with small Maf proteins. The NRF2-Maf heterodimer binds the ARE enhancer and activates ARE-dependent transcription of target genes that serve as enzymatic antioxidants in processes such as electrophile detoxification, glutathione synthesis, and ROS homeostasis (figure 8). The antioxidant pathway regulated by NRF2 is summarized in figure 4.

Major signaling pathways affect the posttranscriptional regulation of ARE-responsive genes by interacting with the KEAP1/NRF2 complex and include the MAPK (mitogen activated protein kinase) cascade, phosphatidylinositol 3-kinase, and protein kinase C (Kensler et al. 2007). Phosphorylation of NRF2 facilitates its dissociation from KEAP1 (figure 8). While phosphorylation is required for NRF2 release from Keap1, it is not necessary for NRF2 stabilization/accumulation in the nucleus (Kensler et al. 2007). Interestingly, studies conducted *in vitro* indicate that the affinity of NRF2 heterodimeric

complexes with Mafs to ARE sequences is similar regardless of the phosphorylation state of NRF2. NRF2 is a substrate of protein kinases associated with the MAPK/ERK signaling cascade (Zipper and Mulcahy 2000).

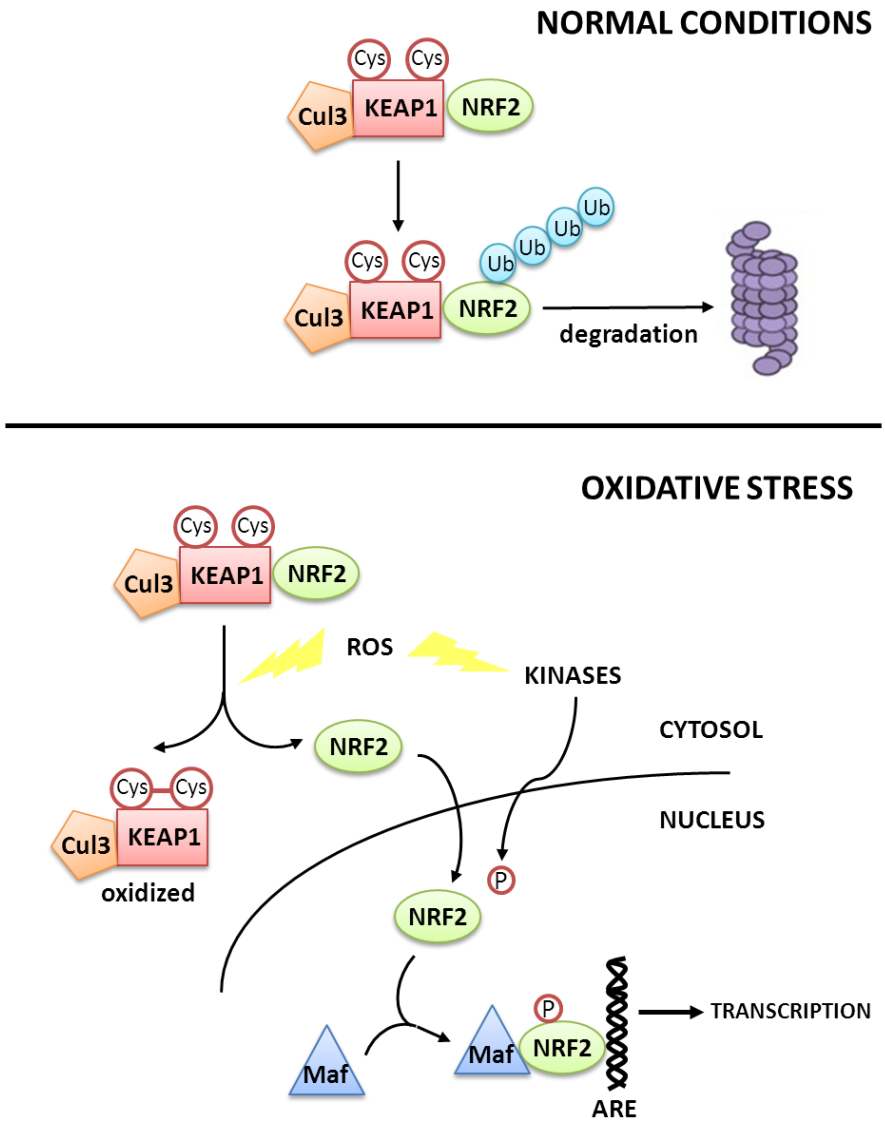


Figure 8. Redox regulation of the NRF2-ARE pathway

5. INFLAMMATORY SIGNALING PATHWAYS

The inflammatory response can be mediated by different signaling pathways, such as the nuclear factor Kappa-B (NF-ƙB) pathway, the janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway, the phosphatidylinositide 3-kinases (PI3K)/AKT pathway, and the mitogen-activated protein kinase (MAPK) pathway.

Nuclear factor Kappa-B (NF-ƙB) is a protein complex that controls transcription of DNA. NF-ƙB is found in almost all animal cell types and is involved in cellular responses to stimuli such as stress, cytokines, free radicals, ultraviolet irradiation and bacterial or viral antigens (Gilmore 1999; Brasier 2006; Gilmore 2006; Perkins 2007). In brief, NF-ƙB is a protein responsible for cytokine production and cell survival. In unstimulated cells, the NF-ƙB dimers are sequestered in the cytoplasm by a family of inhibitors, called IƙBs (inhibitor of ƙB). Activation of the NF-ƙB is initiated by the signal-induced degradation of IƙB proteins.

The JAK-STAT system consists of three main components: a receptor, Janus kinase (JAK) and the signal transducer and activator of transcription (STAT). Cell signaling through the JAK/STAT pathway plays a critical role in the function of cytokines, growth factors, and hormones (Casanova et al. 2012; Ashino et al. 2013). The JAK family members are activated after receptor ligation, which leads to phosphorylation of the STATs, an event central to DNA transcription and cell activation (Levy and Darnell 2002; Ghoreschi et al. 2009).

PI3K/AKT pathway is an intracellular signalling pathway important in apoptosis and cancer (Cortot et al. 2006; LoPiccolo et al. 2008; Yap et al.

2008). It is activated by IGF-1 (insulin-like growth factor-1) and has a number of downstream effects. PI3K stimulation activates AKT which activates mTOR (mammalian target of rapamycin). It has been reported that PI3K/AKT signaling pathway is as a key regulator of cell cycle proliferation, growth, survival, protein synthesis, and glucose metabolism (Yap et al. 2008).

In this thesis, we will focus on the MAPK signaling pathway due to its major role in cell response to stress.

5.1 MITOGEN-ACTIVATED PROTEIN KINASES (MAPKs)

Mitogen-activated protein kinases (MAPKs) are protein Ser/Thr protein kinases that convert extracellular stimuli (including oxidative stress) into a wide range of cellular responses. MAPKs are among the most ancient signal transduction pathways and are widely used throughout evolution in many physiological processes (Widmann et al. 1999). All eukaryotic cells possess multiple MAPK pathways, which coordinately regulate gene expression, mitosis, metabolism, cell motility, survival, apoptosis, and differentiation. Conventional MAPKs comprise the extracellular signal-regulated kinases 1/2 (ERK1/2), c-Jun amino (N)-terminal kinases (JNK) and p38 (Chen et al. 2001; Kyriakis and Avruch 2001; Pearson et al. 2001).

In recent years, it has become clear that multiple parallel mammalian MAPK pathways exist and most of these are pivotal to stress and inflammatory responses in addition to mitogen responses. As mammalian stress-activated signaling pathways have been elucidated, it is becoming evident that these pathways will be important targets for novel anti-inflammatory drugs (Kyriakis and Avruch 2001).

Each group of conventional MAPKs cascade is composed of a set of three evolutionarily conserved, sequentially acting kinases: a MAPK, a MAPK kinase (MAPKK), and a MAPKK kinase (MAPKKK) (figure 9).

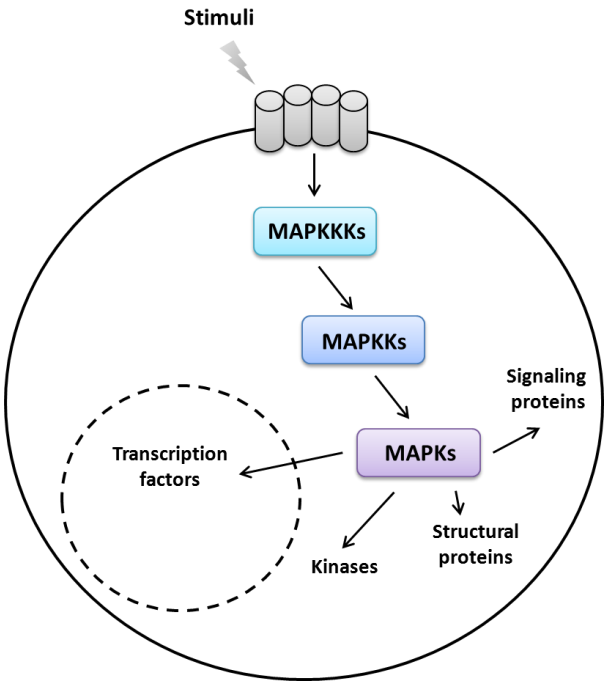


Figure 9. General scheme of the MAP kinase pathways

The MAPKKKs, which are protein Ser/Thr kinases, are often activated through phosphorylation and/or as a result of their interaction with small GTP-binding proteins of the Ras/Rho family in response to extracellular stimuli. MAPKKK activation leads to phosphorylation and activation of a MAPKK, which then stimulates MAPK activity through dual phosphorylation on Thr and Tyr residues within a conserved Thr-X-Tyr motif located in the activation loop of the kinase subdomain VIII (Cargnello and Roux 2011). Phosphorylation of these residues is essential for enzymatic activities.

5.1.1 ERK 1/2

ERK1 was the first mammalian MAPK to be cloned and characterized. It was originally found to be phosphorylated on Tyr and Thr residues in response to growth factors (Cooper et al. 1982; Kazlauskas and Cooper 1988; Ray and Sturgill 1988). ERK1 and ERK2 are isoforms of 44 and 42 KDa, respectively. ERK1 and ERK2 share 83% amino acid identity and are expressed to various extents in all tissues, with particularly high levels in the brain, skeletal muscle, thymus, and heart (Boulton et al. 1990).

ERK1 and ERK2 are activated by growth factors, including platelet-derived growth factor, epidermal growth factor, and nerve growth factor, and in response to insulin (Boulton et al. 1990). They are also activated by ligands for heterotrimeric G protein-coupled receptors, cytokines, and microtubule disorganization (Raman et al. 2007).

The mammalian ERK1/2 pathway consists of the MAPKKKs A-Raf, B-Raf, and C-Raf, the MAPKKs MEK1 and MEK2, and the MAPKs ERK1 and ERK2 (figure 10) (Raman et al. 2007; Shaul and Seger 2007).

In quiescent cells, all components of the ERK1/2 pathway have a cytoplasmic localization, but upon extracellular stimulation a significant proportion of ERK1/2 accumulates in the nucleus (Chen et al. 1992; Lenormand et al. 1993). Upon stimulation, ERK1/2 phosphorylates a large number of substrates (Yoon and Seger 2006). Some of these are localized in the cytoplasm and others in the nucleus. The ERK1/2 module plays a central role in the control of cell proliferation. ERK1/2 activity is rapidly stimulated by mitogenic agents. ERK1/2 controls cell proliferation via several mechanisms, including the

induction of positive regulators of the cell cycle (Meloche and Pouyssegur 2007).

5.1.2 JNK

There are three known JNK isoforms, JNK1, JNK2 and JNK3. JNK isoforms are strongly activated in response to various cellular stresses, including heat shock, ionizing radiation, oxidative stress, DNA-damaging agents, cytokines, UV irradiation, DNA and protein synthesis inhibitors, and growth factor deprivation (Bogoyevitch et al. 2010). Activation of JNK isoforms requires dual phosphorylation on Thr and Tyr residues. The MAPKKs that catalyze this reaction are known as MKK4 and MKK7, which appear to cooperate in the phosphorylation and activation of the JNKs (Lawler et al. 1998). MKK4 and MKK7 are phosphorylated and activated by several MAPKKKs, including MEKK1 to 4, MLK1 to 3 and ASK1/2 (figure 10) (Kyriakis and Avruch 2001; Wagner and Nebreda 2009).

A proportion of activated JNKs have been shown to relocalize from the cytoplasm to the nucleus following stimulation (Mizukami et al. 1997). The transcription factor c-Jun is a well-described substrate for JNKs, as its phosphorylation on N-terminal end was found to increase c-Jun-dependent transcription (Weston and Davis 2002). JNK1 and JNK2 have been shown to play important roles in the control of cell proliferation and in the apoptotic and inflammatory responses to cellular stresses (Dhanasekaran and Reddy 2008).

5.1.3 p38

p38 is a MAPK that is generally responsive to stress stimuli (Han et al. 1994; Lee et al. 1994). Since identification of p38α, three additional isoforms have been found, p38β, p38γ and p38δ (Cuadrado and Nebreda 2010). Whereas p38α and p38β are ubiquitously expressed in cell lineages and tissues, p38γ and p38δ have more restricted expression patterns and may have specialized functions (Jiang et al. 1996). p38α is generally the most highly expressed.

In mammalian cells, the four p38 isoforms are strongly activated by various environmental stresses, including oxidative stress, UV irradiation, hypoxia, ischemia, and inflammatory cytokines, such as interleukin-1 (IL-1), and tumor necrosis factor alpha (TNF-α) (Cuadrado and Nebreda 2010). MKK3 and MKK6 are thought to be the major protein kinases responsible for p38 activation (Derijard et al. 1995; Han et al. 1996), but MKK4 has also been shown to possess some activity toward p38 (Meier et al. 1996). MKK3 and MKK6 are activated by a plethora of MAPKKKs, including MEKK1 to 3, MLK2/3, and TAK1 (figure 10) (Cuadrado and Nebreda 2010). Most stimuli that activate p38 MAPKs also stimulate JNK isoforms, and many MAPKKKs of p38 are shared by JNK.

Like ERK1/2 and JNK MAPKs, p38 isoforms are present in the nuclei and cytoplasm of quiescent cells (Ben-Levy et al. 1998) and have been shown to accumulate in the nuclei of cells subjected to certain stresses (Raingeaud et al. 1995). Upon stimulation, p38 isoforms phosphorylate a large number of substrates in many cellular compartments, including the cytoplasm and the nucleus. p38 plays a critical role in inflammatory responses (Cuadrado and

Nebreda 2010). A major function of p38 isoforms is the production of pro-inflammatory cytokines. p38 isoforms can regulate cytokine expression by modulating transcription factors, such as NF-ƙB (Karin 2006). In addition, they are activated by numerous extracellular mediators of inflammation. p38 MAPKs has also been shown to play roles in cell proliferation and survival. p38 induces apoptosis by cellular stresses and negatively regulates cell cycle progression (Cargnello and Roux 2011).

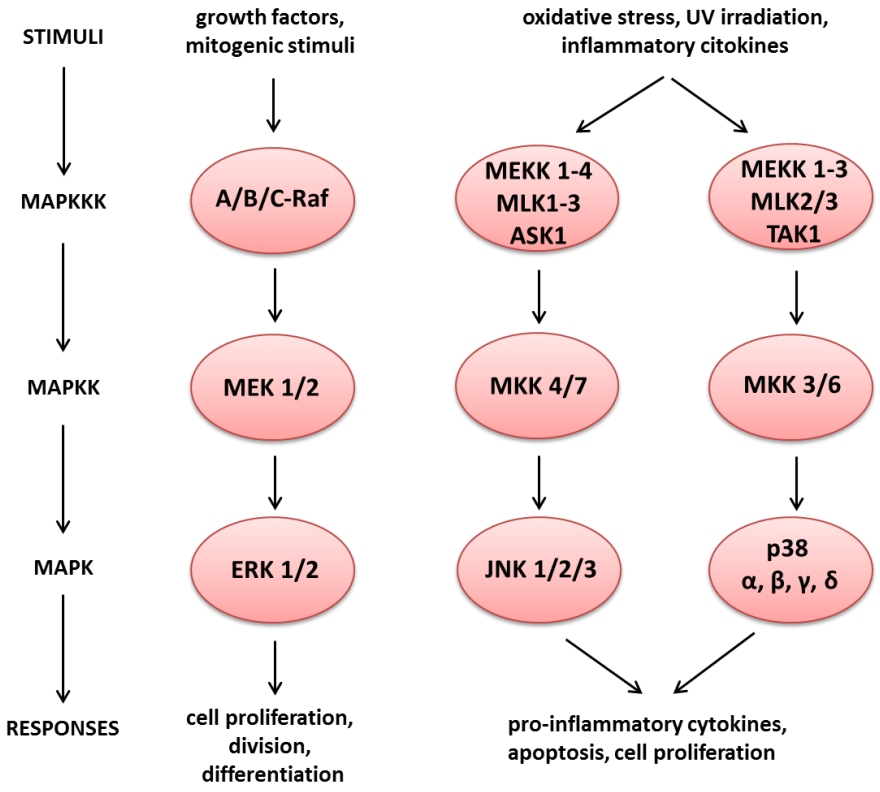


Figure 10. Simplified overview of MAPK cascades

5.2 PHOSPHATASES

MAPKs are important players in signal transduction pathways activated by a range of stimuli and mediate a number of physiological and pathological changes in cell function. This process is reversible even in the continued presence of activating stimuli, indicating that protein phosphatases provide an important mechanism for MAPK control and down-regulation of the inflammatory response (Camps et al. 2000).

Decades of research have shown that reversible phosphorylation of proteins, executed by kinases and phosphatases (figure 11), constitutes a major form of signaling and an essential mechanism of regulation in all living organisms. In eukaryotic cells, phosphorylation mainly occurs on three hydroxyl-containing amino acids, serine, threonine, and tyrosine.

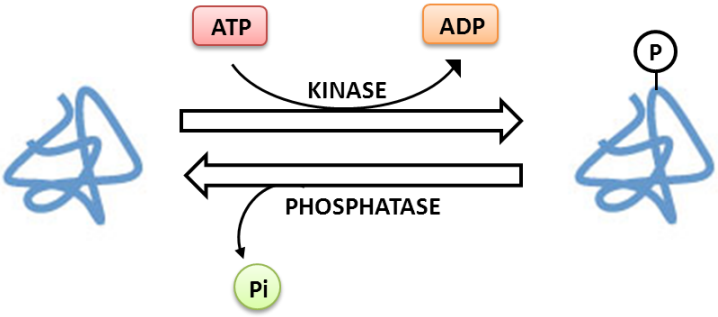


Figure 11. Phosphorylation and dephosphorylation of proteins

The fully sequenced human genome is thought to contain 518 protein kinases (human kinome). The exquisite specificity of signaling and the reversible nature of phosphorylation seem to suggest that there would be a

similar number of protein phosphatases in the human genome. Surprisingly, however, there are only 140 protein phosphatases (human phosphatome) indicating that dephosphorylation is more active than phosphorylation under basal conditions (Lander et al. 2001; Venter et al. 2001; Johnson and Hunter 2005).

Protein phosphatases comprise two major families: tyrosine phosphatases (PTPs) and serine/threonine phosphatases (PPPs). Phosphatases are also distinguished by molecules which inhibit their action. For example, sodium fluoride (NaF) is a specific inhibitor of serine/threonine phosphatases (Oliver and Shenolikar 1998), while vanadate is a specific inhibitor of tyrosine phosphatases (Burke and Zhang 1998).

5.2.1 SERINE/THREONINE PHOSPHATASES

This group dephosphorylates phosphothreonine/phosphoserine residues. Consists of four types of proteins that are classified into two families: PPPs formed by PP1, PP2A and PP2B (also known as calcineurin), and the PPM formed by PP2C. PP1, PP2A, and PP2B are formed by association of several subunits while PP2C is a monomeric protein and, in contrast to PPPs, do not have regulatory subunits. Also it differs in substrate preference, the need for metal cations and sensitivity to different inhibitors (Cohen 1989). PPPs are involved in important processes such as regulation of cell cycle, as well as cell growth and division (Wang et al. 2008).

5.2.1.1 Protein phosphatase 1

PP1 is a major protein Ser/Thr phosphatase and is ubiquitously expressed in all eukaryotic cells. PP1 participates prominently in a wide range of cellular processes, including meiosis and cell division, apoptosis, protein synthesis, metabolism, cytoskeletal reorganization, and the regulation of membrane receptors and channels (Cohen 2002; Ceulemans and Bollen 2004). PP1 needs the presence of manganese to be activated. It is the most active PPPs in cell extracts (Wang et al. 2008). Each functional PP1 enzyme consists of a catalytic subunit and a regulatory subunit (Shi 2009). PP1 shares with PP2A its specific inhibitors such as NaF, microcystin and okadaic acid (Campos et al. 1996) but in contrast to PP2A, PP1 can be specifically inhibited by tautomycetin (Mitsuhashi et al. 2003). The phosphatase activity of PP1 is also regulated by a number of endogenous inhibitory proteins such as inhibitor-1 (Nimmo and Cohen 1978) and inhibitor-2 (Foulkes and Cohen 1980).

5.2.1.2 Protein phosphatases 2

Protein phosphatases 2 differ from PP1 because PP2 don’t respond to inhibitors 1 and 2. The three types of PP2 are differentiated by the divalent cations that require.

5.2.1.2.1 PP2A

PP2A is a family of serine-threonine phosphatases highly conserved and ubiquitously expressed, implicated in the control of diverse cellular processes through the negative regulation of signaling pathways initiated by protein kinases.

The structure of PP2A has been extensively studied. PP2A is a heterotrimer consisting of a 36 KDa catalytic subunit (PP2Ac), a 65 KDa structural subunit (PP2Aa), and a regulatory subunit (PP2Ab) which can vary in size from 50 to 130 kDa. The a and c subunits are ubiquitously expressed and form a catalytic complex that interacts with at least three families of regulatory subunits (b55, b56, and PR72/130).

PP2A regulates a diverse set of cellular processes, such as metabolism, transcription, RNA splicing, translation, DNA replication, cell cycle progression, signal transduction, differentiation, and oncogenic transformation by reversing the actions of protein kinases (Nishida et al. 2000; Eaton et al. 2002; Hess et al. 2005; Wang et al. 2006). In recent years, protein kinases have emerged as an important group of substrates for PP2A. Most of the MAPKs are inactivated by dephosphorylation. PP2A can dephosphorylate and inactivate MEK1 and ERK-family kinases, therefore, inhibiting mitogenic signals (Groen et al. 2005). Other PP2A substrates involved in pro-survival signals are proteins integrated in the signal-dependent activation of the NF-ĸB pathway, which indeed regulates the expression of multiple genes involved in the control of cell growth, division, and survival (Salmeen and Barford 2005). By altering the functions of proteins involved in mitogenic and apoptotic signaling pathways, PP2A is considered to be a tumour suppressor, and many observations support a role for PP2A in tumorigenesis (Groen et al. 2005).

PP2A activity has been shown to be inhibited by oxidative stress (Finkel 1998; Spadaro et al. 2010; Antelmann and Helmann 2011). The inhibition of PP2A activity by oxidative stress in neurons and in other *in vitro* model systems is reversed by disulfide-reducing agents (Salsbury et al. 2008; Spadaro et al. 2010; Antelmann and Helmann 2011) supporting the hypothesis that inhibition

of the phosphatase activity results from reversible oxidation of one or more cysteine residues of PP2A to a disulfide form. $PP2A_C$ contains 10 cysteine residues including a vicinal pair at positions 266 and 269 (Ascenzi et al. 2000; Genereux et al. 2010). Notably, this CXXC motif of $PP2A_C$ is shared by well-established redox-active proteins including thiol-disulfide oxidoreductases of the PDI family, and, theoretically, is capable of undergoing oxidation to form an intramolecular disulfide bond (Stone and Yang 2006). Thus, it is conceivable that $PP2A_C$ may form intermolecular and/or intramolecular disulfide bonds under oxidative stress.

5.2.1.2.2 PP2B

PP2B (also known calcineurin) plays an important role in numerous calcium-dependent biological processes, including neurodevelopment and memory, immune response, cardiac hypertrophy, signal transduction, and muscle development (Rusnak and Mertz 2000). Calcineurin consists of a catalytic subunit and a regulatory subunit. Calcineurin is inactive alone and only gains phosphatase activity upon association with Ca^{2+}-calmodulin (Shi 2009).

5.2.1.2.3 PP2C

PP2C belong to the Mn^{2+}/Mg^{2+}-dependent PPM family. In contrast to the PPP family phosphatases, PP2C is insensitive to inhibition by okadaic acid or microcystin. PP2C represents a large family of highly conserved protein phosphatases, with 16 distinct PP2C genes in the human genome that give rise to at least 22 different isoforms (Lammers and Lavi 2007). The primary function of PP2C appears to be the regulation of stress signaling, although it also plays a

role in cell differentiation, growth, survival, apoptosis, and metabolism (Lu and Wang 2008).

5.2.2 TYROSINE PHOSPHATASES

Protein tyrosine phosphatases (PTPs) dephosphorylate phosphotyrosine residues. PTPs are both membrane and cytoplasmic located. Although tyrosine phosphorylation occurs to a lesser extent than serine/threonine phosphorylation, it has become clear that tyrosine phosphorylation is essential in controlling normal cellular growth survival, differentiation, metabolism, cell cycle, cell-cell communications, cell migration, gene transcription, ion channels, and the immune response (Hunter 2000). Abnormal tyrosine phosphorylation is associated with many human diseases including cancer, diabetes, rheumatoid arthritis, and hypertension (Zhang 2002).

Tyrosine phosphatases play an important role in the regulation of the inflammatory cascade through NF-ƙB, MAPKs, and TNF-α (de Dios et al. 2006; Chong and Maiese 2007; Escobar et al. 2010).

5.2.2.1 Cytoplasmic tyrosine phosphatases

Cytoplasmic PTPs, also called soluble or non-receptor PTPs, have a modular organization. In addition to the highly conserved catalytic domain, they contain non-catalytic regions or domains that play a role in subcellular targeting, in regulation of the enzymatic activity, or in recruiting specific ligands (Alonso et al. 2004).

Within this group it must be highlighted PTP1B, which is localized in the cytoplasm but migrates to the endoplasmic reticulum (Frangioni et al. 1992) and is involved in the negative regulation of MAPK, in the metabolism of glucose, insulin resistance, and weight control, the latter probably negatively regulating the leptin pathway (Koren and Fantus 2007). Others soluble PTPs that should be highlighted are SHP1 (Src homology 1) and SHP2 (Src homology 2); although the latter is structurally similar to the SHP-1 it differs in substrate specificity (Xu et al. 2002). SHP-1, SHP-2 and PTP1B are overexpressed early on during the development of cerulein-induced acute pancreatitis in rats, and their levels can be modulated by some species of MAPKs (Garcia-Hernandez et al. 2013). SHP-1 and SHP-2 are known to govern a host of cellular functions. They modulate progenitor cell development, cellular growth, tissue inflammation, and cellular chemotaxis, but more recently the role of SHP-1 and SHP-2 to directly control cell survival upon oxidative stress has come to light. SHP-1 and SHP-2 modulate cellular signals that involve PI3K/AKT, JAK/STAT, MAPKs and NF-ƙB (Chong and Maiese 2007).

Finally other PTPs are PTPC and PTEN. PTP1C is located in the platelets and it has a high homology with SHP-2 (Catalan et al. 1997). PTEN prevents uncontrolled cell growth that can lead to the formation of tumors.

5.2.2.2 Membrane tyrosine phosphatases

Twenty-one of the 38 classical PTPs identified in the human genome (Alonso et al. 2004) are type I membrane proteins and, because of their domain organization, were termed “receptor-like” PTPs (RPTPs) well before any ligands had been identified (Hunter 1989). Typically, a RPTP has an N-terminal extracellular region (lengths vary from 100 to > 1000 residues), a single

transmembrane region, and one or two intracellular catalytic domains highly conserved within the family and with other classical PTPs (Tonks 2006). Given this architecture, RPTPs appear ideally built to transduce signals across the plasma membrane, triggered by ligands binding to the extracellular region.

5.2.3 DUAL SPECIFICITY PHOSPHATASES

Dual specificity phosphatases (DSPs), also called MAPK phosphatases (MKPs), are an emerging subclass of the protein tyrosine phosphatase gene superfamily, which appears to be selective for dephosphorylating critical phosphothreonine and phosphotyrosine residues within MAPKs. Some DSPs are localized in different subcellular compartments and moreover, certain family members appear highly selective for inactivating distinct MAPK isoforms. This enzymatic specificity is due in part to powerful catalytic activation of the DSP phosphatase after tight binding of its amino-terminal to the target MAPK. DSP gene expression is induced strongly by various growth factors and/or cellular stresses, providing a sophisticated transcriptional mechanism for targeted inactivation of selected MAPK activities (Camps et al. 2000).

5.3 CYTOKINES

Cytokines are proteins released primarily by cells of the immune system which act as nonspecific intercellular signals, whereby certain cells can attract and activate others propagating and amplifying the inflammatory response (Gullberg 2008; Medzhitov 2008). Cytokines are formed in response to a stimulus; its secretion is brief and limited and often acts locally or in the vicinity of cells within a tissue. Its synthesis is activated by up-regulation of gene expression (Lin et al. 2000).

Most cytokine genes are not expressed (at least at the translational level) unless specifically stimulated by noxious events. In fact, the triggering of cytokine gene expression is nearly identical to “cell stressors”. For example, ultraviolet light, heat-shock, oxidative stress, hyperosmolarity or adherences to a foreign surface activate MAPKs, which phosphorylate transcription factors to trigger gene expression. Of course, infection and inflammatory products also activate the MAPK pathway for inducing cytokine gene expression (Dinarello 2000).

Cytokines bind to specific cellular receptors that result in activation of intracellular signaling pathways that regulate gene transcription (Spink and Cohen 1997). By this mechanism, cytokines influence immune cell activity, differentiation, proliferation, and survival. These mediators also regulate the production and activity of other cytokines.

There are presently more than 20 cytokines with the name “interleukin” (IL). Other cytokines have retained their original biological description, such as tumor necrosis factor (TNF). Another way to look at some cytokines is their role in infection and/or inflammation. Some cytokines clearly promote inflammation and are called pro-inflammatory cytokines, whereas other cytokines suppress the activity of pro-inflammatory cytokines and are called anti-inflammatory cytokines (Dinarello 2000).

In this Thesis we will focus on the pro-inflammatory cytokines tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6) and interleukin 8 (IL-8) because they are some of the major cytokines implicated in acute pancreatitis.

5.3.1 TUMOR NECROSIS FACTOR ALPHA

Tumor necrosis factor alpha (TNF-α) is a pleiotropic polypeptide produced from a membrane-bound precursor of 26 kDa. TNF-α can induce its own synthesis, and that of other pro-inflammatory cytokines and adhesion molecules (Papachristou 2008). Monocytes and macrophages are the cells that synthesize the greatest amount of TNF-α, but also other cell types such as acinar cells produce TNF-α. The main stimulus for its production is the lipopolysaccharide, but is also increased in response to bacteria, viruses, cytokines, X radiation and ROS (Muhl and Pfeilschifter 2003). Post-transcriptional regulation of TNF-α mRNA is highly complex and involves the MAPK cascade (Deleault et al. 2008). TNF-α is considered a primary cytokine in acute pancreatitis because it initiates and propagates most of the consequences of the systemic inflammatory response (Norman 1998; Pereda et al. 2006; Escobar et al. 2009). However, it has a short plasma life, of 14-18 min, due to rapid clearance by the liver, gastrointestinal tract and kidney, making its presence difficult to assess by serum levels (Makhija and Kingsnorth 2002).

5.3.2 INTERLEUKIN 6

Interleukin 6 (IL-6) is produced by many cells such as macrophages, endothelial cells, fibroblasts and smooth muscle cells (Montero-Julian 2001). It is also produced in the pancreatic tissue after experimentally induced pancreatitis (Norman et al. 1997). IL-6 is synthesized in response to the stimulation of TNF-α. Its detection in serum is fast and reliable, being detectable from 60 min after the start of production until 10 days later (with a maximum at 4-6 h). IL-6 is the primary inducer of the acute-phase protein response (Papachristou 2008).

IL-6 levels are elevated in pancreatitis and serve as marker of severity of the disease, in addition to paralleling the course of the disease (Leser et al. 1991; Viedma et al. 1992). IL-6 levels can also help to predict the possibility of pulmonary complications (Mayer et al. 2000).

5.3.3 INTERLEUKIN 8

Interleukin 8 (IL-8) belongs to a family of small cytokines ranging from 8 to 10 kDa, termed chemokines, which are responsible for activating and directing leukocytes to damage zones through chemotaxis. IL-8 is a cytokine that activates neutrophils and T lymphocytes, and it is a potent chemotactic agent for these cells. IL-8 is synthesized in several cell types (particularly macrophages and endothelial cells) in response to TNF-α. It is considered the principal mediator of secondary neutrophil activation by TNF-α since it causes migration, expression of adhesion molecules, degranulation and generation of oxygen-derived free radicals by these leukocytes (Wessely-Szponder 2008). However, administration of IL-8 to animals does not cause a state of shock as TNF-α administration does.

IL-8 is a marker of severity in acute pancreatitis (Leser et al. 1991; Norman et al. 1995; Norman 1998) and it participates in neutrophil chemotaxis, activation, and degranulation (Leser et al. 1991; Kusske et al. 1996).

6. ACUTE PANCREATITIS

The pancreas is an organ whose length varies in humans between 15 and 25 cm and weighs between 70 and 150 g in adults. It is located in the retroperitoneum (figure 12), in close contact with other organs such as the

stomach at its upper portion, the duodenum to which it is attached, the spleen in the tail, and the left kidney and the transverse colon ahead (figure 12).

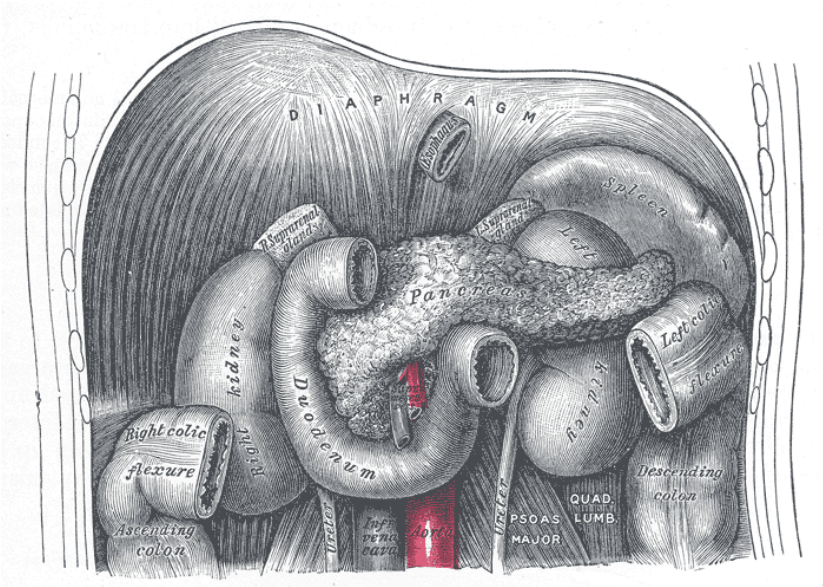


Figure 12. Pancreas location

The pancreas exerts a dual function, endocrine and exocrine. It behaves as an endocrine gland because it has 1-2% of cells capable of secreting hormones, mainly insulin and glucagon. But most of the pancreatic parenchyma is composed of acinar cells that are responsible for the secretion of digestive enzymes, which are secreted through pancreatic ducts (figure 13) into the intestine to digest food. These pancreatic enzymes are trypsin, chymotrypsin, amylases, lipases, elastases, carboxypeptidases A and B, ribonuclease,… Most of them are synthesized and secreted as proenzymes (trypsinogen, chymotrypsinogen, prolipase, proelastase, procarboxypeptidase, etc.) and activated in the lumen of duodenum in order to avoid pancreas

digestion. Trypsin, chymotrypsin and carboxypeptidase are responsible for the degradation of proteins and peptides. Pancreatic lipase hydrolyzes the ester bonds of triacylglycerols ingested releasing their components, fatty acids and glycerol. Amylase acts on glycogen, starch and its components yielding fermentable sugars (maltose) and non-fermentable dextrins.

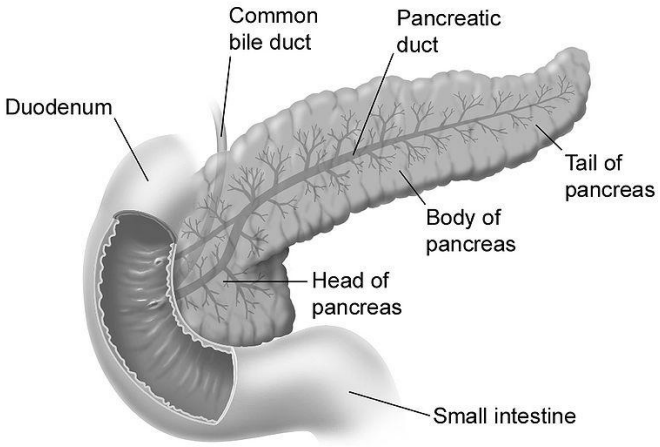


Figure 13. Parts of the pancreas

Among the pathological processes affecting the endocrine part, it highlights diabetes mellitus. Diseases affecting the exocrine area are inflammatory and cancer. In inflammatory pathologies, because of its frequency and impact, it highlights acute pancreatitis.

6.1 DEFINITION AND CLASSIFICATION

Acute pancreatitis (AP) is the acute inflammatory process of the pancreas with variable affectation of local tissues and far organic systems. It is

manifested by the sudden onset of abdominal pain often accompanied by vomiting, fever, tachycardia, leukocytosis, and elevation of pancreatic enzymes in blood and/or urine. Acute pancreatitis can be mild or severe.

- **Mild/edematous acute pancreatitis**

It is acute pancreatitis associated with a slight organic disorder, with complete recovery, and no organ failure neither systemic nor local complications. Mild AP constitutes approximately 75% of cases in clinical practice (Yadav and Lowenfels 2013).

- **Severe/necrotizing acute pancreatitis**

It is acute pancreatitis associated with multiple organ failure and/or local and systemic complications. The mortality rate is around 20-30% (Yadav and Lowenfels 2013).

Acute pancreatitis is a disease in which the clinical spectrum varies widely. While in most cases the disease is mild, with self-limited course and improvement in a few days upon symptomatic therapeutic measures, at other times it is fulminant and evolves to a situation of extreme severity and resistance to all types of treatments. This variability in presentation, in the etiology, and in the clinical course of AP has hindered its study.

6.2 EPIDEMIOLOGY AND ETIOLOGY

Acute pancreatitis is a disease of great importance due to its growing incidence and the relatively high frequency. The incidence of acute pancreatitis

is progressively increasing in parallel with risk factors, such as alcohol abuse, often together with an opulent meal; duct obstruction by gallstones; and obesity (Jha et al. 2009). In hospitals, between 0,15 and 3% of patients seen in the emergency room suffer from acute pancreatitis. According to the Global Organization of Gastroenterology, among 6000 studied episodes of abdominal pain, 2,3% were due to acute pancreatic disease and 1% reached hospital mortality. The average incidence in the European Union countries ranges from 130-450 cases/10^6habitants/year (Yadav and Lowenfels 2013).

The etiology of the disease is varied. Determining the etiology of AP is crucial in the management of an acute episode and in the prevention of recurrent pancreatitis. Two are the main etiologies, biliary disease and alcoholism. 10% are due to causes such as surgical procedures, infections, toxic or ischemia, and in 10% to 30% of cases it is not known the cause being idiopathic. The differences found in the etiology of the disease are due to gender, age, and geographical distribution (Garg 2012). The most common age of onset of acute pancreatitis is between 30 and 70 years old, peaking at 55 years old. The gender distribution has slight variations depending on the etiology. Thus, before 50 years it is more common in men and is related to alcoholic etiology, whereas after this age are the women who suffer mostly this disease in relation to their higher incidence of gallstones (Lankisch et al. 2002; Yadav and Lowenfels 2013).

6.3 PATHOGENY

In 1896, Chiari postulated that pancreatitis was due to autodigestion of the pancreas by succumbing to its own digestive properties (Chiari 1896). Another possibility was that duodenal reflux due to a malfunction of Oddi's

sphincter passes to the pancreas and digests it (McCutcheon and Race 1962). Since the eighties, it is known that even pancreatic duct obstruction can trigger acute pancreatitis due to increased intraductal pressure (Austin et al. 1980). In 1982, the group of Steer noted that there was a colocalization of zymogen granules (granules loaded with inactive digestive enzymes) with lysosomal hydrolases in the early stages of acute pancreatitis induced by ethionine (Koike et al. 1982). Colocalization is produced by a process which involves the fusion of lysosomes with zymogen granules. Other experimental models, both by stimulation with caerulein and pancreatic duct obstruction, showed the same process (Watanabe et al. 1984). Vacuoles formed by the colocalization mechanism have a weak membrane, being able to release destructive enzymes into the cytoplasm. However, other authors have questioned this theory, noting the possibility triggering getting colocalization without producing acute pancreatitis (Luthen et al. 1995). On the other hand, neither the *in vivo* administration of lysosomal enzymes inhibitors prevents the development of acute pancreatitis, nor the *in vitro* administration reduces the levels of trypsinogen activation (Steer 2002). Although limiting the degree of trypsinogen activation in mice decreases acinar necrosis, it is not possible to lower the systemic inflammatory response (Halangk et al. 2000). Therefore, other mechanisms could activate trypsin during acute pancreatitis, as changes in pH or autophagy (Gukovskaya et al. 2002; Hashimoto et al. 2008).

Early events in the pathogenesis are the intrapancreatic activation of zymogen granules, particularly trypsinogen; inhibition of secretion; changes in calcium homeostasis; and activation of cell death pathways (Grady et al. 1996; Pandol et al. 2007). Nowadays, it is accepted that acute pancreatitis is not only caused by the digestion of the pancreas, moreover, these early events trigger

pancreatic cell death that initiates the activation of local inflammatory mediators (Regner et al. 2008).

6.4 PATHOPHYSIOLOGY

The body's inflammatory response to a localised attack on normal conditions remains confined to the organ of origin, due to the action of inhibitors and regulatory mechanisms. However, in inflammatory diseases such as acute pancreatitis, an uncontrolled local inflammatory response can be produced. The clinical response to pancreatitis may eventually lead to a systemic inflammatory response (SIRS), which, if abnormally persistent, develops into a worsening scenario of tissue damage and sepsis resulting in multiple organ dysfunction syndrome (MODS) (figure 14) (Davies and Hagen 1997; Makhija and Kingsnorth 2002). The spectrum of inflammatory responses vary from SIRS that can progress on to MODS or take the more indolent form of a compensatory anti-inflammatory syndrome (CARS) (figure 14) (Bone 1996). The current understanding is that SIRS is the pro-inflammatory response and CARS is the anti-inflammatory response that results in a prolonged period of depressed immune function and increased susceptibility to infections (Bone 1996). The first SIRS cascade occurs over the first week of illness and its potential resolution is the crucial step in deciding the further course of events. The primary mediators of this process are cytokines such TNFα, IL-6 and IL-8 among others. The pro-inflammatory process may be counterbalanced by the anti-inflammatory response that inhibits T-cell mitogenesis and decreases cytokine production (Makhija and Kingsnorth 2002).

The most common extrapancreatic disorder that can appear during acute pancreatitis occurs in the lungs and is considered the most important

cause of immediate death (De Campos et al. 2007). Other complications that can occur in acute pancreatitis are acute renal failure leading to acute tubular necrosis that causes death in many patients (Zhang et al. 2008). The digestive system can be disturbed also by bacterial translocation from the gut to the pancreas causing septic complications (Nakajima et al. 2007). The liver can also be affected producing an increase in cytokines levels that contributes to lung disease (Closa et al. 1996; Closa et al. 1999).

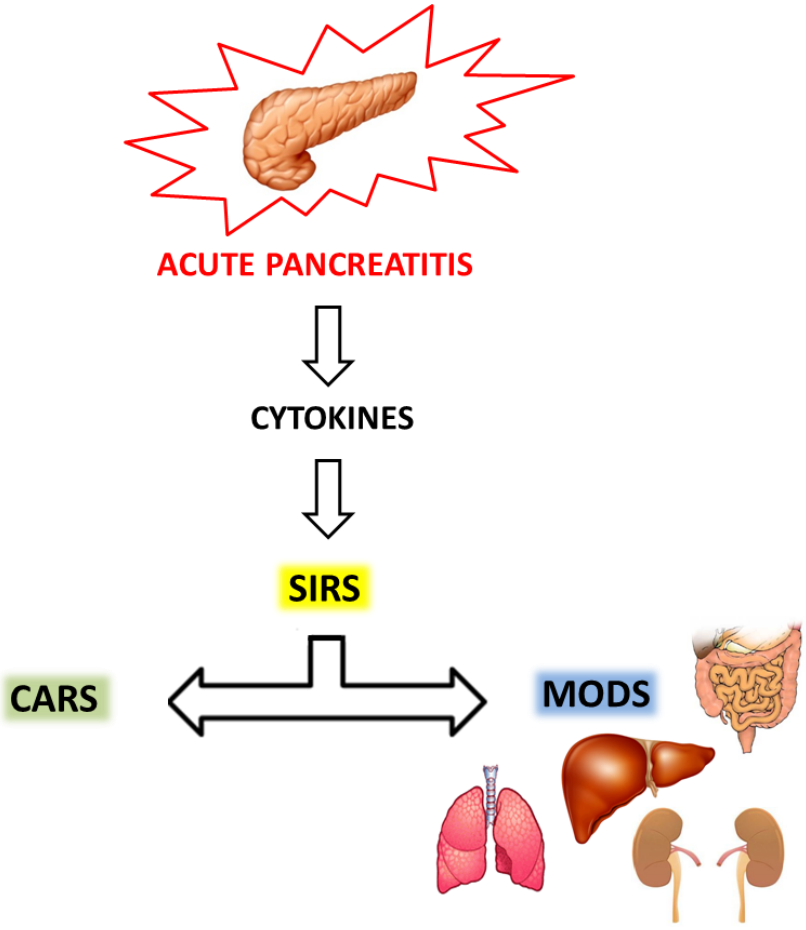


Figure 14. Pathophysiology of acute pancreatitis

6.5 ROLE OF ANTIOXIDANT DEFENSE IN ACUTE PANCREATITIS

Oxidative stress arises during the first phase of acute inflammation because of the generation of ROS that generally leads to glutathione depletion and oxidation (Escobar et al. 2012). Because acute pancreatitis is associated with oxidative stress (Escobar et al. 2012), it is necessary to study the antioxidant defense induced by the pancreas.

The pancreas there appears evolutionarily prepared for defending against oxidative stress. The pancreas is one of the tissues with greatest amount of GSH. In the pancreas there are also other antioxidants such as vitamin C, vitamin E, and vitamin A in considerable amounts (Leung and Chan 2009). The exocrine pancreas has a low activity of glutamate cysteine ligase (GCL), even though is able to synthesize GSH. The pancreas has a high γ-glutamyltranspeptidase (γ-GT) activity and the transsulfuration pathway is intact in pancreatic acini, which promotes synthesis from its amino acids (Leung and Chan 2009). The abundance of precursor amino acids favors the formation of GSH despite the low activity of GCL.

GSH becomes a critical factor in the course of acute pancreatitis. During acute pancreatitis there is a decrease in the levels of GSH and maintaining these low levels of GSH could be responsible for the transformation of mild acute pancreatitis into severe (Rahman et al. 2004; Pereda et al. 2008). GSH depletion in the pancreas is a hallmark of the early course of acute pancreatitis (Neuschwander-Tetri et al. 1992; Schoenberg et al. 1992; Gomez-Cambronero et al. 2000) and it contributes to the severity of the disease (Neuschwander-Tetri et al. 1992; Alsfasser et al. 2002; Pereda et al. 2008). Thus, prevention of glutathione depletion in the pancreas by

administration of glutathione monoethyl ester ameliorated acute pancreatitis (Neuschwander-Tetri et al. 1992).We found that pancreatic GSH levels are rapidly recovered in mild edematous pancreatitis because of up-regulation of GCL, a key enzyme in the control of GSH synthesis (Pereda et al. 2008). However, in severe necrotizing pancreatitis the induction of GCL fails leading to a long-lasting GSH depletion (Pereda et al. 2008). Therefore, the failure in recovery of pancreatic glutathione levels may be a key pathophysiological mechanism differentiating necrotizing from edematous pancreatitis.

The association between enzymatic antioxidants and pancreatic inflammation has been extensively studied over the past decade. Decreased expression and loss of activity of the antioxidant enzymes can enhance the oxidative process. In different models of acute pancreatitis, glutathione peroxidase, catalase and superoxide dismutase decrease, resulting in a redox imbalance (Curran et al. 2000; Sajewicz et al. 2006; Leung and Chan 2009). Moreover, glutathione peroxidase and thioredoxin activities are considered markers for severity of pancreatitis (Leung and Chan 2009).

6.6 ROLE OF MAPK AND PHOSPHATASES IN ACUTE PANCREATITIS

ROS govern the balance between MAPKs and some phosphatases controlling the inflammatory response by redox signaling (Circu and Aw 2010; Naik and Dixit 2011). There is MAPK activation in acute pancreatitis induced by 3,5% sodium taurocholate (Maulik 2002).

The major MAPKs p38, JNK and ERK1/2 are rapidly activated in pancreatic acinar cells *in vitro* upon stimulation with secretagogues, such as cholecystokinin (CCK) or cerulein (Schafer et al. 1998; Wagner et al. 1999).

These MAPKs are also activated early in the course of experimental acute pancreatitis (Schafer et al. 1998; Wagner et al. 1999; Pereda et al. 2004). Blockade of MAPK activation prevented pro-inflammatory gene up-regulation in acinar cells and pancreas during acute pancreatitis (Blinman et al. 2000; Pereda et al. 2004; Ju et al. 2006). This highlights the key role of MAPKs in the inflammatory response in acute pancreatitis.

ROS generated by infiltrated leukocytes could contribute to MAPK activation in pancreas *in vivo* during acute pancreatitis (Leung and Chan 2009). Accordingly, oxypurinol, an inhibitor of xanthine oxidase, inhibited p38 activation in pancreas in necrotizing taurocholate-induced pancreatitis (Pereda et al. 2004), and N-acetylcysteine inhibited p38 activation in pancreas during pancreatitis induced by biliopancreatic duct ligation (Ramudo et al. 2005). Furthermore, the combined treatment of taurocholate-induced acute pancreatitis with pentoxifylline, which inhibits TNF-α production, and oxypurinol led to simultaneous blockade of the three major MAPKs, i.e., p38, JNK, and ERK1/2, in the pancreas, resulting in a remarkable reduction in the inflammatory response (Pereda et al. 2004).

Regarding pulmonary pathology associated with pancreatitis, it has been shown that p38 plays an important role in the induction of TNF-α and lung damage. Thus, administration of CNI-1493 (an inhibitor of macrophage activation) to animals with acute pancreatitis induced by sodium taurocholate inhibited p38 phosphorylation and the secretion of TNF-α in lung. Both effects are associated with less lung damage and less inflammatory infiltrate (Hawkins et al. 2007).

All these findings suggest that there is a cross-talk between oxidative stress and pro-inflammatory cytokines through MAPKs that greatly contributes to amplification of the uncontrolled inflammatory cascade and tissue injury in acute pancreatitis (Escobar et al. 2009).

The role of phosphatases in pancreatitis is really complex. Until the beginning of last decade, attention was focused on the activation of MAPK in the pancreas and in pancreatitis, forgetting its regulation by phosphatases. Wagner's group was the first to study the role of DSPs during acute pancreatitis. They observed that DSPs were encoded by early response genes and their expression changed from zero to very high levels very quickly in acute pancreatitis (Bauer 2002) which should be considered an adaptive protective response that contributes to attenuation of the inflammatory response.

On the other hand, inhibition of tyrosine phosphatase activity induces dissociation of adherens junctions in pancreatic acini as a prerequisite for the development of pancreatic edema (Schnekenburger et al. 2005). In this regard, protein tyrosine phosphatases and particularly SHP1 are critically involved in the regulation and maintenance of cell adhesions in exocrine pancreas (Schnekenburger et al. 2005), and therefore the loss of their activities may decisively contribute to formation of interstitial edema in acute pancreatitis.

Another tyrosine phosphatase critically involved in the inflammatory response in acute pancreatitis is transmembrane tyrosine phosphatase CD45, which is present not only in leukocytes but also in pancreatic acinar cells (De Dios et al. 2005). CD45 regulates the expression of tnf-α in pancreatic acinar cells and accordingly, the decreased expression of CD45 in the course of acute pancreatitis triggered tnf-α upregulation (de Dios et al. 2006). This mechanism

is redox regulated because it was prevented by the antioxidant N-acetylcysteine (de Dios et al. 2006). Consequently, redox signaling through protein tyrosine phosphatases seems to be a key event in the inflammatory response in acute pancreatitis (Escobar et al. 2009).

III. AIMS

The aims proposed in this thesis, based on the background of the research topic, are as follows:

1. To study the mechanisms involved in oxidative stress mediated by thiol oxidation during acute pancreatitis.

1.1. To study the redox state of thiols and mixed disulfides in pancreas during acute pancreatitis.

1.2. To study protein targets that suffer oxidative thiol modification in acute pancreatitis and its relevance in the pathophysiology of the disease.

2. To study the role of redox-sensitive serine/threonine phosphatase PP2A in the inflammatory cascade during acute pancreatitis.

IV. MATERIALES Y MÉTODOS

1. MATERIALES

1.1. ANIMALES DE EXPERIMENTACIÓN

Los animales de experimentación utilizados para la consecución de la presente Tesis fueron ratas Wistar macho jóvenes de 275 a 325 g de peso corporal para la pancreatitis aguda necrótica experimental inducida con taurocolato al 3,5%.

Los animales se mantuvieron en el animalario de la Facultad de Farmacia de la Universitat de València, bajo condiciones de temperatura (23 ± 1ºC), humedad relativa (60%) y ciclos de luz/oscuridad (12/12 h) constantes. Se alimentaron con una dieta de laboratorio estándar que contenía 590 g de carbohidratos, 30 g de lípidos y 160 g de proteínas por kilogramo de dieta (Letica, Barcelona) y agua corriente *ad libitum*.

La inducción de la pancreatitis y el sacrificio de los animales se llevaron a cabo previa inducción anestésica con isoflurano al 2,5% y flujo de oxígeno a 0,5-1 L/min por vía inhalada. El aporte de anestésico se mantuvo durante toda la intervención y extracción de órganos.

La manipulación de los animales y los protocolos de experimentación han sido realizados de acuerdo con las normas de experimentación animal de la Unión Europea (1999) y fueron aprobadas por la Comisión de Ética de Experimentación Animal de la Universitat de València.

La N-Acetilcisteína (NAC) disuelta en agua estéril se administró intraperitonealmente una hora antes de inducir la pancreatitis aguda y una hora después de la misma, a una dosis de 50 mg/Kg de peso corporal por cada inyección.

1.2. APARATOS

- Acquity UPLC acoplado a un detector TQD-Acquity de espectrometría de masas (Waters, Manchester, UK) del SCSIE del Campus de Burjasot de la Universidad de Valencia.
- Agitador magnético Selecta modelo Agimatic-S.
- Aparato de PCR cuantitativa Bio-Rad modelo iQ5.
- Autoclave Selecta modelo Autester-G.
- Balanzas de precisión Sartorius modelo Tecator 6110 y modelo PT 1200.
- Baño termostatizado provisto de agitación automática regulable marca New Brunswick Scientific modelo Innova 4000.
- Bomba de perfusión marca Harvard Instruments modelo Pump 11.
- Campana de flujo laminar vertical Cultair modelo B100.
- Captador de imágenes Bio-Rad modelo Molecular Imager ChemiDoc^{TM} XRS^{+}.
- Centrífuga refrigerada marca Hettich modelo Rotina 420R.
- Cubetas de electroforesis y electrotransferencia de Bio-Rad modelo Mini-PROTEAN 3 Cell.
- Espectrofotómetro Multiskan Spectrum de Thermo Scientific.
- Espectrómetro de masas nanoESI Q-TOF (5600 TripleTOF, ABSCIEX) del SCSIE del Campus de Burjasot de la Universidad de Valencia.
- Estufa termostatizada de cultivos Napco modelo 5415IR, CO_2 System.

- Fuente de alimentación de electroforesis marca Bio-Rad modelo 200/2.0 Power Supply.
- Homogeneizador marca Heidolph modelo RZR 2021.
- Nanodrop marca Thermo Scientific modelo Lite.
- pHmetro marca Crison modelo Microph 2001, con un electrodo incorporado Inglod.
- Pipetas de 1000, 200, 20, 10, 5 y 2 µl de la marca Thermo Scientific.
- Sistema Easy-nLCII acoplado al sistema LTQ-Orbitrap-Velos-Promass (Thermo Scientific, Waltham, MA, USA) de los servicios de proteómica del Centro de Investigación Príncipe Felipe.
- Termobloque con agitación marca Biosan modelo TS-100C
- Transiluminador de ultravioleta.
- Ultracongelador NewBrunswick modelo SC-420.

1.3. REACTIVOS

- Acrilamida, TEMED, persulfato amónico (APS), dodecilsulfato sódico (SDS), Ditiotreitol (DTT), albumina de suero fetal (BSA) (Sigma-Aldrich), dual color protein molecular marker (Bio-Rad).
- Anestesia: Isoflurano (Esteve).
- Anticuerpos frente a: RNH1, SHP1 (Abcam), ERK ½, p-ERK ½, Keap1, PDI, p-p38α, PP2Ac, Prdx1, Ref1/Ape1, SHP2, Trx1, estreptavidina-HRP (Cell Signaling), DNP (Dako), p38α, α-tubulina, mouse-HRP, rabbit-HRP (Santa-Cruz).
- BCA Protein Assay Kit (Thermo Scientific).
- Bradford Protein Assay Kit (Bio-Rad).

- Células acinares 266-6 de pancreas de ratón (European Collection of Cell Cultures).
- Cóctel de inhibidores de proteasas (AEBSF 2 mM, aprotinina 0,3 µM, bestatina 130 µM, EDTA 1 mM, E-64 14 µM, leupeptina 1 µM) (Sigma-Aldrich).
- Filtros de jeringa de nylon 0,45 µm (Nalgene).
- Inhibidores de fosfatasas: Ortovanadato sódico, pirofosfato sódico y fluoruro sódico (Sigma-Aldrich).
- Iodoacetamida biotinilada (Thermo Scientific).
- Kits de actividad fosfatasa: Serine/threonine Phosphatase Assay System y Tyrosine Phosphatase Assay System (Promega).
- Kit de revelado de western blot: HRP Detection kit (Santa Cruz).
- Medio de cultivo "Dubelcco's Eagle Modified", Phosphate Buffer Solution (PBS), estreptomicina/penicilina, suero bovino fetal (Gibco), puromicina (Santa Cruz).
- Membranas de transferencia de nitrocelulosa 0,45 µm (Invitrogen).
- Placas Petri (BD), tubos de plástico de 50 y 15 ml, rascador cell scraper (Corning).
- RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific)
- Sondas TaqMan® para IL-6, TNF-α, CXCL1 y Rplp0 (Applied Biosystems).
- Taurocolato sódico (Sigma-Aldrich).
- Trizol reagent (Ambion)
- Otros: cloruro sódico, ácido etilendiaminotetraacético (EDTA), etilenglicol del ácido tetraacético (EGTA), ácido 4-(2-*hidroxi*-etil)-1- piperazin-etan-sulfónico (HEPES), tris(hidroximetil)aminometano (Tris), glicina, ácido acético, ácido clorihídrico, azul de bromofenol, azul comassie, rojo ponceau, nitrato de plata, metanol, iodoacetamida, N-etilmaleimida (NEM), igepal, glicerol,

acetonitrilo, ácido trifluoroacético (TFA), ácido tricloroacético (TCA), urea, monobromobimano, bicarbonato amónico, Tween-20, β-mercaptoetanol, imidazol.

El resto de reactivos y material fungible se obtuvo de los siguientes laboratorios: Applied Biosystems, Bio-Test, Bromatos, Life Technologies, Merck Biochemica, Panreac, Promega, Sigma-Aldrich, Greiner Bio-One, Takara y Thermo Scientific.

2. MODELOS DE EXPERIMENTACIÓN

2.1. MODELO EXPERIMENTAL DE PANCREATITIS AGUDA NECRÓTICA *IN VIVO* EN RATAS

El modelo de pancreatitis aguda necrótica utilizado en este trabajo ha sido el de infusión retrógrada de la sal biliar taurocolato sódico al 3,5% desarrollado por Aho y colaboradores (Aho et al. 1980), que intenta aproximarse a los fenómenos que ocurren en una pancreatitis de origen biliar. En este modelo, la secuencia de acontecimientos que tienen lugar durante las primeras fases de la pancreatitis aguda parece deberse al efecto detergente del taurocolato, que desestabiliza las membranas celulares. Esto provoca la activación de las enzimas proteolíticas por dos vías: bien incrementando la concentración de calcio en el citoplasma celular (activando las proteasas dependientes de calcio), o bien, como parece más probable, facilitando la fusión de los lisosomas con los gránulos de zimógeno (produciendo el paso de tripsinógeno a tripsina) (Nakae et al. 1995).

Tras anestesiar al animal, la inducción de la pancreatitis aguda se llevó a cabo bajo medidas de asepsia (rasurado y pincelado de los animales con povidona yodada) (figura 15B), como se describe a continuación: se practicó una laparotomía media, se evisceró y localizó el duodeno, el cual se punzó en su borde antimesentérico para canalizar el conducto biliopancreático con una cánula de 0,6 mm de diámetro (Clay Adams PE10) (figura 15C). Después de clampar dicho conducto a su salida del hígado se comenzó la perfusión de taurocolato al 3,5% a un ritmo de 0,1 mL/100 g de peso del animal durante un minuto utilizando una bomba de perfusión (Harvard Instruments, véase figura 15A). Una vez concluida la perfusión, se retiró el *clamp* y la cánula, se reintegraron las asas intestinales al interior de la cavidad abdominal y se cerró la laparotomía con seda de 3/0.

En el modelo de pancreatitis aguda necrótica experimental inducida por taurocolato, la severidad y la mortalidad dependen de la concentración administrada de la sal biliar. Así, con una concentración de taurocolato del 3,5% la mortalidad es de aproximadamente un 25% en las primeras 72 h, mientras que con una concentración del 5% la mortalidad es del 100% (Aho et al. 1980). El modelo empleado tiende a producir una pancreatitis aguda severa, rápidamente destructiva y con una elevada mortalidad, con la ventaja de que dicha mortalidad no se debe sólo a los efectos locales, sino sobre todo a los efectos sistémicos precoces que provoca, tal y como ocurre en los humanos. Además, la homogeneidad de los resultados obtenidos cuando la perfusión se realiza con bomba de perfusión, lo convierte en un modelo idóneo para el estudio de los efectos tanto locales como sistémicos de la pancreatitis aguda necrótica experimental (Lerch and Gorelick 2013).

En el grupo de animales tratados con N-acetilcisteína (NAC), ésta se administró intraperitonealmente 1 hora antes y 1 hora después de la inducción con taurocolato, a una dosis de 50 mg/kg de peso del animal por cada inyección tal y como se utilizó en el trabajo de (Sevillano et al. 2003). En el grupo control se administró suero fisiológico (NaCl al 0,9%) intraperitonealmente para comparar con el grupo tratado.

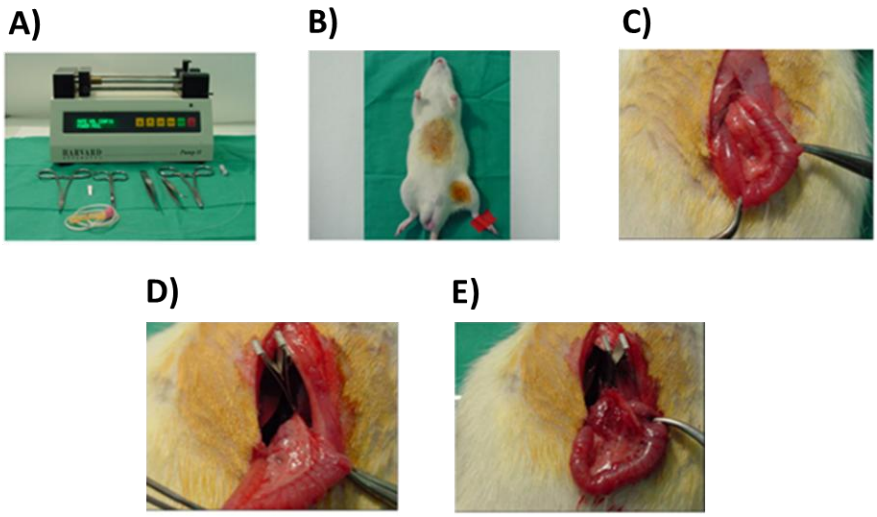


Figura 15. Modelo de pancreatitis necrótica inducida por taurocolato. A) Material necesario, B) preparación del animal, C) cánula insertada en el conducto biliopancreático, D) clampaje proximal del conducto biliopancreático, E) aparición de zonas necróticas en el páncreas.

2.2. MODELO DE CULTIVO DE CÉLULAS 266-6 *IN VITRO*

En el estudio *in vitro*, se ha empleado la línea celular 266-6 proveniente de células acinares de páncreas de ratón. Al provenir de células acinares, su organización y su capacidad de secreción se asemeja a los acinos que forman el

páncreas exocrino de ratón. Se considera la línea celular que más se acerca *in vitro* a las células acinares del páncreas exocrino *in vivo,* además de que son menos complejas de manipular y se transfectan mejor que las células AR42J (células acinares de páncreas de rata).

Las células 266-6 se cultivan según indicaciones de la European Collection of Cell Cultures (ECACC).

Se prepara el medio de cultivo en condiciones de esterilidad. Su composición es la siguiente: Dulbecco´s Modified Eagle Medium (GIBCO), pH 7.4, al cual se le añaden los siguientes compuestos:

Suero bovino fetal	10%
Estreptomicina	100 μg/mL
Penicilina	100 μg/mL
Fungizona	25 μg/mL

Para un frasco de cultivo de 75 cm^2 (T75) se prepara un volumen total de 15 mL. Estos frascos son idóneos para el mantenimiento y la producción de gran número de células.

Una vez preparado el medio de cultivo, se añaden las células en número deseado y se introduce la mezcla en el frasco de cultivo adecuado. Los frascos se incuban a 37^oC en una estufa incubadora bajo una atmósfera con 5% de CO_2.

El medio de cultivo se renueva de dos a tres veces por semana para las células de mantenimiento.

Para la realización de incubaciones de los distintos experimentos fueron empleadas placas Petri de 100 mm de diámetro (BD). Se prepara un volumen total de 8 ml por placa y se siembran 1.000.000 células en cada una.

Se dejan crecer las células sembradas hasta alcanzar un 80%-90% de confluencia, que corresponde a unos 4 días tras la siembra, cambiando el medio de cultivo 2 días antes del experimento. Tras el periodo de incubación con los distintos tratamientos, las placas se lavan con 1 ml de PBS frío, retirando con precaución el PBS y se procede a la recogida de las células mediante rascador *cell scraper* (Corning) una vez añadido el tampón de lisis o de resuspensión correspondiente.

Para la realización de los experimentos *in vitro* se incubaron las células durante 1 hora con TNF-α 10 ng/mL y durante 2 horas con taurocolato al 0,5%.

3. MÉTODOS

3.1. DETERMINACIÓN DE PROTEÍNAS

3.1.1. MÉTODO DEL BCA

La determinación total de proteínas en las muestras de páncreas de rata se realizó mediante el *Pierce® BCA Protein Assay Kit* de Thermo Scientific. Este método combina la reducción del Cu^{2+} a Cu^{1+} por las proteínas en un

medio alcalino con la alta sensibilidad y selectividad colorimétrica de la detección del catión cuproso (Cu^{1+}) usando un reactivo que contiene ácido bicinconínico (BCA). El producto de reacción de color morado se forma por la quelación de dos moléculas de BCA con una de ion cuproso. Este complejo soluble en agua presenta una fuerte absorbancia a 562 nm que es lineal con el aumento de la concentración de proteínas entre un rango de 2-20 mg/mL.

3.1.1.1. Procedimiento

Se añadió una cantidad de muestra de 3 µL y 260 µL de solución reactiva. Después se incubó 30 minutos a 37ºC.

Para la correcta determinación de proteínas, se preparó una recta patrón construida con seroalbúmina bovina a concentraciones comprendidas entre 0,3 y 20 mg/mL, añadiendo el mismo volumen que el de muestra y completando la solución reactiva de la misma forma. Por último, se preparó un blanco con agua. La lectura de la absorbancia se realizó a 562 nm de longitud de onda.

3.1.1.2. Cálculos

La absorbancia de la muestra llamada "blanco" se restó a las muestras problema y a los patrones. El resultado se expresaba en una gráfica de absorbancia en función de la concentración de patrones. Como el sistema sigue la ley de Lambert-Beer, se obtenía una línea recta que pasaba cerca del origen. Una vez obtenida las absorbancias de todas las muestras se realizaba una recta

de regresión lineal con los datos de la recta patrón para interpolar en ésta los datos de las muestras de las que se desconocía su concentración.

3.1.2. MÉTODO DE BRADFORD

Se utilizó el "Bradford Reagent" de la firma BIO-RAD siguiendo el protocolo de Bradford (Bradford 1976) para la determinación de la concentración de proteínas en las muestras de células 266-6. Este método es mucho más sensible que el BCA y dado que la concentración de proteínas en muestras *in vitro* es siempre mucho menor que en muestras procedentes de tejidos, se utilizó el método de Bradford para la cuantificación de la concentración de proteínas en muestras de células 266-6.

La unión de las proteínas con el reactivo de Bradford produce un cambio en el color del reactivo y, por tanto, un cambio en su absorbancia. El reactivo Bradford contiene ácido fosfórico, metanol y también azul de comassie que se une a los residuos aminoacídicos de proteínas, y la absorbancia se mide a 595 nm.

3.1.2.1. Procedimiento

Se añadió una cantidad de muestra de 3 µL y 260 µL de solución reactiva. Se preparó una recta patrón construida con seroalbúmina bovina a concentraciones comprendidas entre 0,12 y 8 mg/mL añadiendo el mismo volumen que el de muestra y completando la solución reactiva de la misma forma. Por último, se preparó un blanco con agua. Se leyó la absorbancia de cada una de las muestras y patrones a 595nm.

3.1.2.2. Cálculos

Se restó a cada muestra la absorbancia del blanco. Las absorbancias obtenidas se interpolaron en la recta construida con los patrones. El valor hallado se expresó en miligramos de proteína por mililitro de muestra (mg/mL).

3.2. DETERMINACIÓN DE LOS NIVELES DE LAS FORMAS LIBRES Y UNIDAS A PROTEÍNAS DE TIOLES DE BAJO PESO MOLECULAR EN PÁNCREAS DE RATA

La cuantificación del contenido de tioles de bajo peso molecular, sus formas reducidas y oxidadas, así como la de puentes disulfuros mixtos (unidos a proteínas) se llevó a cabo después del correspondiente procesado de las muestras procedentes de tejido pancreático fresco de rata y su posterior análisis mediante espectrometría de masas acoplada a cromatografía líquida de alta resolución (HPLC-MS/MS).

3.2.1. PROCESADO DE LAS MUESTRAS

3.2.1.1. Procesado de las muestras para la determinación de tioles libres de bajo peso molecular

1. Se homogeneizaron páncreas frescos de rata en una proporción de 400 µL de PBS NEM 10 mM y acivicina 10 µM por cada 100 mg de tejido (el volumen final era alrededor de 500 µL).

2. Para desproteinizar las muestras se añadieron 50 µL de ácido perclórico (PCA) 44% ($[PCA]_F$=4%) un minuto después de obtener el homogenado.

3. Se centrifugó a 11.000 r.p.m durante 15 minutos a 4ºC y se separó el sobrenadante (en el que se midieron los tioles de bajo peso molecular y sus formas oxidadas) del precipitado.

3.2.1.2. Procesado de las muestras para la determinación de tioles de bajo peso molecular unidos a proteínas

1. Se homogeneizó un trozo de páncreas fresco de rata (25 mg) en ácido tricloroacético (TCA) 10% (a razón de 0,1 mL/100 mg).

2. Se centrifugó durante 2 minutos a 10.000 *g*.

3. El precipitado (donde se encuentran las proteínas tioladas) se lavó 2 veces con TCA 10% (500 µL).

4. Se disolvió el precipitado en tampón Hepes 50 mM, pH 8, dodecilsulfato sódico (SDS) 2% (entre 0,4-1 mL). El SDS se añadió al tampón después de ajustar el pH.

5. Se añadió bicarbonato sódico en polvo hasta saturación.

6. Una vez solubilizado el precipitado, se tomó una alícuota para medir proteínas totales y se comprobó que el pH no fuera superior a 9.

7. Se cogió otra alícuota de 50-100 μL y se le añadió ditiotreitol (DTT) a una concentración final de 2,5 mM.

8. Se incubaron las muestras 1 hora a 40ºC bajo agitación. Se volvió a medir el pH.

9. Una vez comprobado que el pH de la muestra no era excesivamente básico (lo que produciría la hidrólisis alcalina de la muestra) se añadieron 10 μL de NEM 100 mM.

10. Inmediatamente después, a los 110 μL de muestra con NEM se le añadieron 12 μL de PCA al 40% (quedando concentraciones finales de NEM 10 mM y PCA al 3,93%).

11. Se centrifugaron las muestras 10 minutos a 10.000 *g* y a 4ºC, y se separó el sobrenadante (en el que se midieron los tioles de bajo peso molecular unidos a proteínas) del precipitado.

3.2.2. CROMATOGRAFÍA LÍQUIDA DE ALTA RESOLUCIÓN ACOPLADA A ESPECTROMETRÍA DE MASAS (HPLC-MS/MS)

El análisis mediante HPLC-MS/MS se llevó a cabo en el Servicio Central de Soporte a la Investigación Experimental (SCSIE) de la Universidad de Valencia, utilizando un acquity UPLC acoplado a un detector TQD-Acquity de espectrometría de masas (Waters, Manchester, UK). La técnica de espectrometría de masas aparece posteriormente detallada en el apartado 3.8.

La separación analítica se realizó usando una columna UPLC BEH C18 Acquity (2,1 x 50 mm, 1,7 µm, Waters) y un volumen de inyección de 5 µL. El gradiente del solvente consistió en una mezcla del solvente A: agua-ácido fórmico (100:0,5 v/v) y de B: isopropanol-acetonitrilo-ácido fórmico (50:50:0,1 v/v/v). El gradiente de elución fue el siguiente (figura 16): de 0 a 2,52 min 0% B; de 2,52 a 4,4 min de 0 a 65% B; de 4,4 a 6 min 65% B; de 6 a 6,1 min de 65 a 0% B; de 6,1 a 11 min 0% de B. El flujo de la fase móvil fue de 0,35 mL/min y la temperatura de la columna fue la ambiental (23 ± 3^{o}C). La ionización y nebulización de la muestra se llevó a cabo bajo las siguientes condiciones: voltaje capilar de 3,5 kV, temperatura de 120^{o}C, temperatura de desolvatación de 350^{o}C.

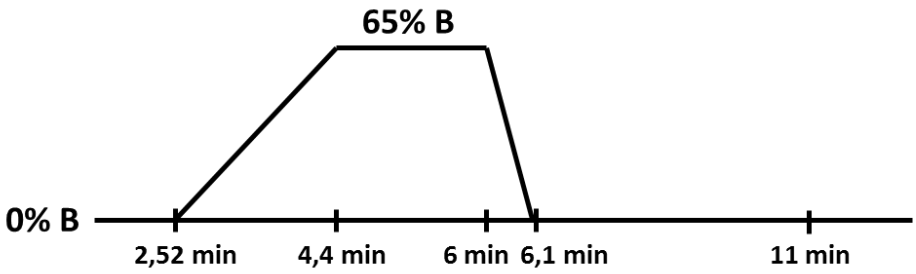


Figura 16. Programa del gradiente de elución

La energía del cono y el voltaje de colisión se optimizaron para cada analito y se muestran en la tabla 2. Las curvas de calibrado que se utilizaron para el GSH, GSSG, cisteína y homocisteína fueron de 0,4 nM a 50 µM, y de 0,1 nM a 12,5 µM para el resto de analitos. El rango de concentraciones se obtuvo mediante el área de cada pico y se utilizó ácido tiosalicílico como patrón interno. Las muestras se analizaron por duplicado y se diluyeron 1:50 para

obtener GSH en el rango de calibración. Las transiciones (m/z) y el tiempo de retención para cada analito se resumen en la tabla 2.

ANALITO	ENERGÍA DEL CONO (eV)	VOLTAJE DE COLISIÓN (V)	TRANSICIÓN (m/z)	TIEMPO DE RETENCIÓN (min)
GSSG	30	25	613 > 355	1,46
GSH (conjugado a NEM)	30	15	433 > 304	4,32
CISTEÍNA (conjugado a NEM)	30	20	247 > 158	1,79
CISTINA	15	15	241 > 152	0,42
HOMOCISTEÍNA-NEM	30	15	261 > 215	4,26
HOMOCISTINA	35	10	269 > 136	0,58
γ-GLUTAMILCISTEÍNA-NEM	40	20	376 > 314	4,33
BIS-γ-GLUTAMILCISTEÍNA	20	18	499 > 241	1,13
ÁCIDO TIOSALICÍLICO-NEM	25	10	278 > 153	5,03

Tabla 2. Condiciones del HPLC/MS-MS para cada analito

3.3. ELECTROFORESIS UNIDIMENSIONAL

La electroforesis en geles verticales con una matriz de poliacrilamida es la que se utiliza mayoritariamente para la separación de proteínas. Se realizó la que se denomina “SDS-PAGE” (electroforesis en gel de poliacrilamida con dodecilsulfato sódico) en condiciones desnaturalizantes. De esta forma, la separación se produce por el peso molecular de cada proteína.

Se cargaron las muestras en los pocillos, acompañadas de tampón de carga (Tris-HCl 130 mM, pH 6,8, glicerol 10%, azul de bromofenol 0,05%, SDS 2%). En el caso de las muestras en condiciones reductoras, este tampón

contenía 100 mM de DTT. A continuación se cargó un patrón de pesos moleculares (Bio-rad). Los geles utilizados eran geles discontinuos, ya que tienen una zona de concentración (*staking)* de baja concentración de acrilamida y una zona de separación (*running)* de alta concentración de acrilamida. El gel concentrador tiene un porcentaje de acrilamida menor, facilitando la entrada y la compactación de las muestras en el gel. El *gel separador* se compone de acrilamida (29:1 acrilamida:bisacrilamida. El gel se somete a un campo eléctrico de voltaje constante (alrededor de 100 voltios) durante al menos 2 h en tampón Tris-glicina (Tris 25 mM, glicina 200 mM, SDS 0,1%, pH 8,3) (figura 17).

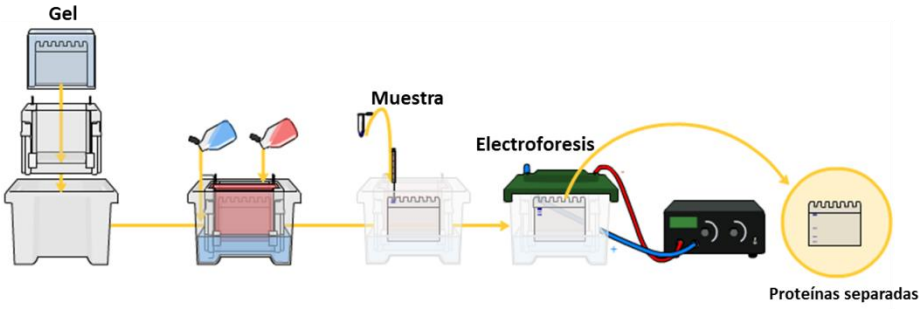


Figura 17. Esquema de electroforesis unidimensional

3.3.1. ELECTROFORESIS UNIDIMENSIONAL CON INTERCAMBIO CON MONOBROMOBIMANO

El monobromobimano (MBrB) es un compuesto heterocíclico que se utiliza como sonda gracias a sus propiedades fluorescentes. Reacciona con los grupos tiol y por ello se usa en la determinación del estado redox de tioles de bajo peso molecular, así como los que forman parte de proteínas en los

sistemas biológicos. Por tanto, esta técnica se basa en la derivatización de la muestra con monobromobimano y la posterior detección mediante espectroscopía de fluorescencia.

3.3.1.1. Procesado de las muestras

Las formas reducidas y oxidadas de glutatión, así como otros disulfuros de proteínas son estables y se pueden determinar correctamente ya sea en tejido fresco o congelado. De hecho, no hay diferencias en este sentido entre el tejido fresco y congelado. Sin embargo, la cisteína libre es inestable y por tanto, la congelación y descongelación puede dar lugar a su oxidación artificial. Por ello, en la determinación de los niveles de tioles de bajo peso molecular se utilizaron páncreas frescos mientras que en el resto de técnicas se han utilizado páncreas congelados.

Se partió de páncreas congelados que se homogenizaron en el tampón que contenía RIPA 1X (Tris 20 mM pH 7,5, NaCl 150 mM, EDTA 1 mM, SDS 0,1%, Igepal 1%) NEM 50 mM y cóctel de inhibidores de proteasas 10 µL/mL. Se utilizó 1 mL de tampón por cada 125 mg de tejido. A continuación, los homogenados se incubaron durante 15 min bajo agitación a temperatura ambiente y se procedió de la siguiente manera:

1. Se desproteinizaron las muestras con TCA 10%.

2. Se centrifugaron a 12.000 *g* durante 2 min a 4ºC.

3. Se lavaron los precipitados dos veces, la primera con TCA 10% y la segunda con acetona para eliminar el exceso de NEM.

4. Se resuspendieron los precipitados en una solución de PBS y DTT 50 mM, y se llevaron a un pH de 7-7,5. Las muestras se dejaron 1 hora incubándose a 42ºC.

5. Se precipitaron de nuevo las muestras con TCA 10% y se centrifugaron a 12.000 *g* durante 2 min a 4ºC para eliminar el exceso de DTT.

6. Se lavaron de nuevo los precipitados con TCA 10% y acetona.

7. Se resuspendieron los precipitados en tampón PBS con urea 8 M, (Rogers et al. 2006)) y se ajustó el pH a 7-7,5.

8. Se añadieron 5 µL de monobromobimano 18,4 mM por cada 90 µL de muestra.

9. Posteriormente, las muestras se incubaron en oscuridad a 37ºC, bajo agitación y durante 30 min (Giustarini et al. 2009).

Una vez que las muestras fueron tratadas adecuadamente y los residuos de cisteína de las proteínas llevaban incorporado el monobromobimano (como agente alquilante de los grupos tiol), se procedió a la separación de las proteínas en función de su peso molecular mediante una electroforesis desnaturalizante en gel de poliacrilamida (SDS-PAGE).

Una vez completada la electroforesis, el gel se destiñó durante 24 h con 200 mL de etanol 40%, ácido acético 10% y agua 50%, con al menos tres cambios en el lavado (Rogers et al. 2006). Finalmente, el gel se visualizó y fotografió con luz UV.

3.4. *WESTERN BLOTTING*

El *western blotting* es un procedimiento de Biología Molecular que combina varias técnicas para conseguir finalmente evaluar semicuantitativamente la cantidad de una determinada proteína en una muestra problema. Comprende la electroforesis, la electrotransferencia a membrana de nitrocelulosa y la detección de la proteína deseada por inmunoquimioluminiscencia.

3.4.1. PROCESADO DE LAS MUESTRAS

Los *western blots* se realizaron a partir de tejido pancreático congelado de rata. Se utilizó 1 mL de tampón por cada 100 mg de tejido. En el caso de los *western blots* que se realizaron bajo condiciones no reductoras, el tampón de homogenización contenía además de lo que se expone a continuación, NEM 10 mM. El tampón de homogenización que se utilizó fue el siguiente:

Tris-HCl pH 7,5	20 mM
NaCl	150 mM
EDTA	1 mM
SDS	0,1%
Igepal	1%
Cóctel de inhibidores de proteasas	10 µL/mL
NaF	50 mM
Pirofosfato sódico	30 mM
Ortovanadato sódico	200 mM

Cuando los *western blots* se realizaron a partir de células, se añadieron 100 µl de tampón de lisis celular por cada placa Petri de 100 mm. Se incubaron en hielo durante 30 min y se recogió el lisado con un *cell scraper*.

El tampón de lisis llevaba la siguiente composición:

Hepes pH 7,4	50 mM
NaCl	150 mM
EGTA	1 mM
$MgCl_2$	1,5 mM
Igepal	1%
Glicerol	10%
Cóctel de inhibidores de proteasas	10 µL/mL
NaF	50 mM
Pirofosfato sódico	30 mM
Ortovanadato sódico	1 mM
DTT	1 mM

El cóctel de inhibidores de proteasas contenía AEBSF 2 mM, aprotinina 0,3 µM, bestatina 130 µM, EDTA 1 mM, E-64 14 µM y leupeptina 1 µM (Sigma-Aldrich).

El procesado de las muestras, tanto de tejido como de células, se realizó siempre en frío y una vez homogeneizadas se centrifugaron a 15.000 *g*, recogiendo el sobrenadante para la determinación de proteínas.

3.4.2. ELECTROFORESIS

Se realizó de acuerdo a lo expuesto en el apartado 3.3. Se cargaron 40 µg de proteína en cada uno de los pocillos.

3.4.3. ELECTROTRANSFERENCIA

Una vez migradas las proteínas en el gel de poliacrilamida, han de ser fijadas y expuestas para su posterior detección mediante anticuerpos. Para ello se realizó una electrotransferencia de las proteínas. Se trata de poner el gel de acrilamida en contacto en toda su longitud con una membrana de nitrocelulosa y crear un campo eléctrico que empuje a las proteínas a salir del gel. De esta forma, quedan retenidas sobre la membrana y serán susceptibles de unirse a los anticuerpos específicos correspondientes.

La electrotransferencia se realizó en seco (figura 18) por medio del sistema iBlot de Invitrogen. El proceso se desarrolló durante 9 min, a temperatura ambiente y una intensidad constante de 2 voltios (programa 3).

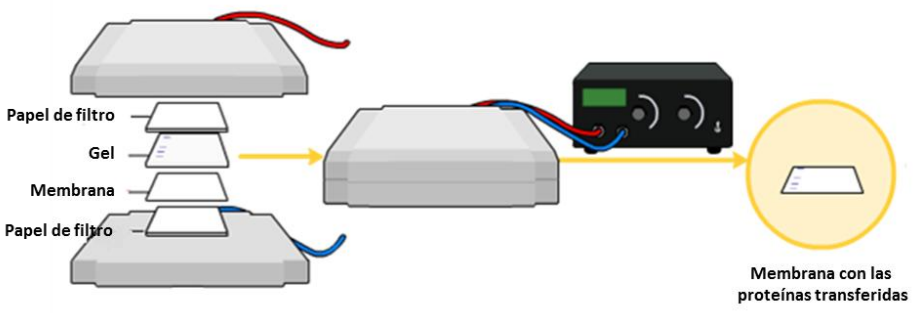


Figura 18. Esquema de la electrotransferencia

3.4.4. INMUNOQUIMIOLUMINISCENCIA

Tras la transferencia se empapó la membrana en una solución de bloqueo para impedir las uniones inespecíficas de los anticuerpos, y a continuación se bañó en la solución que contenía el anticuerpo primario, que se une específicamente a la proteína que se quiere determinar. Tras varios lavados se incubó con el anticuerpo secundario (se fija al primario) que lleva unida una enzima, la HRP (peroxidasa de rábano). Por último y tras lavados, se incubó la membrana con la solución de revelado que contiene peróxido de hidrógeno y luminol. (Santa Cruz). Ambos reaccionan con ayuda de la peroxidasa dando quimioluminiscencia que se puede medir.

Las condiciones de la inmunoquimioluminiscencia fueron:

1. Incubación durante 1 hora a temperatura ambiente en tampón de bloqueo: 5% de leche desnatada en polvo (o albúmina de suero bovino) disuelta en TBS-T (Tris 20 mM, NaCl 137 mM, pH 7,6, Tween-20 0,05%).

2. Tres lavados de 5 min con 15 mL de TBS-T.

3. Las membranas se incubaron 1 hora a temperatura ambiente, o bien a 4ºC toda la noche *(over night*, o/n), con su correspondiente anticuerpo primario (véase tabla 3) bajo agitación orbital en tampón de anticuerpo (1% BSA en TBS-T) y con la correspondiente dilución de trabajo para el anticuerpo primario utilizado (véase tabla 3).

4. Tres lavados de 5 min con 15 mL de TBS-T.

5. Incubación de las membranas durante 1 hora con anticuerpo secundario conjugado con peroxidasa de rábano, disuelto en tampón de bloqueo a la concentración correspondiente (véase tabla 3).

6. Se realizaron 3 lavados de 5 min con 15 mL de TBS-T.

3.4.5. VISUALIZACIÓN

La membrana se incubó 1 min con solución de revelado ECL (Enhanced Chemiluminescence; Santa Cruz). Esta solución es una mezcla 1:1 del reactivo 1 (solución con peróxido de hidrógeno) y reactivo 2 (solución que contiene luminol). La luz emitida se captó mediante una cámara CCD (Gel Doc-Bio-Rad).

3.4.6. CUANTIFICACIÓN DE LOS RESULTADOS

Las imágenes obtenidas se analizaron mediante un programa de análisis de imágenes computarizado (Image Lab-Bio-Rad).

ANTICUERPO	CASA COMERCIAL	MW (Kda)	FUENTE	DILUCIÓN	TIEMPO-Tª
Anti-DNP	Dako	-	Conejo	1:10000	1h. Tª amb
Anti-ERK 1/2	Cell Signaling	44/42	Conejo	1:1000	1h. Tª amb
Anti-JNK	Cell Signaling	54/46	Conejo	1:1000	1h. Tª amb
Anti-Keap1	Cell Signaling	60-64	Conejo	1:1000	o/n 4ºC
Anti-p38α	Santa Cruz	43	Conejo	1:1000	1h. Tª amb
Anti-PDI	Cell Signaling	57	Conejo	1:1000	o/n 4ºC
Anti-p-ERK 1/2	Cell Signaling	44/42	Conejo	1:1000	1h. Tª amb
Anti-p-JNK	Cell Signaling	54/46	Conejo	1:1000	1h. Tª amb
Anti-p-p38α	Cell Signaling	43	Conejo	1:1000	1h. Tª amb
Anti-PP2Ac	Cell Signaling	37	Conejo	1:1000	1h. Tª amb
Anti-Prdx1	Cell Signaling	21	Conejo	1:1000	1h. Tª amb
Anti-Ref1/Ape1	Cell Signaling	34	Conejo	1:1000	o/n 4ºC
Anti-RNH1	Abcam	50	Conejo	1:1000	o/n 4ºC
Anti-SHP1	Abcam	67	Ratón	1:1000	o/n 4ºC
Anti-SHP2	Cell Signaling	72	Conejo	1:1000	o/n 4ºC
Anti-Trx1	Cell Signaling	12	Conejo	1:1000	1h. Tª amb
Anti-α-tubulina	Santa Cruz	55	Ratón	1:1000	1h. Tª amb
Estreptavidina-HRP	Cell Signaling	-	*S. avidinii*	1:2000	o/n 4ºC
Anti-Mouse-HRP	Santa Cruz	-	Cabra	1:20000	1h. Tª amb
Anti-Rabbit-HRP	Santa Cruz	-	Ratón	1:20000	1h. Tª amb

Tabla 3. Anticuerpos utilizados en los *western blotting*

3.4.7. *WESTERN BLOTTING* CON INTERCAMBIO DE BIOTINA: DETERMINACIÓN DE S-NITROSILACIÓN

En 2001, Jaffrey y sus colaboradores publicaron un método para derivatizar específicamente tioles nitrosilados con residuos biotinilados (Jaffrey et al. 2001). Llamaron a este método el "*biotin switch*". Consiste en tres pasos:

- Bloqueo de tioles libres utilizando MMTS (metilmetanotiosulfonato).

- Reducción específica de nitrosotioles utilizando ascorbato.

- Marcaje de los nuevos tioles con un agente de biotinilación (biotina-HPDP), el cual se incorpora a los tioles formando un puente disulfuro.

3.4.7.1. Procesado de las muestras

Se partió de páncreas congelados que se homogenizaron en el tampón que contenía RIPA 1X (Tris 20 mM pH 7,5, NaCl 150 mM, EDTA 1 mM, SDS 0,1%, Igepal 1%) y cóctel de inhibidores de proteasas 10 µL/mL. Se utilizó 1 mL de tampón por cada 100 mg de tejido. Una vez homogeneizadas las muestras se centrifugaron a 15.000 *g*, recogiendo el sobrenadante para la determinación de proteínas.

3.4.7.2. "Biotin switch"

1. Añadir 4 volúmenes de tampón quelante (HEPES 225 mM pH 7,7, EDTA 0,9 mM, neocuproína 90 µM, SDS 2,5%, MMTS 20 mM) e incubar a 50ºC durante 20 min bajo suave agitación.

2. Precipitar con acetona. Añadir 2 volúmenes de acetona guardada a -20ºC. Dejar a temperatura ambiente durante 15 min.

3. Centrifugar a 2000 *g* durante 5 min a 4ºC. Descartar el sobrenadante y lavar el precipitado con acetona fría y volver a centrifugar. Descartar el sobrenadante con cuidado de no afectar al precipitado y dejar que se seque.

4. Resuspender el precipitado en 0,1 mL de tampón HENS (HEPES 250 mM pH 7,7, EDTA 1 mM, neocuproína 0,1 mM, SDS 1%) por mg de proteína en la muestra de partida. Hasta este punto, todos los pasos se llevaron a cabo en oscuridad.

5. Añadir 1/3 de volumen de solución de biotina-HPDP (4 mM en *N,N*-dimetilformamida) y 1/100 de volumen de ácido ascórbico 100 mM. Utilizar un control negativo (muestra a la que sólo se le añade dimetilformamida para determinar la biotinilación endógena de las proteínas.

6. Incubar 1 hora a temperatura ambiente.

7. Precipitar con acetona tal y como se describió anteriormente.

8. Resuspender en tampón HENS como se describió anteriormente.

Una vez alcanzado este punto se procedió a la separación de las proteínas en función de su peso molecular mediante una electroforesis desnaturalizante en gel de poliacrilamida (SDS-PAGE). Una vez completada la electroforesis, se llevó a cabo una electrotransferencia y tras el bloqueo de la membrana se incubó durante toda la noche a 4ºC con estreptavidina-HRP (Cell Signaling) a una dilución 1:2000 bajo agitación orbital en tampón 5% de BSA en TBS-T. La posterior visualización de las proteínas biotiniladas se llevó a cabo utilizando la solución de revelado ECL.

3.4.8. ELECTROFORESIS BIDIMENSIONAL ACOPLADA A *WESTERN BLOTTING* PARA LA DETERMINACIÓN DE CARBONILACIÓN

La electroforesis bidimensional es una técnica de alta resolución cuyo objetivo es la separación de mezclas de proteínas altamente complejas. La base de su elevado poder de resolución está precisamente en la bidimensionalidad, es decir, en que las proteínas son separadas secuencialmente por dos criterios físicos. En primer lugar las proteínas son separadas en un gel con gradiente de pH en condiciones desnaturalizantes de acuerdo con su punto isoeléctrico (isoelectroenfoque). Tras esta separación por carga, las proteínas son separadas de acuerdo con su masa molecular por electroforesis discontinua en gel de poliacrilamida en presencia de SDS (SDS-PAGE). Tras la tinción del gel, las proteínas aparecen formando manchas circulares (spots) (figura 19).

La mayoría de los ensayos para la detección de proteínas carboniladas implican la derivatización del grupo carbonilo con 2,4-dinitrofenilhidrazina (DNPH) que conduce a la formación de un producto estable, dinitrofenil hidrazona que puede ser detectado mediante un anticuerpo anti-DNP.

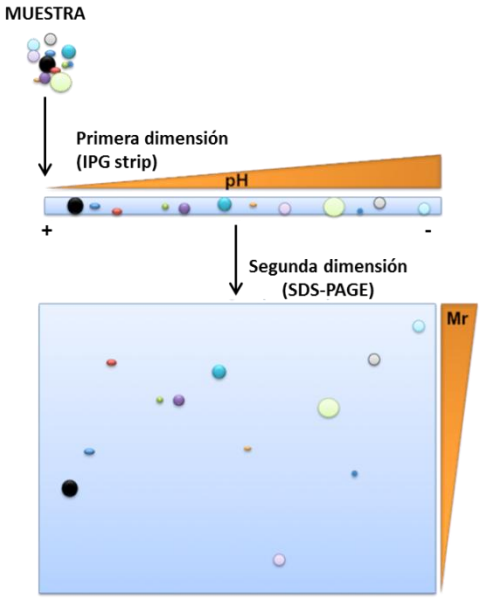


Figura 19. Esquema de electroforesis bidimensional

3.4.8.1. Procesado de las muestras

Se partió de páncreas de rata congelados que se homogenizaron en tampón que contenía Tris-HCl 50 mM pH 7,5, SDS 6% y cóctel de inhibidores de proteasas 10 µL/mL. Se utilizó 1 mL de tampón por cada 100 mg de tejido. A continuación, los homogenados se incubaron durante 3 minutos a 100ºC y se centrifugaron a 10.000 r.p.m. durante 5 minutos. Se recogieron los sobrenadantes y se ajustó la concentración de proteínas a 4 µg/µL.

3.4.8.2. Procedimiento

La electroforesis bidimensional se llevó a cabo según el siguiente protocolo:

1. Se descongelaron las tiras para la realización del isoelectroenfoque. Se utilizaron tiras IPG de 11 cm, pH 5-8 (Bio Rad).

2. Se rehidrató cada tira con 200 µL de tampón de rehidratación (solución de anfolitos y DTT 1M) durante 14 h a 20ºC. Se aplicó la muestra durante la rehidratación. Se cargaron 15 µg de proteína en las tiras que iban ser posteriormente transferidas a una membrana, mientras que se cargaron 40 µg de proteína en las tiras que posteriormente serían sometidas a tinción de plata.

3. Una vez rehidratadas las tiras, se procedió a la realización del isoelectroenfoque. Programa de isoelectroenfoque: a 20ºC; corriente máxima 50 mA/tira. Se siguieron las siguientes fases: 30 minutos a 500 v; 1 h a 1000 v; 1 h a 6000 v; 12 h as 500 v.

4. Tras el isoelectroenfoque, se lavaron las tiras con agua MilliQ para derivatizarse. Se incubaron las tiras con solución de derivatización (DNPH 10 mM en TFA 10%) durante 20 minutos a temperatura ambiente.

5. Lavado de las tiras con solución equilibradora I (urea 6M, Tris-HCl 0,375 M pH 8,8, SDS 2%, glicerol 20% y DTT 2%) durante 15 min y posteriormente con solución equilibradora II (urea 6M, Tris-HCl 0,375 M pH 8,8, SDS 2%, glicerol 20% y IAA 2,5%) durante el mismo tiempo.

6. Se preparó un gel de poliacrilamida al 11%.

7. Se colocaron las tiras sobre el gel de electroforesis. El lado ácido de la tira debe estar orientado hacia el ánodo. A continuación se cubrieron las tiras con aceite mineral.

8. El gel se sometió a un campo eléctrico de voltaje constante (alrededor de 100 voltios) hasta que el frente alcanzó el final del gel.

Tras realizarse la electroforesis, la mitad de los geles se sometieron a tinción de plata, y con la otra mitad se llevó a cabo un *western blot* anti-DNP.

3.5. ELECTROFORESIS DIAGONAL

La electroforesis diagonal es un tipo de electroforesis bidimensional que consiste en una doble separación de las proteínas de una muestra por su tamaño mediante SDS-PAGE, con un tratamiento de la muestras entre ambas electroforesis, que permite analizar diversas modificaciones post-traduccionales, entre ellas la oxidación reversible de proteínas por formación de puentes disulfuro inter- y/o intra-moleculares (Wait et al. 2005). En la primera dimensión, la mezcla de proteínas se somete a una electroforesis bajo condiciones no reductoras en un tampón alquilante, para prevenir la oxidación de los tioles libres. A continuación, se extrae el carril del gel y se trata con un agente reductor. El carril escindido se coloca en la parte superior de un segundo gel y se lleva a cabo una electroforesis bajo condiciones reductoras. La única diferencia que existe entre la primera y la segunda dimensión son las condiciones redox de la electroforesis. Después, se tiñe el gel y se visualizan las proteínas.

Una vez que las proteínas de las muestras en estudio han sido separadas dos veces por su tamaño, aquellas que no contengan puentes disulfuro se dispondrán a lo largo de la diagonal, mientras que aquellas que presenten puentes disulfuro quedarán fuera de la diagonal (Eaton 2006). Las proteínas con puentes disulfuro intermoleculares caen por debajo de la diagonal, ya que cada subunidad migra más rápido que la proteína completa debido a su menor peso. Por el contrario, las proteínas con puentes disulfuro intramoleculares quedan por encima de la diagonal (figura 20), ya que tras la reducción la proteína se despliega y migra más lentamente que cuando está compactada su estructura (Rinalducci et al. 2008).

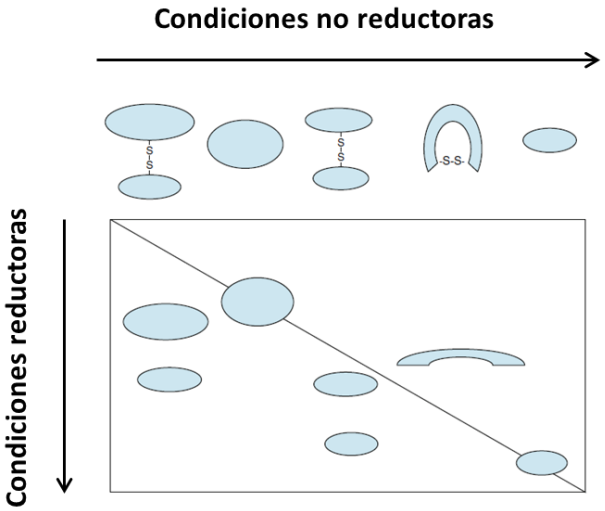


Figura 20. Esquema de separación de las proteínas mediante electroforesis diagonal (McDonagh, 2009)

3.5.1. PROCESADO DE LAS MUESTRAS

Partiendo de páncreas congelados, se homogenizaron los tejidos en tampón RIPA 1X que contenía yodoacetamida (IAM) 75 mM y cóctel de inhibidores de proteasas 10 µL/ml (AEBSF 2 mM, aprotinina 0,3 µM, bestatina 130 µM, EDTA 1 mM, E-64 14 µM, leupeptina 1 µM).

Se utilizó 1 mL de tampón por cada 140 mg de tejido. Se centrifugaron los homogenados a 4 ºC durante 15 min a 15.000 *g.* Se recogieron los sobrenadantes que se incubaron durante 1 hora a 4 ºC para que la IAM actuase.

3.5.2. PROCEDIMIENTO

La electroforesis diagonal se llevó a cabo según el siguiente protocolo (McDonagh, 2009):

1. Se preparó un gel de poliacrilamida al 12% en su zona de separación (*running)* y al 4% en la zona de concentración (*stacking).*

2. Se añadieron 100 µg de proteína en 12 µL de tampón de carga no reductor a cada pocillo. Las muestras se cargaron cada 2 pocillos, es decir dejando un pocillo en medio en el que se cargó sólo tampón de carga, para facilitar la escisión posterior de los carriles del gel.

3. Se realizó la electroforesis en la primera dimensión con un voltaje constante de 90 V hasta que el frente llegó al final del gel. Entonces se escindió el carril entero con un bisturí.

4. El carril entero se incubó con tampón de equilibrio (urea 6 M, Tris-HCl 0,375 M, pH 8,8, SDS 2%, glicerol 20%) con DTT 2% durante 30 min a 42 ^{o}C y agitación suave.

5. Se volvió a incubar el carril en tampón de equilibrio, pero sustituyendo el DTT por IAM 2,5% durante 30 min a 42 ^{o}C y agitación suave.

6. El carril del gel se lavó en tampón de electroforesis (Tris 0,025 M, glicina 0,2 M, SDS 0,1%, pH 8,3).

7. Se calentó una solución de agarosa 0,1% (en tampón de electroforesis) que contenía azul de bromofenol y se colocó una fina capa sobre un gel de separación de poliacrilamida al 12%. Se colocó horizontalmente el carril entero del gel asegurándose de que no quedaban burbujas de aire entre el carril y el gel ya que podrían interferir en la separación proteica.

8. Se realizó la electroforesis hasta que el frente alcanzó el final del gel.

3.5.3. ELECTROFORESIS DIAGONAL CON IODOACETAMIDA BIOTINILADA

La iodoacetamida (IAM) es un agente alquilante que se une covalentemente al grupo tiol de las cisteínas, de forma que las proteínas ya no pueden formar puentes disulfuro. La iodoacetamida se conjuga con la biotina para facilitar la detección de los grupos –SH de las proteínas. La estreptavidina, al igual que la avidina, tiene una gran afinidad por la biotina, lo que permite la detección de proteínas biotiniladas. Además, la unión de la estreptavidina a la biotina es mucho más específica que la de la avidina.

El protocolo fue el mismo que el descrito anteriormente con la única diferencia de que la segunda vez que se incuba el carril en tampón de equilibrio, se hace con iodoacetamida biotinilada (IAM-B) 98mM (Thermo Scientific) en lugar de IAM, y se incrementó el tiempo de incubación a 2 horas para facilitar la interacción de la IAM-B con los grupos tioles.

3.5.4. ELECTROFORESIS DIAGONAL CON IODOACETAMIDA BIOTINILADA Y ELECTROTRANSFERENCIA

El procedimiento fue el mismo que el descrito en el apartado 3.5.3, pero una vez finalizada la electroforesis diagonal se llevó a cabo una electrotransferencia a una membrana de nitrocelulosa que tras su bloqueo con BSA al 5% se incubó durante toda la noche a 4ºC con estreptavidina-HRP (Cell Signaling) a una dilución 1:2000 bajo agitación orbital en tampón 5% de BSA en TBS-T. La posterior visualización de las proteínas biotiniladas se llevó a cabo como en un *western blot* utilizando la solución de revelado ECL.

3.5.5. ELECTROFORESIS DIAGONAL ACOPLADA A *WESTERN BLOTTING*

Una vez realizada la electroforesis diagonal, las proteínas pueden ser electrotransferidas a una membrana de nitrocelulosa para su detección mediante anticuerpos. Así, se siguió el protocolo de la electroforesis diagonal y a continuación se llevó a cabo una inmunodetección siguiendo los mismos pasos que se han descrito anteriormente en el apartado *western blotting* (3.4).

3.6. TINCIONES

3.6.1. TINCIÓN DE PLATA

La tinción de plata se utiliza para la detección de proteínas después de su separación electroforética en geles de poliacrilamida (Chevallet et al. 2006). Esta técnica presenta una excelente sensibilidad (rango de nanogramos), a la vez que es sencilla. Es compatible con el análisis por espectrometría de masas tras la digestión proteica. Las fases de la tinción de plata consisten en una primera fijación de las proteínas seguida de sensibilización e impregnación con plata.

El protocolo seguido para la tinción de plata era compatible para el posterior análisis proteico mediante espectrometría de masas.

3.6.1.1. Fijación

El gel se sumerge durante 30 min en una solución de ácido acético al 5 % y metanol al 50%.

3.6.1.2. Lavado

Dos lavados de 5 min con agua MilliQ.

3.6.1.3. Sensibilización

1 min en una solución de tiosulfato sódico al 0,01%.

3.6.1.4. Lavado

Dos lavados de 1 min con agua MilliQ.

3.6.1.5. Tinción

Se incuba el gel en una solución de nitrato de plata al 0,1% y se mantiene en agitación constante durante 20 min.

3.6.1.6. Lavado

Dos lavados de 1 min con agua MilliQ.

3.6.1.7. Revelado

Incubar el gel en carbonato de sódio al 2% y formalina al 0,04% (formaldehído 35%). Agitar 20-30 segundos (se pone amarilla la solución) y cambiar por solución fresca. Repetir este proceso 3 veces.

3.6.1.8. Fijación

5 min en una solución de ácido acético al 5%.

3.6.2. TINCIÓN DE COOMASSIE

El azul de coomassie es un colorante derivado del trifenilmetano. Se emplea para la tinción de geles de electroforesis, pero presenta una

sensibilidad mucho menor que la tinción de plata. Por tanto, en función de la cantidad de muestra de partida de la que se disponía y del grado de sensibilidad que se quería obtener en la detección de las proteínas en los geles de poliacrilamida, se utilizó la tinción de plata o la de azul de coomassie.

Las fases de la tinción de coomassie consisten en una primera fijación de las proteínas seguida de la tinción con azul de coomassie coloidal G-250.

3.6.2.1. Fijación

1 hora a temperatura ambiente en una solución de etanol al 50% y 10% de ácido acético glacial.

3.6.2.2. Lavado

Tres lavados de 5 min con una solución de metanol al 50% y ácido acético glacial al 10%.

3.6.2.3. Tinción

Se incuba el gel durante 3-4 horas a temperatura ambiente en una solución de metanol al 20%, ácido acético glacial al 10% y azul de coomassie al 0,1%.

3.6.2.4. Destinción

Desteñir el gel mediante 4 lavados de 15 min con una solución de metanol al 25% y ácido acético glacial al 10%.

3.6.3. TINCIÓN DE PONCEAU

La tinción de Ponceau se utiliza para la detección de proteínas después de su transferencia a membranas de nitrocelulosa, acetato de celulosa o PVDF. El tinte Ponceau tiene una sensibilidad similar a la del azul de coomassie, pero al contrario que éste, presenta la capacidad de teñir reversiblemente las proteínas. La tinción es rápida pero no es permanente y puede desaparecer en el procesamiento posterior. Como la tinción desaparece, es compatible con casi todos los procedimientos de detección con anticuerpos.

3.6.3.1. Procedimiento

1. Después de la electroforesis, se sumerge la membrana de nitrocelulosa en una solución de rojo Ponceau al 0,1% en ácido acético al 5%.

2. Desteñir la membrana mediante 3 lavados de 15 segundos con TBS-T.

3.7. DIGESTIÓN DE GELES DE POLIACRILAMIDA

La digestión en gel forma parte de la preparación de la muestra para la identificación de las proteínas por espectrometría de masas en el curso del análisis proteómico. La digestión en gel comprende principalmente la

destinción, la extracción de las proteínas del gel y la escisión proteolítica de las mismas.

3.7.1. DESTINCIÓN

Después de la escisión de la banda de la proteína de interés a partir del gel, es necesario decolorar el trozo de gel que previamente ha sido teñido con azul de coomassie o plata. Para ello se realizan tres lavados de 10 minutos con acetonitrilo:agua (1:1).

3.7.2. EXTRACCIÓN DE LAS PROTEÍNAS DEL GEL

Para la digestión de las proteínas fijadas en la matriz del gel, éstas tienen que ser accesibles a la proteasa. Para ello se procede de la siguiente manera:

1. Se añade al gel una solución de acetonitrilo al 50% durante 30 minutos y bajo agitación para reducir el tamaño de los trozos.

2. Eliminar el disolvente y secar los trozos de gel en un evaporador de vacío.

3.7.3. ESCISIÓN PROTEOLÍTICA DE LAS PROTEÍNAS

En esta fase del proceso, las proteínas son cortadas enzimáticamente en un número limitado de péptidos que permiten su identificación mediante su masa y el patrón de digestión característico de la enzima o huella peptídica. La tripsina es la enzima más comúnmente utilizada en el análisis de proteínas. La

tripsina corta específicamente el enlace peptídico en el extremo carboxilo de los aminoácidos básicos arginina y lisina.

1. Añadir la solución de tripsina (Promega, Madison, WI) a una concentración de 60 ng/µL disuelta en bicarbonato amónico 50 mM pH 8,8 y mantener durante 2 horas en hielo.

2. Dejar incubando 12 horas a 37^{o}C. La digestión se para mediante la adición de TFA al 10%.

3. Secar los sobrenadantes y resuspender las proteínas digeridas en 10 µL de ácido fórmico al 0,1%.

3.8. IDENTIFICACIÓN PROTEICA MEDIANTE ESPECTROMETRÍA DE MASAS

La espectrometría de masas es una técnica instrumental universal y específica que permite la identificación inequívoca de una sustancia.

El principio de la espectrometría de masas es la producción de iones a partir de compuestos neutros y la observación de la subsiguiente descomposición de esos iones. Estos iones descompuestos se mueven rápidamente y son separados en función de su relación masa/carga (m/z). El espectrómetro de masas no sólo caracteriza los fragmentos, sino que además mide la cantidad de ellos que se forman.

Los espectrómetros de masas son instrumentos sofisticados que en general constan de (figura 21):

• **Un sistema de introducción de la muestra,** que puede ser entre otros un cromatógrafo de líquidos.

• **Una fuente de ionización,** que es donde se produce la fragmentación molecular característica de cada compuesto.

• **Un analizador** que separa los fragmentos iónicos generados en función de su relación masa/carga.

• **Un detector** que recoge y caracteriza los fragmentos iónicos que salen del analizador.

En los distintos análisis por espectrometría de masas que se llevaron a cabo para la realización de los experimentos en la presente Tesis se utilizó como sistema de introducción de la muestra un cromatógrafo de líquidos de alta resolución (HPLC). Las técnica de HPLC (si fue en fase reversa o no) y la separación variaron en función del tipo de muestra de partida y del servicio de proteómica en el cual se llevó a cabo el proceso.

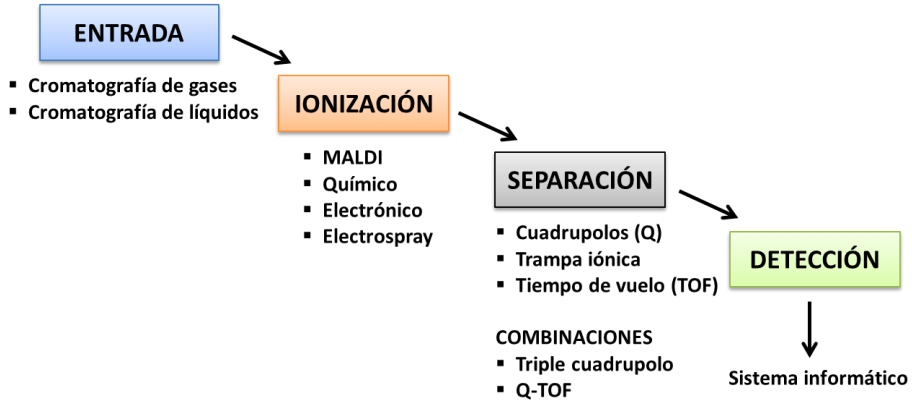


Figura 21. Esquema de los componentes de los espectrómetros de masas

3.8.1. LC-MS/MS EN FASE REVERSA

La identificación de las proteínas por LC-MS/MS en fase reversa se llevó a cabo en el servicio de proteómica perteneciente al Centro de Investigación Príncipe Felipe.

La cromatografía líquida en fase reversa (LC-RP) consiste en una fase estacionaria apolar y una fase móvil de polaridad moderada.

El análisis mediante LC-RP-MS/MS se llevó a cabo en el sistema Easy-nLCII acoplado al sistema de separación mediante trampa iónica LTQ-Orbitrap-Velos-Promass (Thermo Scientific, Waltham, MA, USA).

El analizador de masas tipo trampa iónica se caracteriza porque tanto la ionización, como la separación y la detección de los iones tiene lugar en el mismo espacio, produciéndose de forma secuencial en el tiempo. Este tipo de analizador es muy útil para obtener espectros de masas en los que se filtran todos los iones comprendidos en un rango masa/carga determinado. A diferencia de los analizadores de cuadrupolo, en los analizadores de trampa de iones, los espectros se obtienen sin que se produzca una considerable pérdida de sensibilidad.

Los péptidos de la muestra se cargaron en un cromatógrafo de líquidos de alta resolución (HPLC) en fase reversa que utilizaba una precolumna 0,1-20 mm C18-RF (Proxeon) y después se separaron en una columna 0,075-100 mm C18-FR con un flujo de 0,3 µl/min. Los péptidos fueron eluídos durante 90 min mediante un gradiente de 5 a 40% de B (A: ácido fórmico al 0,1%; B: acetonitrilo al 80%). La ionización se realizó mediante un electrospray de 30

micras de diámetro. La resolución de la separación del Orbitrap se fijó en 30.000.

3.8.2. LC-MS/MS EN FASE NORMAL

La identificación de las proteínas mediante LC-MS/MS en fase normal (LC-NP) se llevó a cabo en el Servicio Central de Soporte a la Investigación Experimental (SCSIE) de la Universidad de Valencia.

La cromatografía líquida en fase normal fue el primer tipo de sistema HPLC utilizado en el campo de la química, y se caracteriza por separar los compuestos en base a su polaridad. Esta técnica utiliza una fase estacionaria polar y una fase móvil apolar.

Las muestras que iban a ser analizadas por LC-NP-MS/MS fueron purificadas previamente mediante el kit Dynabeads® MyOne™ Streptavidin T1 (Invitrogen) para enriquecer la muestra en las proteínas que contenían BIAM (iodoacetamida biotinilada).

La muestra se cargó en una columna de trampa iónica (NanoLC, 3µ C18-CL, 75 µm x 15 cm; Eksigen) y fue desalada con TFA al 0,1% durante 10 min a un flujo de 2 µL/min. A continuación, los péptidos atravesaron una columna analítica (LC, 3µ C18-CL, 75 µm x 25 cm, Eksigen) equilibrada con acetonitrilo al 5% y ácido fórmico al 0,1%. La elución se llevó a cabo mediante un gradiente lineal de 5 a 40% de B (A: ácido fórmico al 0,1%; B: acetonitrilo) con un flujo constante de 300 nL/min. Los péptidos se analizaron en un espectrómetro de masas nanoESI Q-TOF (5600 TripleTOF, ABSCIEX).

3.8.3. PROCESADO DE LOS DATOS OBTENIDOS POR ESPECTROMETRÍA DE MASAS. IDENTIFICACIÓN PROTEICA USANDO EL PROGRAMA MASCOT

El MASCOT es un potente programa desarrollado por *Matrix Science* para el análisis de datos proteómicos en red. Este programa tiene recogida numerosa información sobre masas asignadas a infinidad de péptidos. El MASCOT utiliza todas las bases de datos de péptidos y proteínas disponibles (NCBInr, Trembl, Swissprot, Java script) y compara los datos recogidos mediante análisis con todos los patrones de péptidos y proteínas conocidos hasta la fecha. Los datos integrados en MASCOT son analizados estadísticamente y al resultado de las proteínas identificadas se le asigna un valor o índice de probabilidad.

El valor o índice de probabilidad es 10*Log (p), donde "p" es la probabilidad de que el resultado sea un evento casual ($p<0,05$). Si el péptido o la proteína identificada presentan un score ≥ al asignado por la búsqueda, indica que los datos del análisis coinciden con la proteína asignada o presenta una alta homología.

El MASCOT permite realizar varios tipos de rastreos de proteínas, pero en esta Tesis se ha realizado a partir del espectro iónico de masas. Mediante este sistema, el MASCOT realiza una búsqueda a partir de los picos obtenidos en la espectometría de masas y se comparan con un listado de picos conocidos. Concretamente, las listas de picos fueron generados directamente desde archivos Wiff QSTAR por MASCOT Daemon versión 2.2.2 (Matrix Science) con opciones de filtro de importación utilizando los parámetros por defecto. La búsqueda de base de datos se hizo en la base de datos de proteínas Expasy (515.203 secuencias; 181.334.896 residuos). Los parámetros de búsqueda se

fijaron sin ninguna restricción taxonomía, dos rupturas perdidas y una tolerancia en la medición de la masa de 50 ppm.

3.9. DETERMINACIÓN DE LAS ACTIVIDADES DE PROTEÍN FOSFATASAS

Las actividades de las proteín fosfatasas *in vivo* se determinaron en páncreas de rata a partir de tejido fresco, y se utilizaron 5 ratas por condición.

Para llevar a cabo este análisis, se utilizaron kits comerciales para determinar las actividades serín/treonín y tirosín fosfatasas (Promega) según el protocolo descrito anteriormente por nuestro laboratorio en (Sandoval et al. 2009). El método utilizado para cuantificar el fosfato libre generado en la reacción es un método no radioactivo que mide espectrofotométricamente la absorbancia de un complejo formado por una sonda de molibdato/verde malaquita unida a fosfatos.

El rango de sensibilidad en la liberación de fosfatos en este sistema es de 100-4000 picomoles de fosfato. La parte inferior de este rango es menos sensible que los métodos radioactivos. Trabajando en la parte alta del rango se reduce la posibilidad de detectar actividades inespecíficas producidas por la gran reactividad de algunos fosfatos. En este rango de sensibilidad, se pueden detectar niveles de fosfato del orden nanomolar. Con este método se puede medir la actividad de diversas fosfatasas en muestras parcialmente purificadas y también en extractos de tejidos celulares.

El principio del método es igual para medir la actividad de todas las fosfatasas citosólicas. No obstante, lo que determinará la especificidad de una

fosfatasa u otra será el sustrato fosforilado, el tampón de reacción y los correspondientes inhibidores de fosfatasas utilizados.

3.9.1. TAMPONES PARA LA DETERMINACIÓN DE LAS ACTIVIDADES PROTEÍN FOSFATASAS

Como la mayoría de las familias de enzimas, las fosfatasas poseen diversas condiciones óptimas y no existe un tampón universal para todas ellas.

Los tampones fosfato no son compatibles y pueden interferir con el análisis dependiendo de su concentración, pureza y estabilidad en medio ácido.

Algunos detergentes pueden usarse a una concentración ≤0,1%, pero concentraciones superiores pueden generar señales inespecíficas. Si fuera necesario utilizar una concentración elevada de un detergente, la misma cantidad del mismo se debería añadir al patrón para corregir las posibles señales inespecíficas.

Los tampones idóneos para fosfatasas generalmente incluyen un agente reductor a bajas concentraciones, un quelante de cationes divalentes y distintos inhibidores de proteasas. Hasta un 1% de detergente (ej. Tritón X-100) puede ser usado para analizar proteínas de membrana, si la dilución de la muestra es apropiada.

Se escoge el tampón adecuado dependiendo de si se van a analizar fosfatasas de membrana o intracelulares.

El método que se ha utilizado en la presente Tesis es compatible con numerosos tampones, agentes reductores, detergente y glicerol.

3.9.2. PROCESADO DE LAS MUESTRAS

En primer lugar, para la medida de las actividades fosfatasas se partió de tejido pancreático de rata congelado a -80ºC, el cual fue homogeneizado en el siguiente tampón de homogeneización:

Tris-HCl (pH 7,4)	50 mM
NaCl	150 mM
EDTA	2 mM
Tritón X-100	0,1%
Cóctel de inhibidores de proteasas	10 µL/mL

Este tampón de homogeneización no lleva DTT, ya que se pretendía estudiar si los cambios en las actividades fosfatasas durante la pancreatitis aguda son debidos a la oxidación de estas proteínas.

El tejido se homogeneizó sobre hielo durante 30 segundos usando 1 g de tejido en 3 mL de tampón de homogeneización. A continuación se centrifugaron los homogenados a 25.000 *g* a 4ºC durante 1 h, y se recogió el sobrenadante.

Los extractos de tejidos suelen contener concentraciones milimolares de fosfato libre que podrían interferir con el análisis. Además, altas concentraciones de ATP pueden incrementar el ruido de fondo y producir fosforilación por contaminación de quinasas. Por ello, tras la homogeneización

se eliminaron estos componentes de las muestras utilizando cromatografía de exclusión.

La cromatografía de exclusión (también llamada cromatografía de filtración en gel) se basa en la diferencia de penetración de las moléculas en los poros de la fase estacionaria porque la separación obtenida depende del tamaño de la molécula. El tiempo de elución es proporcional al peso molecular de las mismas, por lo que no es muy usada con los compuestos de alto peso molecular. Este tipo de separación por tamaño difiere de las demás técnicas de cromatografía en que no existen interacciones físicas o químicas entre el analito y la fase estacionaria. Es una técnica reproducible y rápida.

La fase estacionaria está formada por partículas poliméricas o de sílice que contienen una red uniforme de poros por los que pueden penetrar las moléculas de pequeño tamaño. Las moléculas de tamaño grande se excluyen totalmente y son eluídas en primer lugar, mientras que las de pequeño tamaño tienen acceso a todo el volumen poroso y son las últimas que se eluyen. Los diferentes tipos de partículas usadas deben ser estables mecánica y químicamente, tener bajo contenido en grupos iónicos, uniformidad de poro y tamaño. Los compuestos pueden ser derivados de dextranos (Sephadex), derivados de agarosa (Sepharosa), derivados de acrilamidas (Biogel P) y esferas de vidrio. Hay diferentes tamaños de partícula para un gel: a menor tamaño mayor resolución.

Las columnas utilizadas presentaban una fase estacionaria compuesta por Sephadex® G-25. Los kits proporcionan las columnas y resinas para realizar este paso.

Para la eliminación de los fosfatos por cromatografía de exclusión se procedió de la siguiente manera:

1. Se resuspendió la resina Sephadex® G-25 con agitación suave (no vortear).

2. Se pipetearon 10 mL de la suspensión de Sephadex® y se colocaron en la columna. Se dejó que drenara por gravedad en un tubo de 50 mL. Se eliminó el eluído.

3. Se añadieron 10 mL de tampón de equilibrio (Tris-HCl 50 mM, pH 7,4, NaCl 150 mM, EDTA 2 mM, Tritón X-100 0,1%) frío a la columna. Se dejó que eluyera por gravedad. Se centrifugó a 600 *g* durante 2 min en otro tubo de 50 mL para eliminar el exceso de tampón en la resina.

4. Se colocó la columna con el adaptador en un tubo de 50 mL y se añadieron 250 µl de homogenado. No usar volúmenes más grandes de muestra porque se reduce la eficacia en la eliminación de los fosfatos.

5. Se centrifugó a 600 *g* durante 2 min a 4ºC. La muestra salió al final del tubo.

6. Las columnas se lavaron dos veces con 25 mL de agua bidestilada y se guardaron a 4ºC en tampón de almacenamiento (Tris-HCl 10 mM, pH 7,5, EDTA 1 mM, azida sódica 0,02%) de forma que pueden reutilizarse.

3.9.3. SUSTRATOS FOSFORILADOS

El kit para medir la actividad serín/treonín fosfatasa (serine/threonine phosphatase assay system, Promega) proporciona dos fosfopéptidos que son

reconocidos por estas enzimas, y el kit para medir la actividad tirosín fosfatasa (tyrosine phosphatase assay system, Promega) proporciona un sustrato general para tirosín fosfatasas.

Otros sustratos naturales de las fosfatasas de interés pueden ser usados (Sandoval et al. 2009). Algunas de estas proteínas sustrato vienen purificadas parcialmente de la casa comercial, pero usualmente requieren una diálisis para remover los fosfatos libres antes de su uso.

3.9.4. INHIBIDORES DE FOSFATASAS

Existe una variedad de inhibidores de fosfatasas con espectros de acción distintos que permiten modificar de forma sencilla las condiciones experimentales para inhibir unas fosfatasas u otras de forma más o menos específica. La tabla 4 muestra la acción de los inhibidores más utilizados sobre las actividades fosfatasas (Mitsuhashi et al. 2001; Swingle et al. 2007).

ENZIMA	ACTIVIDAD ENZIMÁTICA EN PRESENCIA DEL INHIBIDOR						
CLASE DE FOSFATASA	VANADATO	NaF	EDTA (Sin Mg^{2+})	EGTA (Sin Ca^{2+})	ÁCIDO OKADAICO	TRIFLUORO-PERAZINA	TAUTOMICETINA
PTPs	-	++++	++++	++++	++++	++++	++++
PP1	++++	++++	++++	++++	++++	++++	-
PP2A	++++	-	++++	++++	-	++++	++++
PP2B	++	-	++++	-	++++	-	++++
PP2C	++++	-	-	++++	++++	++++	++++

Tabla 4. Efecto de inhibidores de fosfatasas sobre la actividad de las tirosín fosfatasas (PTPs) y sobre serín/treonín fosfatasas (PPPs). ++++ alta actividad; ++ moderada actividad; - actividad nula.

3.9.5. PROCEDIMIENTO

1. Se determinó la concentración de proteínas de las muestras mediante el método del BCA y se igualaron las concentraciones de todas las muestras a 285,7 ng/ µL.

2. Se preparó la dilución de la sonda de molibdato. Se añadieron 10 µL de la sonda de molibdato a 1 mL de la solución de dilución de la sonda.

3. Se preparó el fosfopéptido que se utilizó como sustrato a una concentración 1 mM con agua libre de fosfatos (proporcionada en el Kit). Por lo general, se usan 5 µL del sustrato reconstituído en 50 µL de reacción (100 µM), dando como resultado una liberación de fosfatos superior a 5 nanomoles.

4. Se construyó una curva patrón de fosfatos, diluyendo el estándar de fosfato 1 mM (proporcionado en el kit) en agua libre de fosfatos. Se generó una solución 50 pmol/µL (50 µM) y se prepararon diluciones que contenían 0, 100, 200, 500, 1000 y 2000 pmol/µL en 50 µL de tampón de reacción 1X.

5. Se prepararon 50 µL de cada punto de la recta patrón en los pocillos de reacción. Se realizaron triplicados de cada punto de la recta.

6. Se añadieron 10 µL de tampón 5X de la fosfatasa en estudio y 5 µL del fosfopéptido directamente en los pocillos de una placa de 96 pocillos de fondo cónico (Greiner Bio-One), evitando la formación de burbujas. Se realizaron triplicados de cada muestra.

7. Se atemperaron las muestras a temperatura ambiente durante dos minutos y se empezó la reacción añadiendo 35 µL de la dilución de la muestra en el pocillo de reacción.

8. Incubación de la reacción durante 30 min.

9. Se paró la reacción añadiendo 50 µL de la solución de la sonda de molibdato (1 volumen de reacción). También se añadieron 50 µL de la solución de la sonda en los pocillos de la recta patrón.

10. Se colocó la placa de 96 pocillos a temperatura ambiente. Una vez la coloración de la solución se ha completado, el color se mantiene estable durante al menos 2 h.

11. Lectura de la densidad óptica de las muestras a 630 nm a los 15 y 30 min después de haber añadido la sonda.

3.9.6. CÁLCULOS

Con la ecuación de la recta obtenida a partir de los datos de la recta patrón de fosfatos, se interpolaron las concentraciones de fosfatos en las muestras. Dividiendo la concentración de fosfatos por el tiempo de incubación y por la concentración de proteínas se obtuvo la actividad fosfatasa de cada muestra.

La actividad fosfatasa se expresa en nmol de PO_4^{2-} liberados/min x mg proteína.

3.9.7. TAMPÓN Y SUSTRATO UTILIZADOS PARA DETERMINAR LA ACTIVIDAD TIROSÍN FOSFATASA CITOSÓLICA

La actividad tirosín fosfatasa citosólica total se determinó en homogenado de páncreas de rata centrifugados a 100.000 *g*, eliminando así las tirosín fosfatasas de membrana. Se utilizó un sustrato con afinidad por las tirosín fosfatasas. Este sustrato viene proporcionado por el kit de actividad tirosín fosfatasa (Promega), y se trata de un péptido de 1,33 KDa con un residuo de tirosina fosforilado y cuya secuencia es D-A-D-E-pY-L-I-P-Q-Q-G.

TAMPÓN DE REACCIÓN 5X PARA LA ACTIVIDAD TIROSÍN FOSFATASA CITOSÓLICA (Jarvis et al. 2006)

Tris-HCl	100 mM, pH 6,8
EDTA	5 mM
EGTA	5 mM
NaF	125 mM

3.9.8. TAMPONES Y SUSTRATO UTILIZADOS PARA DETERMINAR LA ACTIVIDAD PP1, PP2A, PP2B (CALCINEURINA) Y PP2C.

El sustrato utilizado para determinar la actividad de las serín/treonín fosfatasas analizadas fue el mismo en los cuatro casos. Este sustrato viene proporcionado en el kit para medir la actividad serín/treonín fosfatasa (Promega), y es un péptido de 0,75 KDa fosforilado en un residuo de treonina, cuya secuencia es R-R-A-pT-V-A.

Lo que diferenció la determinación de las actividades de las serín/treonín fosfatasas fueron los tampones de reacción, que fueron los siguientes:

TAMPÓN DE REACCIÓN 5X PARA LA ACTIVIDAD PP1

Imidazol	250 mM, pH 7,2
EDTA	1 mM
β-mercaptoetanol	0,1%
BSA	0,5 mg/mL

TAMPÓN DE REACCIÓN 5X PARA LA ACTIVIDAD PP2A

Imidazol	250 mM, pH 7,2
EDTA	1 mM
β-mercaptoetanol	0,1%
BSA	0,5 mg/mL
Tautomicetina	50 nM

TAMPÓN DE REACCIÓN 5X PARA LA ACTIVIDAD PP2B

Imidazol	250 mM, pH 7,2
EGTA	1 mM
β-mercaptoetanol	0,1%
Calmodulina	250 µg/mL
$NiCl_2$	50 mM

TAMPÓN DE REACCIÓN 5X PARA LA ACTIVIDAD PP2C

Imidazol	250 mM, pH 7,2
EGTA	1 mM
β-mercaptoetanol	0,1%
BSA	0,5 mg/mL

Estos tampones fueron utilizados de acuerdo con las instrucciones del fabricante. En el caso del tampón de la PP2A, se añadió la tautomicetina porque a una concentración final de 10 nM es capaz de inhibir específicamente a la PP1 sin alterar la función de la PP2A.

3.10. TRANSFECCIÓN DE CÉLULAS 266-6

La transfección consiste en la introducción de material genético externo en células eucariotas mediante plásmidos u otros mecanismos. La transfección de células animales generalmente se lleva a cabo abriendo poros transitorios en la membrana plasmática mediante electroporación para permitir el paso del material genético. Además de la electroporación, se pueden utilizar otras técnicas para efectuar la transfección, como por ejemplo liposomas que se fusionan con la membrana plasmática celular y depositarán su carga dentro de la célula.

Cuando la transfección es llevada a cabo mediante vectores virales se denomina transducción. En estos casos, la eficiencia de la transfección es mucho mayor. La transducción puede llevarse a cabo mediante distintos tipos de virus como adenovirus, retrovirus y lentivirus. En este trabajo se ha ultilizado el sistema de lentivirus pGIPZ shRNAmir de Thermo Scientific.

Las principales características del vector pGIPZ se resumen en la figura 22:

- Expresión de TurboGFP, que permite visualizar al microscopio de fluorescencia si se ha producido la transducción.

- Expresión de un shRNAmir que se une al RNA mensajero diana y así se silencia la proteína de interés.

- Presentar resistencia a la puromicina, lo que permite la selección estable de las células transfectadas.

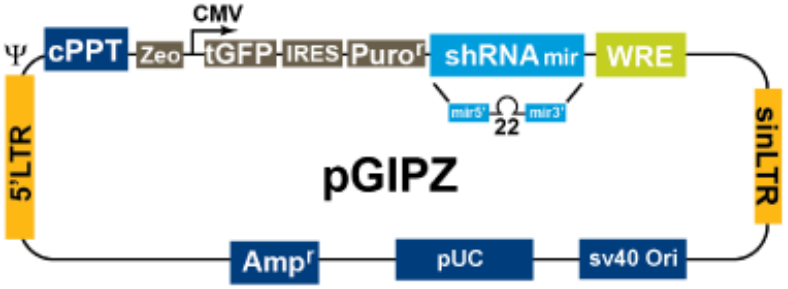


Figura 22. Vector pGIPZ del lentivirus

3.10.1. SIEMBRA Y TRANSFECCIÓN

Las células 266-6 fueron transfectadas con partículas víricas que habían sido sintetizadas por Thermo Scientific Open Biosystems y cuyo vector pGIPZ expresaba un shRNAmir que se unía al RNA mensajero de la subunidad

catalítica de la PP2A y así anulaba su expresión. Como control negativo se utilizó un vector pGIPZ que no expresaba ningún shRNAmir.

1. El día 1 se siembran 50.000 células 266-6 por pocillo en una placa de 24 pocillos. Se deja que se fijen a la placa y se repliquen hasta el día siguiente.

2. Al día siguiente (día 2), se quita el medio en el que se encuentran las células. Se añade medio nuevo sin suero ya que éste puede disminuir la eficiencia de la transducción. A continuación, se añade una dilución 1/10 de las partículas víricas.

3. A las 6 horas post-transducción se añadió 1 mL de medio completo (que contenía suero).

3.10.2. SELECCIÓN CON PUROMICINA

El día 4 se examinan las células al microscopio de fluorescencia para comprobar que la transducción ha sido efectiva. Es muy difícil que el 100% de las células hayan sido transfectadas. Para eliminar las células que no hayan incorporado el vector, se añade al medio de cultivo el antibiótico puromicina (Santa Cruz) a una concentración de 4 µg/mL. De esta forma, las células que hayan incorporado el vector, y que por tanto hayan sido transducidas, presentarán resistencia a la puromicina y sobrevivirán mientras que el resto de células morirá. Cada 2-3 días se reemplaza el medio de selección.

Una vez se haya conseguido eliminar todas las células no transfectadas (a la semana de añadir la puromicina), se baja la concentración de puromicina a 1 µg/mL.

3.10.3. SELECCIÓN MEDIANTE CITOMETRÍA DE FLUJO

Una vez se ha conseguido la selección estable con puromicina, se analizaron las células por citometría de flujo para seleccionar aquellas que expresaban con mayor intensidad la fluorescencia del GFP. TurboGFP y shRNAmir son parte de un transcrito bicistrónico, lo que permite la visualización de las células que expresan el shRNAmir. De esta forma, cuanto mayor sea la intensidad de la fluorescencia observada, la transducción habrá sido más eficaz y mayor será la expresión del shRNAmir.

La citometría de flujo es una tecnología biofísica basada en la utilización de luz láser para el recuento y clasificación de células según sus características morfológicas y presencia de biomarcadores. En los citómetros de flujo, las células en suspensión circulan de una en una a través de un finísimo capilar transparente sobre el que incide un delgado haz de luz láser, y la luz transmitida y dispersada por la hilera de células a través del capilar se recoge por medio de unos dispositivos de detección, permitiendo hacer inferencias en cuanto a tamaño y complejidad de las células. Permite el análisis multiparamétrico simultáneo de diversas características físicas y químicas, evaluando en promedio más de dos mil partículas por segundo.

El citómetro de flujo utilizado en este trabajo fue el MoFlo^{TM} XDP de la marca Beckman coulter (figura 23), que permite, además de separar las células, cuantificarlas.

Figura 23. MoFlo™ XDP de Beckman coulter

El protocolo de selección fue el siguiente:

1. A partir de una placa Petri de 100 mm de diámetro con células control 266-6 confluentes y otra placa Petri con células transducidas confluentes, se despegaron las células de la placa tras incubarse durante 3 min con 20 µL de tripsina-EDTA.

2. Se centrifugaron las células durante 5 minutos a 2500 r.p.m.

3. Se descartó el sobrenadante y se resuspendieron las células en medio completo sin puromicina.

4. Se contaron las células mediante un contador Coulter.

5. Se introdujeron 2.000.000 de células control 266-6 en el citómetro para eliminar el ruido de fondo que pueda generar la propia autoflorescencia que presentan las células 266-6.

6. Una vez fueron pasadas por el citómetro las células control, se introdujeron en él 2.000.000 de células transducidas.

7. Una vez el aparato generó el espectro de fluorescencia, se seleccionaron el 5% de células transducidas que presentaban mayor intensidad de fluorescencia en la zona verde del espectro visible.

8. Del 5% de células seleccionadas, se sembró una sola célula por pocillo en una placa de 96 pocillos con medio de selección y se esperó a que fueran creciendo.

9. Una vez que la población de células fue suficiente, se realizó un *Western blot* para comprobar que se había conseguido silenciar la PP2Ac. Cuando se confirmó el silenciamiento se procedió a la realización del resto de experimentos.

3.11. AISLAMIENTO DEL ARN DE CÉLULAS

Para la extracción de ARN total de células se utilizó como reactivo TRIZOL® Reagent (Ambion). Este método se basa en el uso de una solución monofásica de fenol e isotiocianato de guanidina para la lisis de las células y la separación de la muestra en dos fases (acuosa y orgánica).

3.11.1. PROCEDIMIENTO

Para la correcta extracción del ARN, todo el material como puntas, tubos eppendorfs, etc., estaban debidamente limpios (libres de RNasas) y esterilizados. Los tampones deben estar autoclavados o filtrados. Siempre que sea posible, trabajar en campana.

Para la extracción del ARN de células se siguió el siguiente protocolo:

1. Tras el periodo de incubación de las células con los distintos tratamientos, las placas Petri de 100 mm se lavaron con 1 ml de PBS y se añadió 0,5 mL de trizol mediante el cual se despegaron y recogieron las células.

2. Se añadió 100 µL de cloroformo.

3. Se agitó la mezcla durante 15 segundos y se dejó reposar 3 min.

4. Centrifugación a 13.000 r.p.m, 10 min a 4ºC.

5. Se recogió la fase de arriba sin apurar demasiado para evitar la contaminación del ARN.

6. Precipitación con 250 µL de isopropanol.

7. Se agitó y dejó reposar a temperatura ambiente durante 7 min.

8. Centrifugación a 13.000 r.p.m, 10 min a 4ºC.

9. Se quitó el isopropanol y se lavó el precipitado con 500 µL de etanol al 75%.

10. Centrifugación a 13.000 r.p.m, 10 min a 4ºC.

11. Se eliminó el etanol y se dejó secar el ARN.

12. Se resuspendió el precipitado de ARN con 25 µL de agua destilada tratada con dietilpirocarbonato (DEPC) 0,1% (agua DEPC).

13. Mediante el Nanodrop se midió la absorbancia de 1 µL de muestra a 260 nm y se utilizó como blanco 1 µL de agua DEPC para conocer la concentración de ARN. Además, el Nanodrop proporciona la pureza de la muestra mediante el cociente entre las absorbancias a 260 nm (longitud a la que absorbe el ARN) y a 280 nm (longitud a la que absorben las proteínas). La pureza del ARN era apropiada si el valor del cociente 260/280 estaba por encima de 1,7 y por debajo de 2,4.

3.12. RETROTRANSCRIPCIÓN DEL ARN

Consiste en la obtención de cDNA a partir de un ARN mensajero, el proceso inverso de la transcripción. Para ello son necesarias unas ADN polimerasas específicas, llamadas transcriptasas inversas o retrotranscriptasas. Las enzimas utilizadas proceden de algunos retrovirus (virus que presentan ARN como genoma, en vez de ADN). La transcipción reversa fue realizada utilizando RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific).

3.12.1. PROCEDIMIENTO

1. Se prepara una mezcla que contenga los componentes del kit expuestos en la tabla 5 en función del número de muestras a analizar. Se cargan 6 µl en cada eppendorf de 0,2 mL.

COMPONENTES DEL KIT	VOLUMEN (µl) POR MUESTRA
dNTP mix 10 mM	1
Reaction buffer 5X	2
Random hexamer primers	0,83
RiboLock RNase inhibitor	0,5
RevertAid H Minus M-MuLV Reverse Transcriptase	0,5
Agua libre de RNasas	1,17
TOTAL	**6**

Tabla 5. Reactivos y sus volúmenes para la retrotranscripción

2. El volumen de muestra necesario para una correcta transcripción es de 4 µl con una concentración de partida de ARN de 500 ng/µL. De esta forma, la concentración final de ARN en la mezcla es de 200 ng/µL.

3. Se agita suavemente para que se mezclen bien todos los componentes de la mezcla con la muestra y se centrifuga para evitar posibles burbujas de aire que puedan interferir con una buena retrotranscripción. Es importante que durante todo el proceso tanto la placa como los reactivos se encuentren en hielo en todo momento.

4. Se llevan los eppendorfs al termociclador.

5. Una vez finalizado todo el proceso, la concentración de cDNA obtenido en cada muestra es de 200 ng/µL en un volumen de 10 µL.

3.12.2. CICLOS TÉRMICOS DE LA AMPLIFICACIÓN

Los ciclos térmicos de la retrotranscripción son las que se muestran en la figura 24.

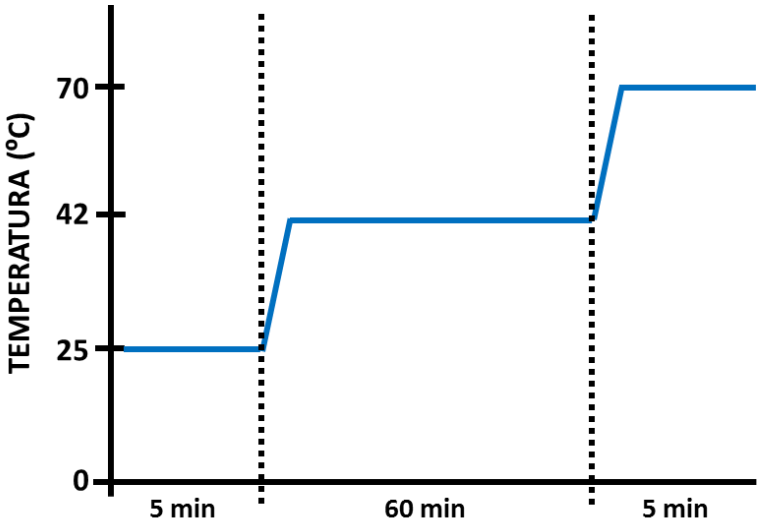


Figura 24. Ciclos térmicos de la retrotranscripción

3.13. AMPLIFICACIÓN CUANTITATIVA DEL ADN (PCR EN TIEMPO REAL)

La reacción en cadena de la polimerasa (PCR) cuantitativa o también llamada PCR en tiempo real (RT-PCR) es uno de los métodos más sensibles y exactos para la detección de niveles de ADN tanto en células como en tejidos. A diferencia de la PCR convencional (en punto final), que mide la acumulación del ADN al final de un número predeterminado de ciclos, con la PCR cuantitativa tiene lugar un proceso de amplificación usando una sonda fluorescente, de forma que su aumento es proporcional a la cantidad de ADN formado hasta llegar a saturación.

La reacción en cadena de la polimerasa utiliza dos fragmentos cortos de ADN (oligonucleótidos) como cebadores de la síntesis. Estos cebadores o *"primers"* se unen específicamente a las secuencias que rodean la región a amplificar, uno en cada cadena del ADN.

El proceso se desarrolla siguiendo tres pasos que se repiten tantas veces como sea necesario para el gen a amplificar (figura 25):

- **Desnaturalización:** Separación de las cadenas complementarias del ADN.

- **Unión de los *"primers"* específicos**, que se realiza a una temperatura específica (según el primer).

- **Extensión:** Síntesis de la hebra complementaria.

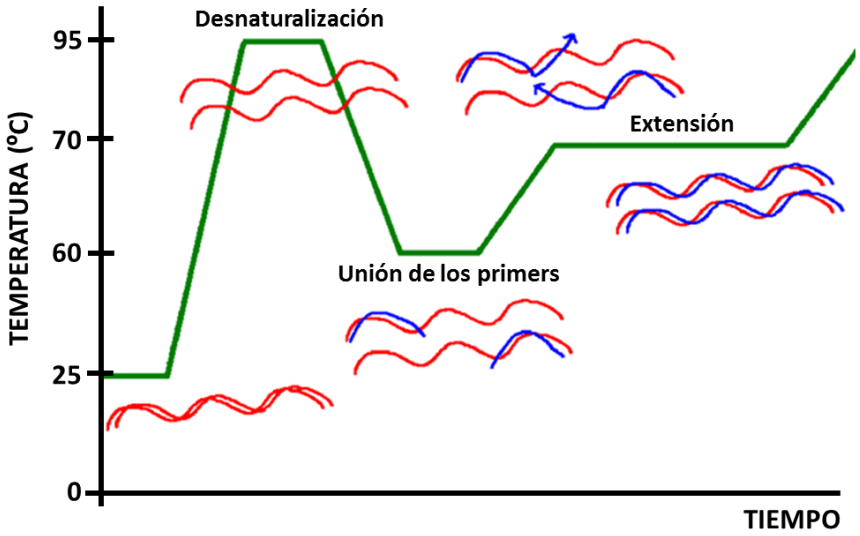


Figura 25. Perfil de temperaturas en un ciclo de la PCR

La repetición de este ciclo un determinado número de veces produce un aumento exponencial de la cantidad de la región diana del ADN, que viene dado por la expresión 2^n (siendo n el nº de ciclos) hasta que se llega a un punto en que disminuye la eficiencia de la enzima, y la reacción deja de ser exponencial.

El parámetro a tener en cuenta como dato de medida de la expresión del gen deseado es el ciclo en el que la amplificación comienza a ser exponencial. Este ciclo es el denominado ciclo umbral ("threshold cycle", Ct), y es a partir de éste cuando la amplificación es apreciable. Así, cuanto mayor sea la cantidad de ARNm que tenga la muestra, mayor será la cantidad de cDNA que se obtendrá en la transcripción; y, por tanto, la amplificación se obtendrá

antes. En consecuencia, cuanto menor sea el Ct, mayor será la cantidad de cDNA y también mayor será la expresión del gen a estudiar.

Las muestras se analizaron por triplicado junto con un control endógeno y un control negativo (sin cDNA) para así tener una mayor fiabilidad.

La PCR cuantitativa se puede clasificar en función de las sondas utilizadas que pueden ser fluorocromos no específicos o sondas específicas.

En las técnicas basadas en fluorocromos no específicos, el ADN, que ve multiplicada su cantidad con cada ciclo, se une al fluorocromo (generalmente SYBR Green) produciendo fluorescencia que es cuantificada mediante un termociclador propio para las PCR en tiempo real. Permite cuantificar sólo una secuencia por reacción, pero tiene la ventaja de utilizar cebadores normales para su realización. Es mucho más económica que la que usa sondas específicas.

Las técnicas basadas en sondas TaqMan® permiten medir la producción de productos de PCR mediante un sistema de sondas marcadas con dos fluorocromos. Su utilidad radica en que poseen un fluoróforo en su extremo 5' y otra molécula en el 3' que bloquea su emisión de fluorescencia ("quencher"); esta sonda marcada híbrida específicamente en la parte central del producto de PCR a obtener. De este modo, cuando se efectúa la PCR (con la sonda más el par de cebadores específicos), la sonda hibrida en el amplicón pero debido a la cercanía del fluoróforo al quencher, no se emite fluorescencia; cuando la polimerasa se topa con la sonda la hidroliza mediante su actividad exonucleasa, lo cual provoca la separación del quencher del fluorocromo y, por tanto comienza la emisión de fluorescencia (figura 26). La

fluorescencia está relacionada con la cantidad de amplicón producido. Hay que resaltar dos ventajas primordiales frente a las sondas inespecíficas, como el SYBR GREEN, pues con las sondas Taqman la fluorescencia que se produce es totalmente específica de la amplificación que estemos estudiando y la eficiencia es óptima, además de permitir que se usen varios fluorocromos en la misma reacción y detectar varios ADN/ARN al mismo tiempo.

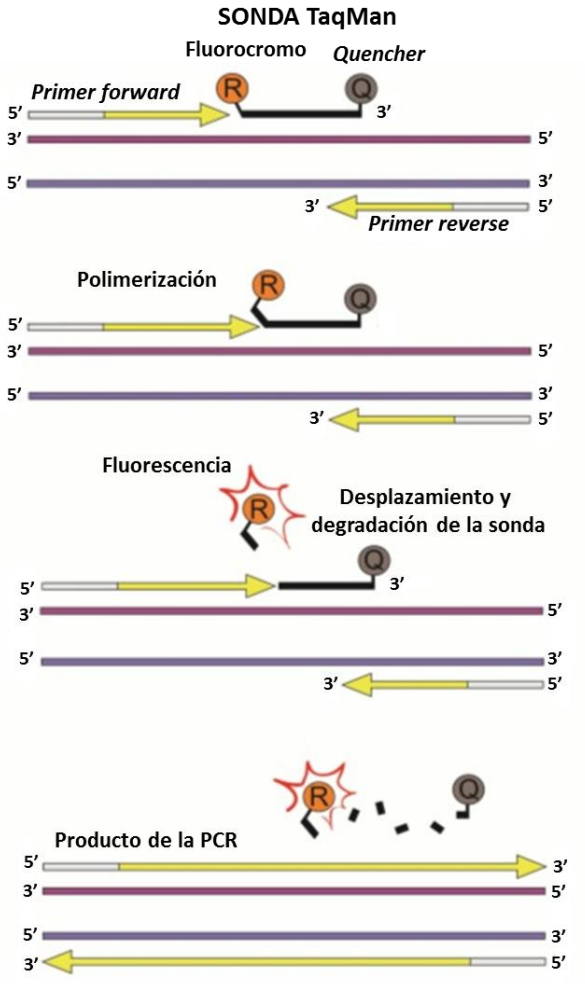


Figura 26. Mecanismo de acción de las sondas TaqMan®

En la presente Tesis se han utilizado sondas TaqMan®, copyright de la casa comercial Applied Biosystems y para la realización de la PCR cuantitativa se utilizó el sistema iQ5 Real-Time PCR System de Bio-Rad.

3.13.1. SONDAS TaqMan®

Para la realización de la PCR se utilizaron sondas TaqMan® de Applied Biosystems para los genes de ratón *il-6*, *tnf-α* y *cxcl1* (el equivalente en ratón al humano *il-8*) (tabla 6). Como gen de referencia se utilizó r*plp0* ya que se expresa a un nivel constante en muchos tejidos (tabla 6).

GEN	SONDA TaqMan®
cxcl1 (il-8)	Mm04207460_m1
il-6	Mm00446190_m1
rplp0	Mm00725448_s1
tnf-α	Mm00443260_g1

Tabla 6. Sondas TaqMan® utilizadas

3.13.2. PROCEDIMIENTO

1. Se prepara una mezcla de reacción (“mix”) que contenga los componentes expuestos en la tabla 7 y en función del número de muestras que se tenga que analizar. Se cargan 9 µl en cada pocillo de la placa.

COMPONENTES DE LA "MIX"	VOLUMEN (µl) POR MUESTRA
Sonda TaqMan® 20X	0,6
TaqMan® Reaction buffer 2X	6
Agua libre de RNasas	2,4
TOTAL	**9**

Tabla 7. Reactivos y sus volúmenes para la PCR cuantitativa

2. El volumen de muestra necesario para una correcta amplificación es de 3 µl con una concentración de cDNA de 1-100 ng/µL. Después de cargar la "mix", se cargan 50 ng/µL de cDNA en la placa de 96 pocillos.

3. Se sella la placa

4. Se agita suavemente para que se mezclen bien todos los componentes de la mezcla con la muestra y se centrifuga para que no queden burbujas de aire que puedan interferir con una buena amplificación. Es importante que durante todo el procesado anterior tanto la placa como los reactivos se encuentren en hielo en todo momento.

5. Se centrifuga para que todos los componentes de la reacción se mezclen bien. Se lleva la placa al termociclador y se lanza la reacción.

3.13.3. CICLOS DE TEMPERATURA DE AMPLIFICACIÓN

Los ciclos de temperatura en las que se llevó a cabo la PCR cuantitativa para los genes estudiados se muestran en la figura 27.

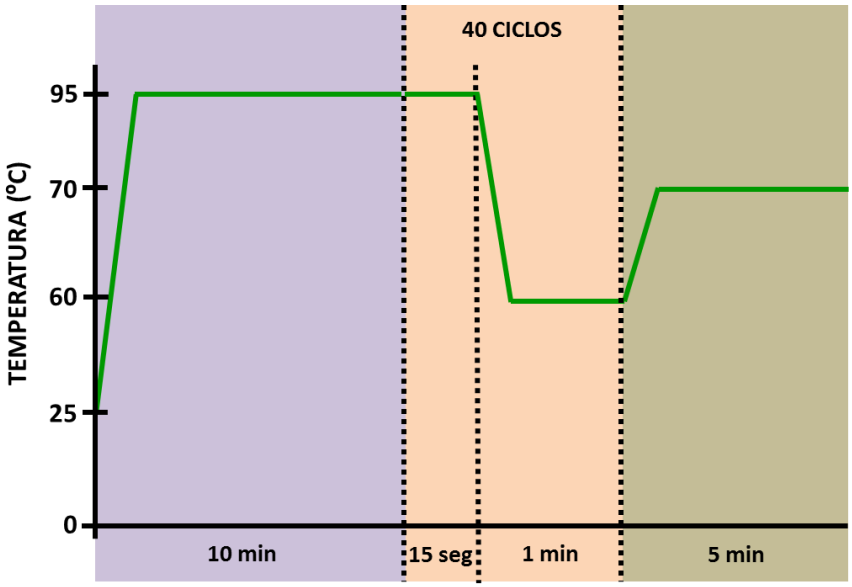


Figura 27. Ciclos de temperatura de la amplificación

3.13.4. CÁLCULOS

En la presente Tesis se analizaron los resultados utilizando el método de comparación de Ct (Livak and Schmittgen 2001) que, como se ha comentado anteriormente, es cuando el sistema comienza a detectar el aumento de la señal fluorescente asociada con un crecimiento exponencial del producto de PCR durante la fase log-lineal.

Todas las muestras se relativizan o se normalizan con el gen de referencia, que en la presente Tesis fue *rplp0*.

$$\Delta Ct = Ct \text{ gen en estudio} - Ct \text{ gen de referencia}$$

A continuación se normaliza comparando el ΔCt de cada muestra problema con el ΔCt de las muestras del grupo control.

$$\Delta\Delta Ct = \Delta Ct \text{ muestra problema} - \Delta Ct \text{ control}$$

Finalmente, los resultados se expresan en unas unidades arbitrarias, también llamadas "fold change", que reflejan cuantas veces está más o menos expresado el gen estudiado en las distintas muestras problema en comparación con las muestras control. El "fold change" se calcula de la siguiente manera:

$$\text{Fold change} = 2^{(-\Delta\Delta Ct)}$$

4. ANÁLISIS ESTADÍSTICO DE LOS RESULTADOS

Los resultados se han expresado como media ± desviación estándar. El análisis estadístico se llevó a cabo en dos pasos. En primer lugar, los datos de los diferentes grupos se compararon utilizando el análisis de la varianza. Cuando la comparación global entre grupos fue significativa, la diferencia entre grupos individuales se determinó aplicando la prueba de *Tukey* cuando los grupos tuvieron igual número de muestras y la prueba de *Scheffe* cuando el tamaño de los grupos fue diferente, considerando que las diferencias fueron significativas para una $p<0,05$ y $p<0,01$.

V. RESULTS

1. LEVELS OF FREE LOW MOLECULAR WEIGHT THIOLS AND MIXED DISULFIDES IN PANCREAS OF RATS WITH ACUTE PANCREATITIS

1.1. LEVELS OF REDUCED GLUTATHIONE (GSH) AND OXIDIZED GLUTATHIONE (GSSG)

Changes in glutathione levels provide a measure of the cellular redox status (Jones et al. 2000; Therond et al. 2000; Presnell et al. 2013). Therefore, we measured the levels of the reduced and oxidized forms of glutathione in pancreas of control rats and in pancreas of rats 1 hour and 6 hours after the induction of acute necrotizing pancreatitis.

GSH levels in pancreas of control rats were 149 ± 53,53 nmol/mg of protein, while it decreased to 76 ± 31,35 nmol/mg of protein at 1h after acute pancreatitis and to 78 ± 46,85 at 6h (figure 28). The GSH depletion was around 50%.

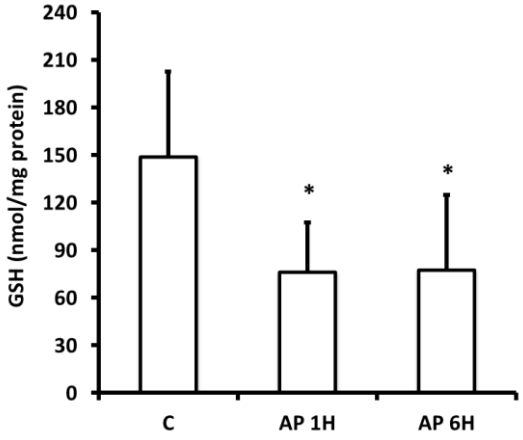


Figure 28. Levels of reduced glutathione (GSH) in pancreas of rats with acute pancreatitis (AP). The number of rats tested was 8 for each condition. Statistical significance is expressed as follows: *p<0,05 vs control.

Despite GSH depletion, pancreatic GSSG levels were maintained without significant changes in the course of the disease (figure 29). The pancreas of control rats had 2,82 ± 1,00 nmol of GSSG/mg of protein. At 1h post-induction of acute pancreatitis, GSSG levels were 1,37 ± 0,40 nmol/mg of protein, and at 6h they were 1,86 ± 1,05 nmol/mg of protein.

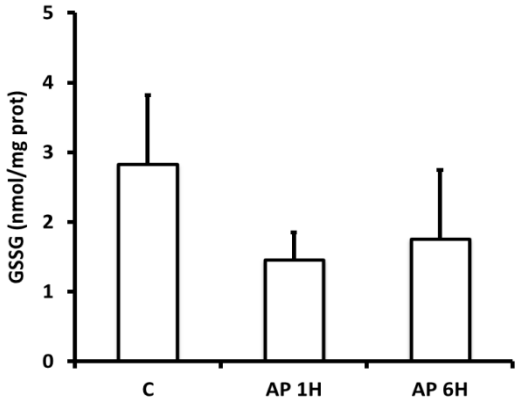


Figure 29. Levels of oxidized glutathione (GSSG) in pancreas of rats with acute pancreatitis (AP). The number of rats tested was 8 for each condition.

Importantly, the GSH/GSSG ratio did not change significantly after pancreatitis induction (figure 30). The GSH/GSSG ratio was 59 ± 18 in pancreas from control rats, 58 ± 23 in pancreas from rats at 1h post-induction of acute pancreatitis, and 51 ± 20 at 6h (figure 30).

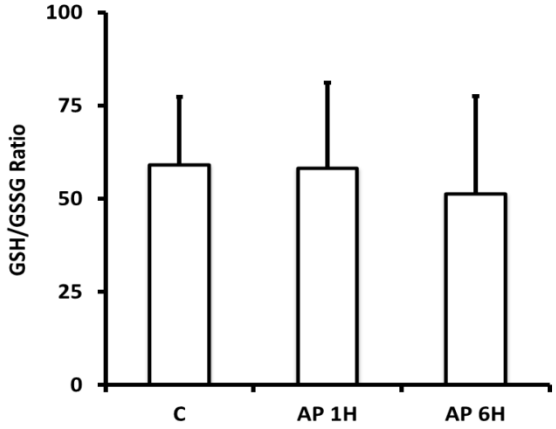


Figure 30. GSH/GSSG ratio in pancreas of rats with acute pancreatitis (AP). The number of rats tested was 8 for each condition

1.2. LEVELS OF CYSTEINE AND CISTINE

Levels of the amino acid cysteine and its oxidized form cystine were also measured because they are another key redox couple in cellular redox balance. They were determined in pancreas of control rats and in rat pancreas at 1 and 6 hours post-induction of acute necrotizing pancreatitis.

Pancreatic cysteine levels of control rats were 11,19 ± 2,09 nmol/mg of protein. After 1 h of the beginning of the disease, cysteine levels markedly increased to 17,21 ± 5,15 nmol/mg of protein, coming back to 10,24 ± 3,97 nmol/mg of protein (the basal levels) at 6h (figure 31).

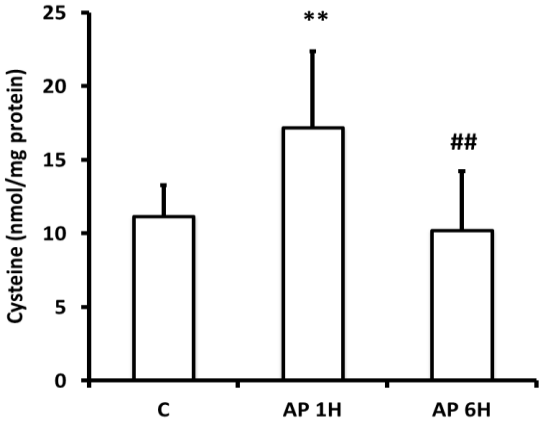


Figure 31. Cysteine levels in pancreas of rats with acute pancreatitis (AP). The number of rats tested was 8 for each condition. Statistical significance is expressed as follows: **p<0,01 vs control; $^{\#\#}$p<0,01 vs AP 1H.

Pancreatic cystine levels markedly increased during the course of acute pancreatitis (figure 32). Under control conditions, cystine levels were very low (0,007 ± 0,010 nmol/mg of protein), but then increased to 0,08 ± 0,09 nmol/mg of protein at 1h after the disease started. At 6h, cystine levels markedly rose to 0,18 ± 0,14 nmol/mg of protein (figure 32).

The cysteine/cystine ratio decreased from 4186 ± 3159 in controls to 1941 ± 2193 in rats at 1 h post-induction and 308 ± 438 at 6h (figure 33).

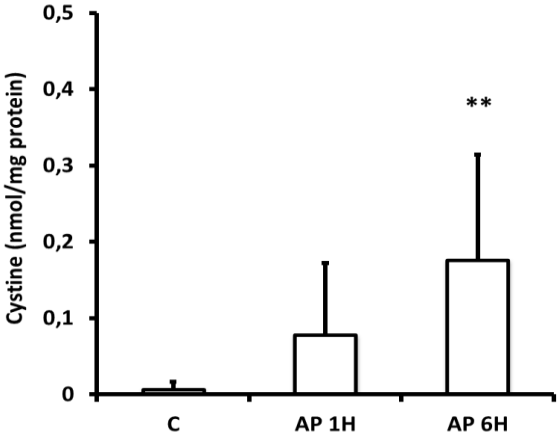


Figure 32. Cystine levels in pancreas of rats with acute pancreatitis (AP). The number of rats tested was 8 for each condition. Statistical significance is expressed as follows: **p<0,01 vs control.

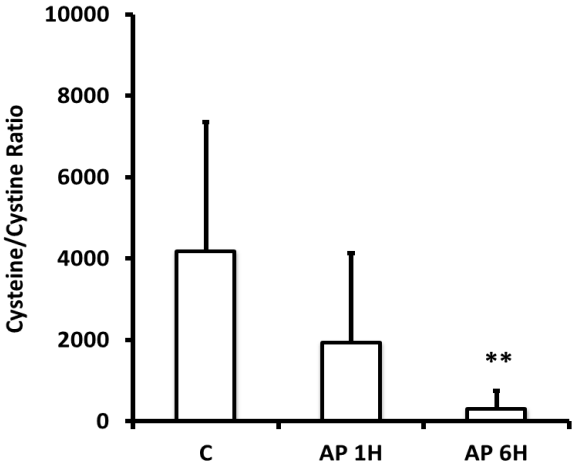


Figure 33. Cysteine/cystine ratio in pancreas of rats with acute pancreatitis (AP). The number of rats tested was 8 for each condition. Statistical significance is expressed as follows: **p<0,01 vs control.

1.3. LEVELS OF HOMOCYSTEINE AND HOMOCYSTINE

Homocysteine is an homologue of the amino acid cysteine, differing by an additional methylene bridge (-CH_2-). It can be recycled into methionine or converted into cysteine. Homocysteine levels were determined in pancreas of control rats and at 1 and 6 hours after the induction of acute pancreatitis.

Homocysteine levels in pancreas of control rats were 59 ± 31 pmol/mg of protein, and it significantly decreased to 33 ± 9 pmol/mg of protein at 1h after induction of acute pancreatitis. At 6h, homocysteine levels were maintained lower than in control rats with a value of 39 ± 15 pmol/mg of protein (figure 34).

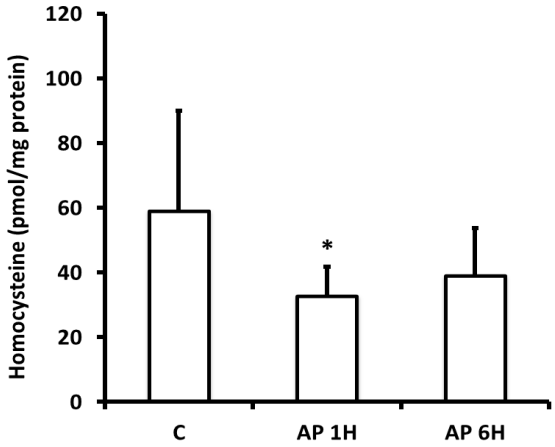


Figure 34. Homocysteine levels in pancreas of rats with acute pancreatitis (AP). The number of rats tested was 8 for each condition. Statistical significance is expressed as follows: *p<0,05 vs control.

Homocystine levels markedly increased in pancreas after induction of pancreatitis. Under basal conditions, the pancreatic levels of homocystine were undetectable, but at 1h after induction of acute pancreatitis they raised to 0,86 ± 0,68 pmol/mg of protein. At 6h they significantly increased to 1,78 ± 0,61 pmol/mg of protein (figure 35).

Changes in homocystine levels followed the same profile as cystine, being the latter always several orders of magnitude higher (figures 32 and 35).

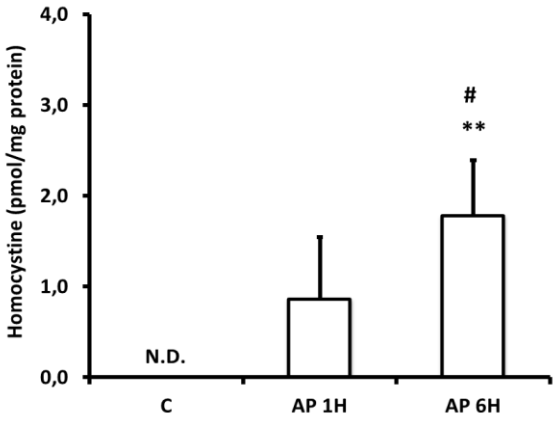


Figure 35. Homocystine levels in pancreas of rats with acute pancreatitis (AP). The number of rats tested was 8 for each condition. N.D: Non detectable. Statistical significance is expressed as follows: **p<0,01 vs control; #p<0,05 vs AP 1H.

Homocysteine/homocystine ratio was 37 ± 30 in pancreas of rats at 1 h post-induction and 16 ± 2 at 6h (figure 36).

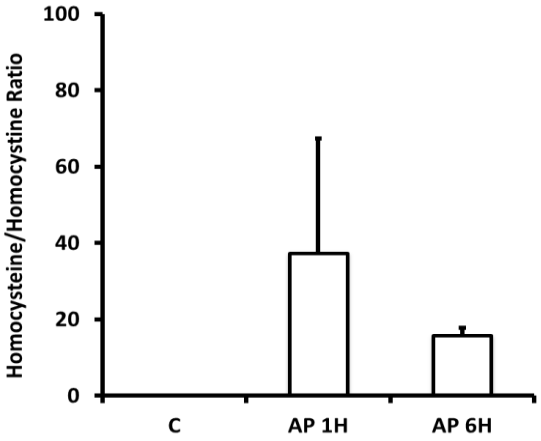


Figure 36. Homocysteine/homocystine ratio in pancreas of rats with acute pancreatitis (AP). The number of rats tested was 8 for each condition.

1.4. LEVELS OF Γ-GLUTAMYLCYSTEINE AND BIS-Γ-GLUTAMYLCYSTINE

γ-Glutamylcysteine and bis-γ-glutamylcystine are also metabolites of the glutathione synthesis pathway. γ-Glutamylcysteine levels were 520 ± 145 pmol/mg of protein in pancreas of control rats, and 500 ± 116 pmol/mg of protein at 1h after pancreatitis induction. Hence, at the early stage of the disease, γ-glutamylcysteine levels didn't change. However, at 6h after the onset of the disease, pancreatic levels of γ-glutamylcysteine significantly decreased to 363 ± 108 pmol/mg of protein (figure 37).

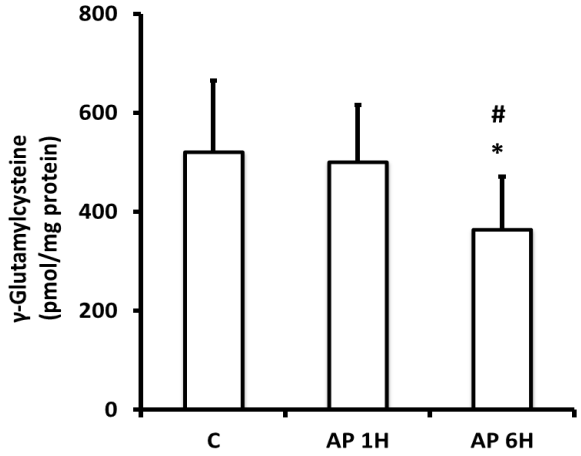


Figure 37. γ-Glutamylcysteine levels in pancreas of rats with acute pancreatitis (AP). The number of rats tested was 8 for each condition. Statistical significance is expressed as folloews: *p<0,05 vs control; #p<0,05 vs AP 1H.

In the case of bis-γ-glutamylcystine, it was observed the opposite profile to γ-glutamylcysteine. In control rats, bis-γ-glutamylcystine pancreatic levels were 17 ± 3 pmol/mg of protein. After acute pancreatitis had started, its levels increased to 26 ± 13 pmol/mg of protein at 1h and to 24 ± 13 pmol/mg of protein at 6h (figure 38).

γ-Glutamylcysteine/bis-γ-glutamylcystine ratio decreased in pancreas upon pancreatitis (figure 39). Indeed, it was 30,2 ± 12,5 in controls vs. 13,8 ± 3,8 in rats at 1h and 14,1 ± 2,2 in rats at 6 h post-induction.

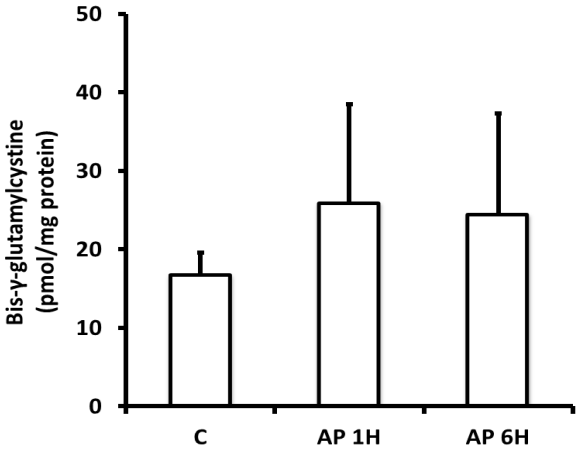


Figure 38. Bis-γ-glutamylcystine levels in pancreas of rats with acute pancreatitis (AP). The number of rats tested was 8 for each condition.

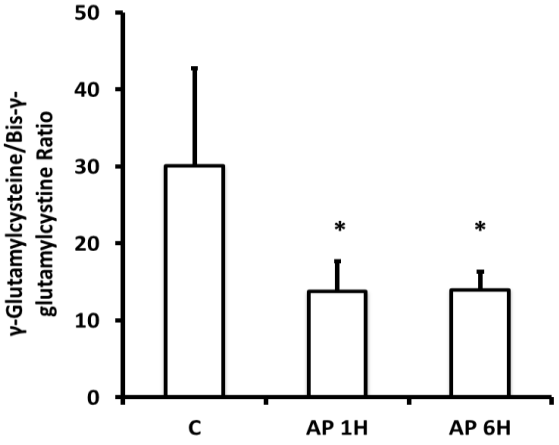


Figure 39. γ-Glutamylcysteine/Bis-γ-glutamylcystine ratio in pancreas of rats with acute pancreatitis (AP). The number of rats tested was 8 for each condition. Statistical significance is expressed as follows: *p<0,05 vs control.

2. LEVELS OF MIXED DISULFIDES IN PANCREAS OF RATS WITH ACUTE PANCREATITIS

Glutathione, cysteine and γ-glutamylcysteine can form mixed disulfides with proteins. Therefore, in this work we also determined the levels of glutathionylation, cysteinylation and γ-glutamilcysteinylation of proteins in rat pancreas during pancreatitis.

2.1. LEVELS OF PROTEIN GLUTATHIONYLATION

Protein glutathionylation did not change significantly after pancreatitis induction (figure 40). Note that there was a basal level of glutathionylation of proteins in pancreas, and during the course of acute pancreatitis these levels did not change.

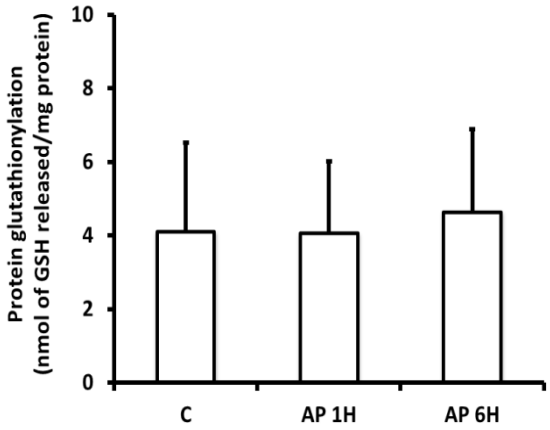


Figure 40. GSH released from mixed disulfides in pancreas of rats with acute pancreatitis (AP). The number of rats tested was 8 for each condition.

Under basal conditions, GSH released from mixed disulfides in rat pancreas was 4,1 ± 2,4 nmol/mg protein. During the course of the disease, levels of protein glutathionylation were maintained close to this value. Specifically, at 1h, 4 ± 2 nmol of GSH/mg of protein were released from proteins and at 6h they were 4,6 ± 2,3 nmol/mg of protein (figure 40).

2.2. LEVELS OF PROTEIN CYSTEINYLATION

Protein cysteinylation was non-detectable in pancreas under basal conditions but it increased markedly after induction of pancreatitis (figure 41), particularly at 6h when the cysteine/cystine ratio was lower.

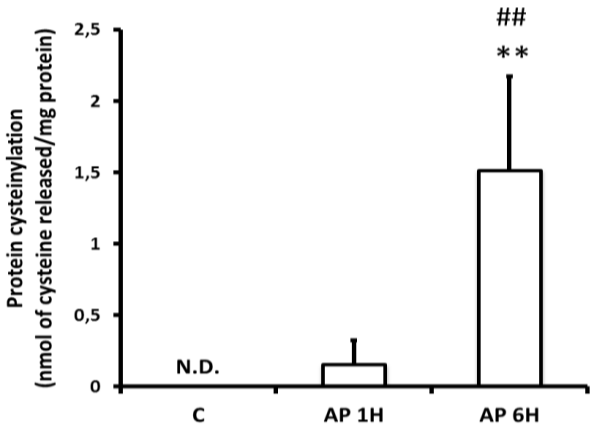


Figure 41. Cysteine released from mixed disulfides in pancreas of rats with acute pancreatitis (AP). The number of rats tested was 8 for each condition. N.D: Non detectable. Statistical significance is expressed as follows: **p<0,01 vs control; ##p<0,01 vs AP 1H.

At 1h after acute pancreatitis induction, cysteine released from mixed disulfides in rat pancreas was 0,15 ± 0,17 nmol/mg protein. At 6h it significantly increased to 1,5 ± 0,7 pmol/mg of protein (figure 41). Between 1 and 6h after pancreatitis induction there was a tenfold increase in protein cysteinylation.

2.3. LEVELS OF PROTEIN Γ-GLUTAMYLCYSTEINYLATION

In line with cysteinylation, protein γ-glutamylcysteinylation rose progressively after induction of pancreatitis (figure 42), while γ-glutamylcysteine/bis-γ-glutamylcystine ratio decreased upon pancreatitis.

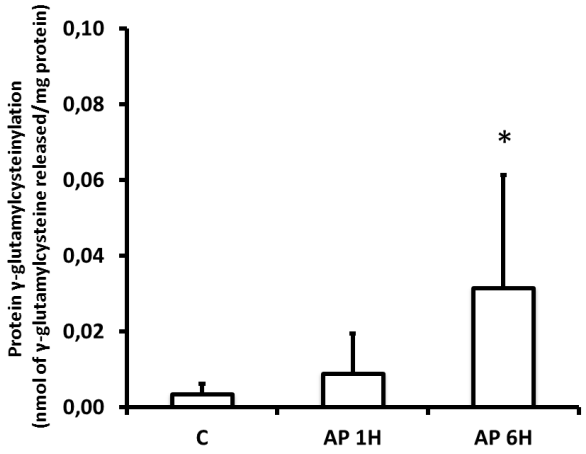


Figure 42. γ-Glutamylcysteine released from mixed disulfides in pancreas of rats with acute pancreatitis (AP). The number of rats tested was 8 for each condition. Statistical significance is expressed as follows: *p<0,05 vs control.

γ-Glutamylcysteine levels released from mixed disulfides in pancreas of control rats were 0,003 ± 0,002 nmol/mg of protein. After 1h of the beginning of the disease, protein γ-glutamylcysteinylation increased three times to 0,009

± 0,011 nmol of γ-glutamylcysteine/mg of protein rising to 6h 0,03 ± 0,03 nmol/mg of protein at 6h (figure 42). Therefore, γ-glutamylcysteinylation increased ten times during the course of acute pancreatitis.

3. PROTEIN DISULFIDE FORMATION IN ACUTE PANCREATITIS

To assess the formation of disulfide bridges during the course of acute pancreatitis, it was performed the "redox monobromobimane switch" in which free cysteines were first blocked with NEM and subsequently oxidized cysteines were reduced with DTT and labeled with monobromobimane (figure 43). Thiol modifications were assessed by fluorescent detection in gel electrophoresis of control rat pancreas of rats after 1h and 6h of acute pancreatitis induction. The increase in fluorescence after pancreatitis onset (figure 43) showed that reversible thiol oxidation is formed in the course of the disease.

After confirming disulfide formation during acute pancreatitis, it was investigated whether disulfides were formed with low molecular free thiols, such as GSH, cysteine, etc., or between proteins. Samples from control and 6h acute pancreatitis rat pancreas were assayed. Free thiols of proteins were blocked with iodoacetamide. Oxidized cysteines were reduced with DTT and alkylated with biotinylated iodoacetamide. Proteins with biotinylated iodoacetamide were detected by streptavidin HRP. Membranes were stained with Ponceau as loading control. The majority of protein disulfides seem to be ascribed to low molecular weight thiols since the diagonal electrophoresis performed after the redox switch revealed a marked increase in chemiluminescence along the diagonal at 6h after the induction of acute pancreatitis (figure 44).

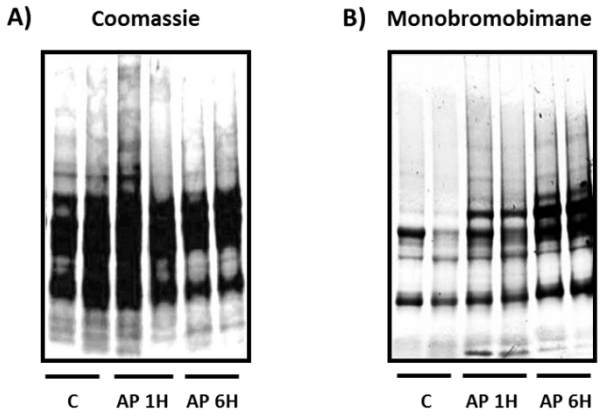


Figure 43. Formation of protein disulfides in pancreas of rats with acute pancreatitis analyzed by the redox monobromobimane switch. The figures show representative electrophoresis with the redox monobromobimane assay for 3 different experiments. AP: Acute pancreatitis. A) Coomassie staining as loading control. B) Monobromobimane switch.

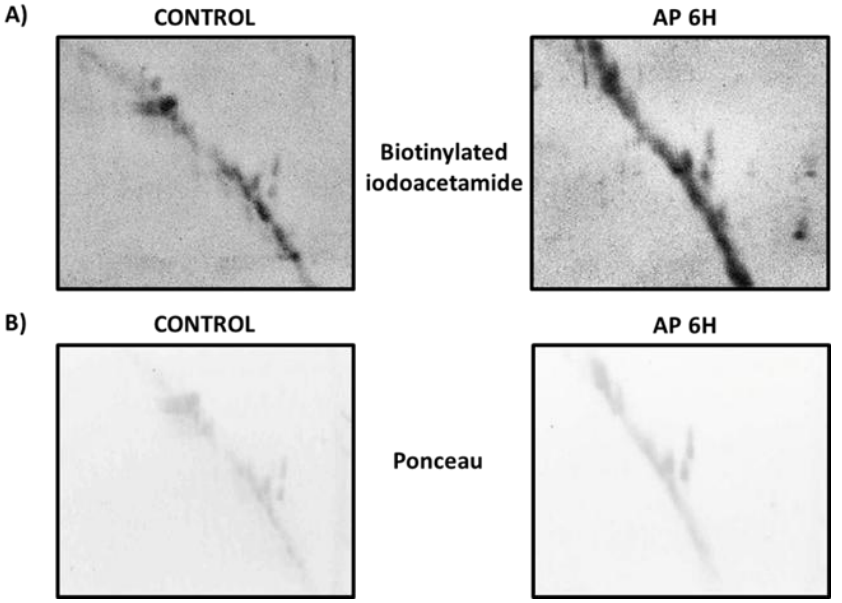


Figure 44. Formation of protein disulfides in pancreas of rats with acute pancreatitis analyzed by the biotinylated iodoacetamide switch. AP: Acute pancreatitis. A) Biotinylated iodoacetamide switch. B) Ponceau staining as loading control.

4. PROTEIN S-NITROSYLATION IN PANCREAS OF RATS WITH ACUTE PANCREATITIS

After studying and confirming the formation of disulfide bridges in acute pancreatitis, it was evaluated another oxidative modification of proteins, S-nitrosylation. S-nitrosylation was assessed by "biotin switch" in which nitrosylated cysteines were labeled with biotin. Pancreatic samples from control rats and after 1h, 3h and 6h of the induction of acute pancreatitis were assayed. As it can be observed in figure 45, there were no significant changes in protein S-nitrosylation during the course of acute pancreatitis.

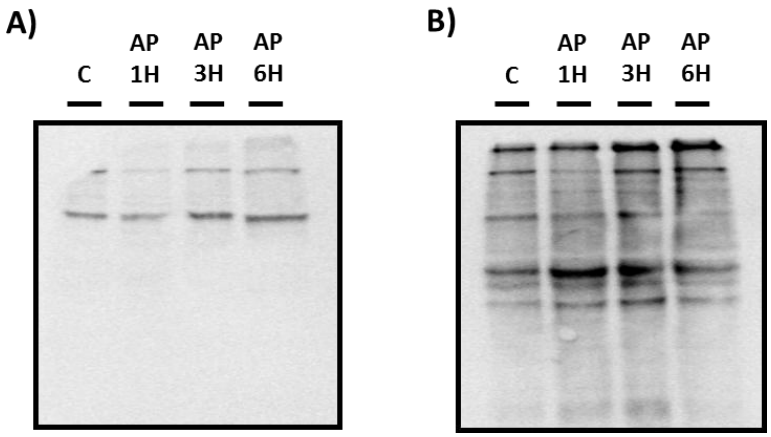


Figure 45. S-nitrosylation of proteins in pancreas of rats with acute pancreatitis analyzed by the redox biotin switch. The images show representative electrophoresis with the redox biotin switch assay for 4 different experiments. AP: Acute Pancreatitis. A) Endogenous biotinylation of proteins (negative control). B) Biotin switch.

5. PROTEIN CARBONYLATION IN PANCREAS OF RATS WITH ACUTE PANCREATITIS

Carbonylation is another oxidative modification that proteins can undergo. Protein carbonylation of control pancreas and of pancreas at 6h after pancreatitis induction was measured by bidimensional electrophoresis using an antibody against carbonylated residues (figure 46). It was not found any difference between protein carbonylation of control rats and rats with acute pancreatitis (figure 46).

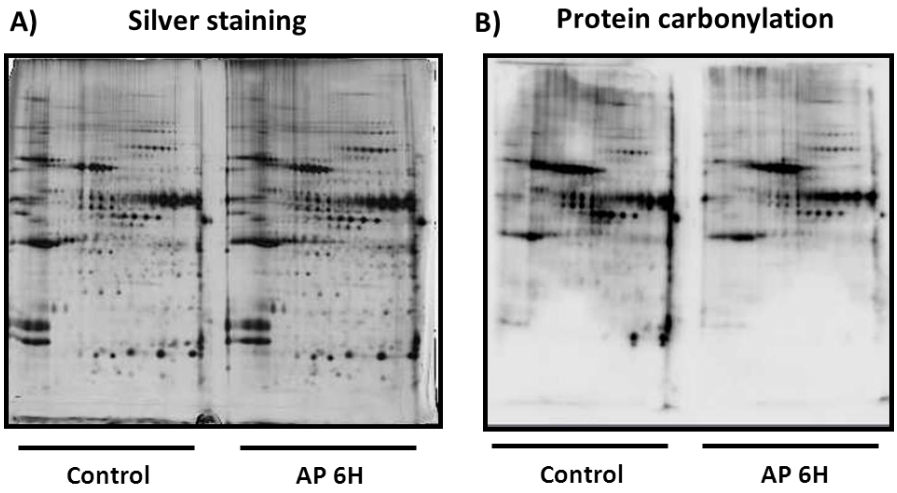


Figure 46. Protein carbonylation in pancreas of rats with acute pancreatitis analyzed by bidimensional electrophoresis. The images show representative bidimensional electrophoresis carbonylation assay for 8 different experiments A) Silver staining of the gel as loading control. B) Carbonylated proteins detected by anti-DNP.

6. IDENTIFICATION OF TARGETS OF DISULFIDE FORMATION IN PANCREAS OF RATS WITH ACUTE PANCREATITIS

6.1. TARGETS OF DISULFIDE FORMATION IN PANCREAS OF RATS WITH ACUTE PANCREATITIS IDENTIFIED BY WESTERN BLOTTING UNDER REDUCING AND NON-REDUCING CONDITIONS

The next step in this work was to identify targets that suffer thiol oxidation with formation in the course of acute pancreatitis. To this end western blottings of target candidates were performed using reducing (with DTT) or non-reducing conditions, in all cases in presence of NEM as thiol modifier. Decreased intensity of a western blot band under non-reducing conditions relative to reducing conditions gives an indirect indication of oxidation of the protein by disulfide formation.

Under reducing conditions, protein disulfide bridges are broken and they remain as thiol groups (-SH), so that the protein is unfolded and the epitopes are accessible and can be detected by the antibody. Thus, a decrease in the levels of protein in a western blotting under reducing conditions indicates that it has been degraded. In the case of western blottings carried out under non-reducing conditions, proteins that are oxidized and form disulfide bonds are still folded, and their epitopes are not accessible so the antibody cannot detect them. Therefore, a decrease in band intensity indicates protein oxidation.

Western blotting of each protein is shown in figures 47-64 with its corresponding loading control, the extracellular signal-regulated kinase 1/2

(ERK1/2). Densitometries are normalized by ERK1/2. Each blot includes a set of three different pancreatic rat samples obtained at time 0, and 30 min, 1, 3 and 6 hours after taurocholate-induced pancreatitis.

6.1.1. SH2-CONTAINING TYROSINE PHOSPHATASE 1 (SHP1)

In the the western blotting under reducing conditions, the levels of SHP1 did not vary along the disease (figure 47).

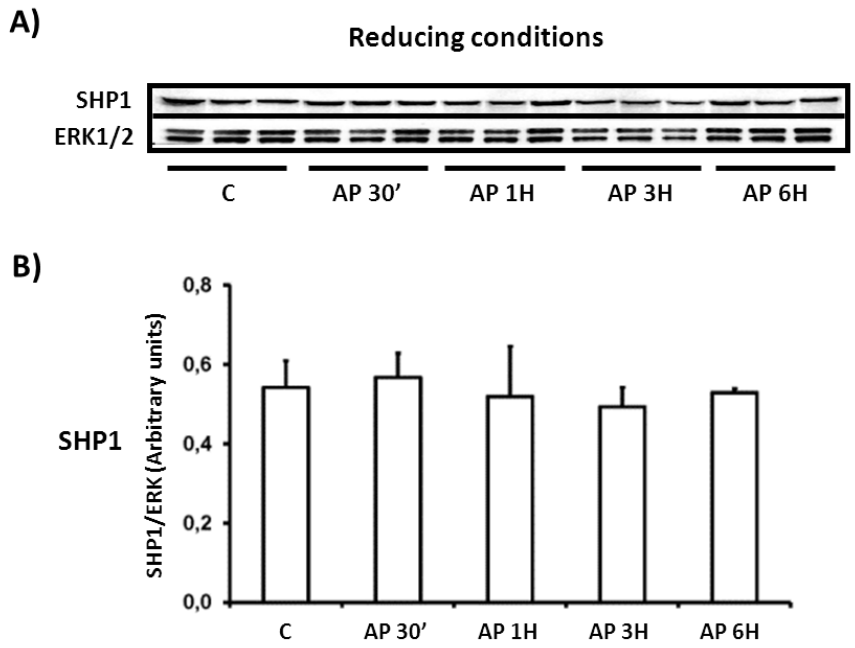


Figure 47. Protein levels of SHP1 analyzed by western blotting under reducing conditions in pancreas of rats with acute pancreatitis (AP). ERK1/2 was used as loading control. A) Western blotting under reducing conditions. The blot includes a set of three different pancreatic samples obtained at time 0, 30 min, 1, 3 and 6 hours after pancreatitis induction B) Densitometries normalized by ERK1/2.

Comparing figures 47 and 48, it can be observed that SHP1 is oxidized during the course of acute pancreatitis, especially at 1 hour. Thus, the level of oxidation is maintained during the disease.

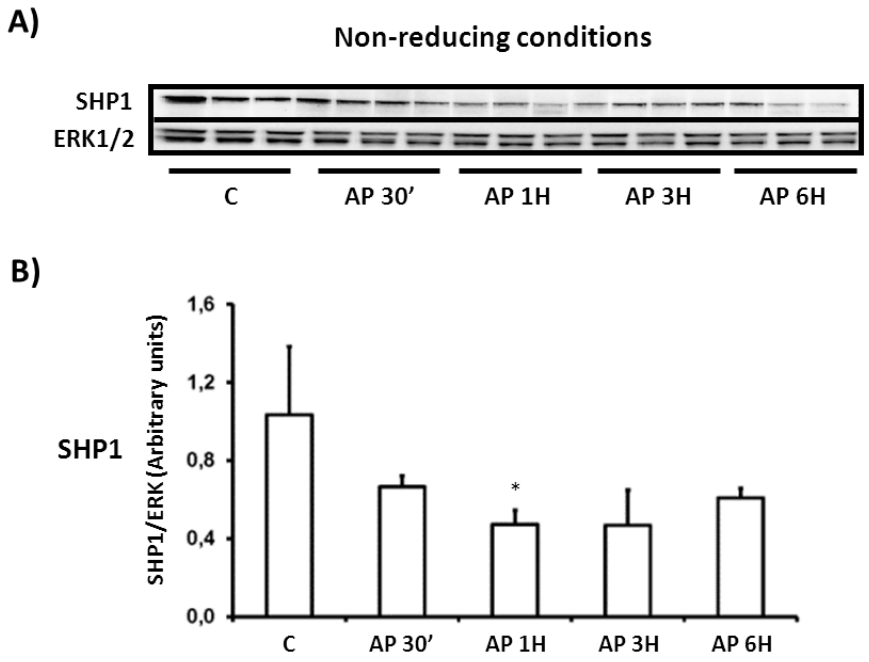


Figure 48. Protein levels of SHP1 analyzed by western blotting under non-reducing conditions in pancreas of rats with acute pancreatitis (AP). ERK1/2 was used as loading control. A) Western blotting under non-reducing conditions. The blot includes a set of three different pancreatic samples obtained at time 0, 30 min, 1, 3 and 6 hours after pancreatitis induction B) Densitometries normalized by ERK1/2. Statistical significance is expressed as follows: *p<0,05 vs control.

6.1.2. SH2-CONTAINING TYROSINE PHOSPHATASE 2 (SHP2)

Another tyrosine phosphatase that may play a role in the regulation of the inflammatory cascade is SHP2. In its western blotting under reducing conditions the intensity of the bands did not change during the time course of acute pancreatitis as it can be observed in the densitometry (figure 49).

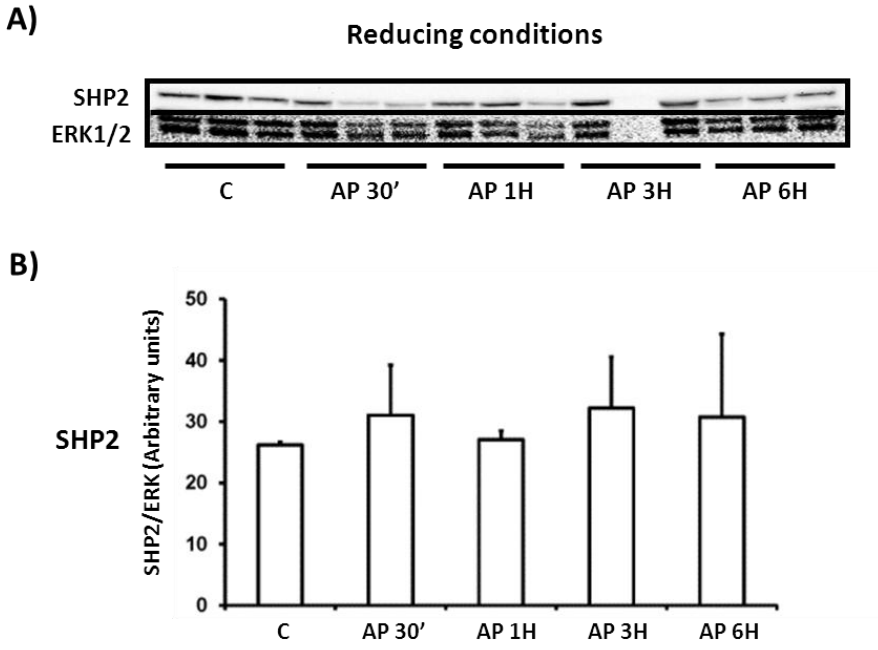


Figure 49. Protein levels of SHP2 analyzed by western blotting under reducing conditions in pancreas of rats with acute pancreatitis (AP). ERK1/2 was used as loading control. A) Western blotting under reducing conditions. The blot includes a set of three different pancreatic samples obtained at time 0, 30 min, 1, 3 and 6 hours after pancreatitis induction B) Densitometries normalized by ERK1/2.

When the western blotting was performed under non-reducing conditions it showed that there is a decrease in the intensity of the bands during the course of acute pancreatitis (figure 50). Hence, SHP2 is a target of disulfide oxidation. The oxidation of the protein starts at the beginning of the disease and increases progressively reaching its maximum at 6h (figure 50).

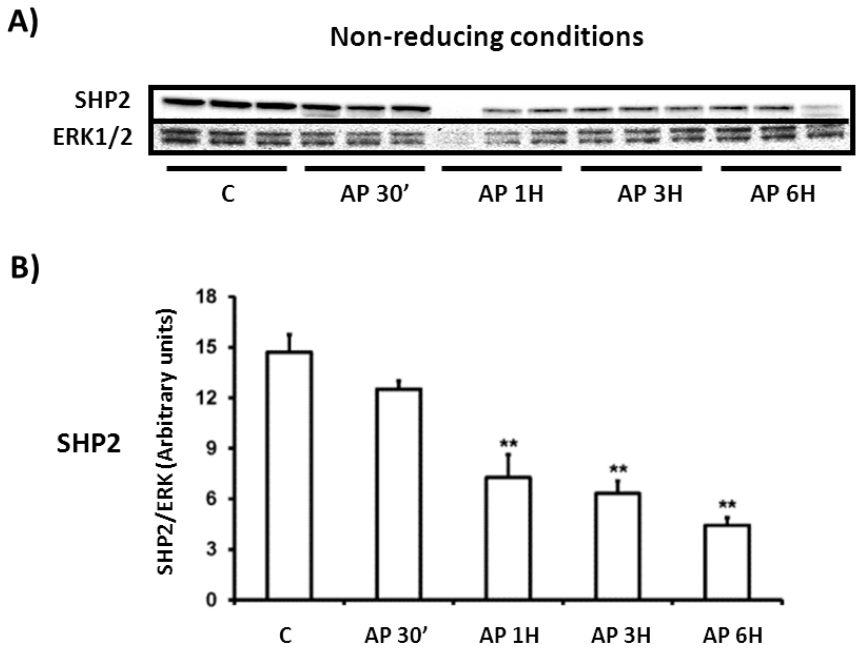


Figure 50. Protein levels of SHP2 analyzed by western blotting under non-reducing conditions in pancreas of rats with acute pancreatitis (AP). ERK1/2 was used as loading control. A) Western blotting under non-reducing conditions. The blot includes a set of three different pancreatic samples obtained at time 0, 30 min, 1, 3 and 6 hours after pancreatitis induction B) Densitometries normalized by ERK1/2. Statistical significance is expressed as follows: **p<0,01 vs control.

6.1.3. SERINE/THREONINE PROTEIN PHOSPHATASE 2A (PP2A)

Other protein phosphatase studied was the serine/threonine phosphatase PP2A, more concretely its catalytic subunit (PP2Ac). When the western blotting under reducing conditions was performed, the levels of PP2Ac significantly decreased at 30 min post-induction of acute pancreatitis (figure 51).

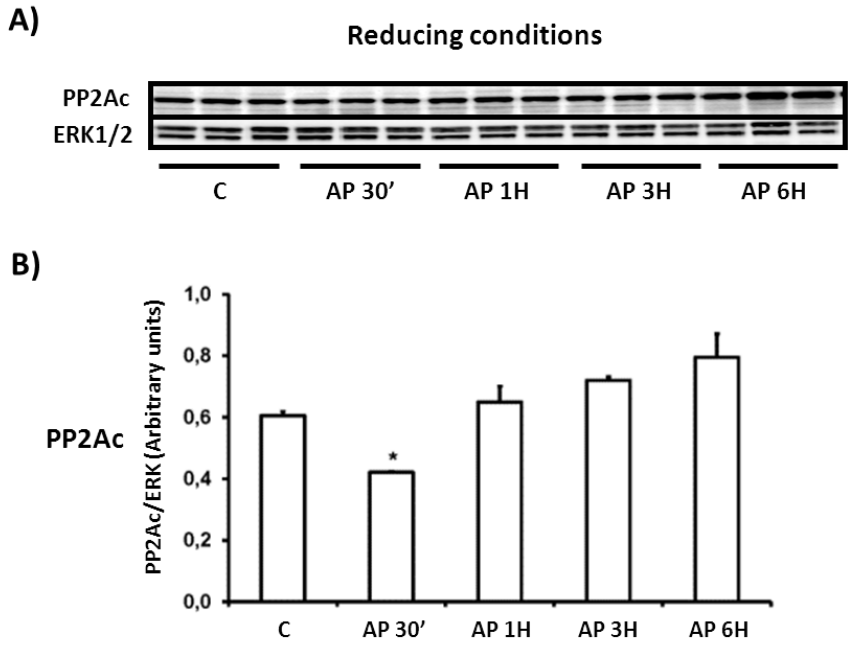


Figure 51. Protein levels of PP2Ac analyzed by western blotting under reducing conditions in pancreas of rats with acute pancreatitis (AP). ERK1/2 was used as loading control. A) Western blotting under reducing conditions. The blot includes a set of three different pancreatic samples obtained at time 0, 30 min, 1, 3 and 6 hours after pancreatitis induction B) Densitometries normalized by ERK1/2. Statistical significance is expressed as follows: *p<0,05 vs control.

On the other hand, when PP2Ac was analyzed by western blotting under non-reducing conditions, it showed oxidation at 3h post-induction (figure 52).

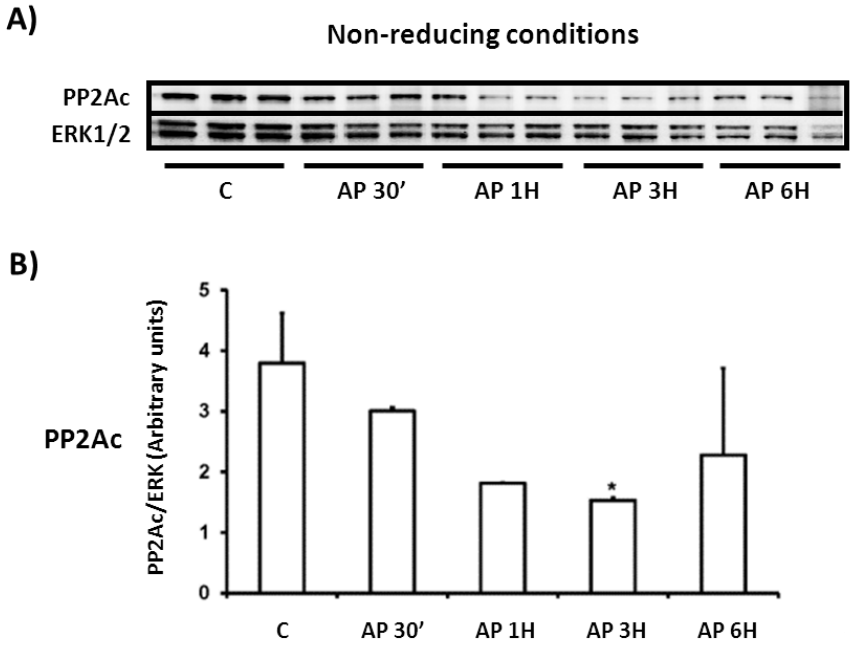


Figure 52. Protein levels of PP2Ac analyzed by western blotting under non-reducing conditions in pancreas of rats with acute pancreatitis (AP). ERK1/2 was used as loading control. A) Western blotting under non-reducing conditions. The blot includes a set of three different pancreatic samples obtained at time 0, 30 min, 1, 3 and 6 hours after pancreatitis induction B) Densitometries normalized by ERK1/2. Statistical significance is expressed as follows: *p<0,05 vs control.

6.1.4. PROTEIN DISULFIDE ISOMERASE (PDI)

Protein disulfide isomerase is an enzyme of the endoplasmic reticulum that catalyzes the formation and breakage of disulfide bonds between cysteine residues within protein as they fold. Therefore, PDI is a protein related to thiol equilibrium. When the western blotting of PDI was carried out under reducing conditions, it was found that there wasn't any significant decrease in the intensity of the bands during the course of the disease (figure 53).

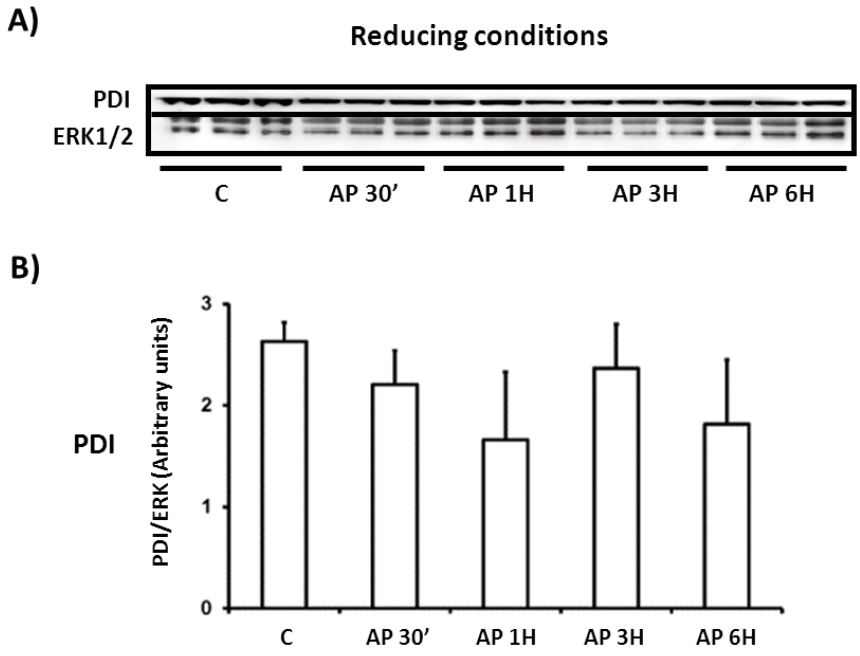


Figure 53. Protein levels of PDI analyzed by western blotting under reducing conditions in pancreas of rats with acute pancreatitis (AP). ERK1/2 was used as loading control. A) Western blotting under reducing conditions. The blot includes a set of three different pancreatic samples obtained at time 0, 30 min, 1, 3 and 6 hours after pancreatitis induction B) Densitometries normalized by ERK1/2.

In the case of the PDI western blotting under non-reducing conditions, this protein was rapidly and progressively oxidized during the course of acute pancreatitis and reached the higher degree of oxidation at 6h (figure 54).

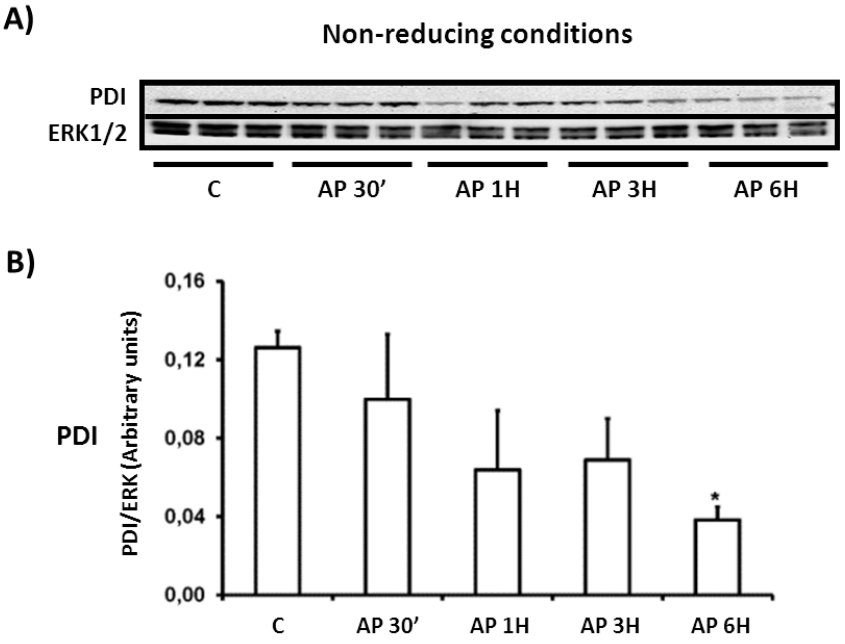


Figure 54. Protein levels of PDI analyzed by western blotting under non-reducing conditions in pancreas of rats with acute pancreatitis (AP). ERK1/2 was used as loading control. A) Western blotting under non-reducing conditions. The blot includes a set of three different pancreatic samples obtained at time 0, 30 min, 1, 3 and 6 hours after pancreatitis induction B) Densitometries normalized by ERK1/2. Statistical significance is expressed as follows: *p<0,05 vs control.

6.1.5. THIOREDOXIN 1 (Trx1)

Trx1 is also involved in thiol equilibrium in proteins including redox signalling. Thus, it was analysed by western blotting under reducing and non-reducing conditions. In the first case, there were no changes in Trx1 expression during the time course of acute pancreatitis (figure 55).

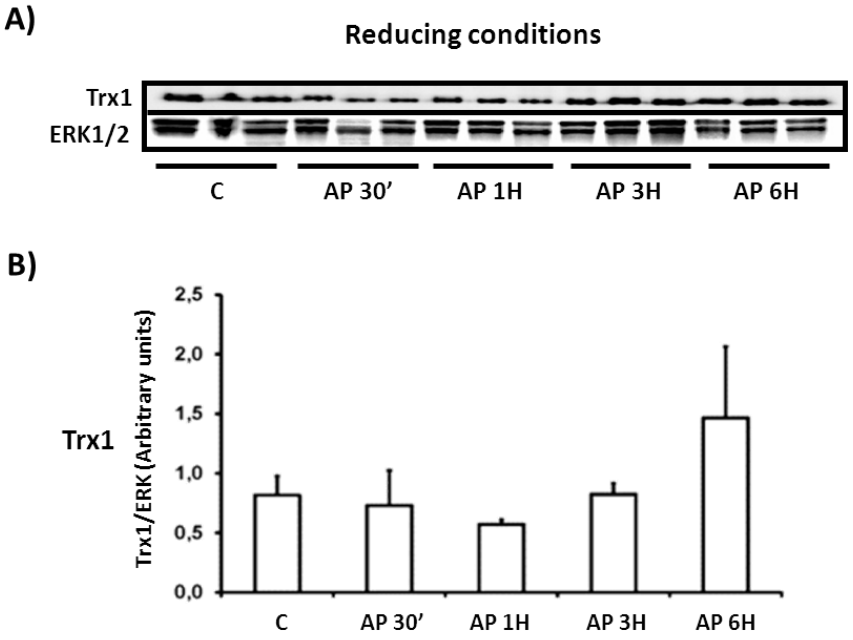


Figure 55. Protein levels of Trx1 analyzed by western blotting under reducing conditions in pancreas of rats with acute pancreatitis (AP). ERK1/2 was used as loading control. A) Western blotting under reducing conditions. The blot includes a set of three different pancreatic samples obtained at time 0, 30 min, 1, 3 and 6 hours after pancreatitis induction B) Densitometries normalized by ERK1/2.

In the case of the western blotting under non-reducing conditions, there was a marked oxidation of Trx1 at 3h post-induction (figure 56).

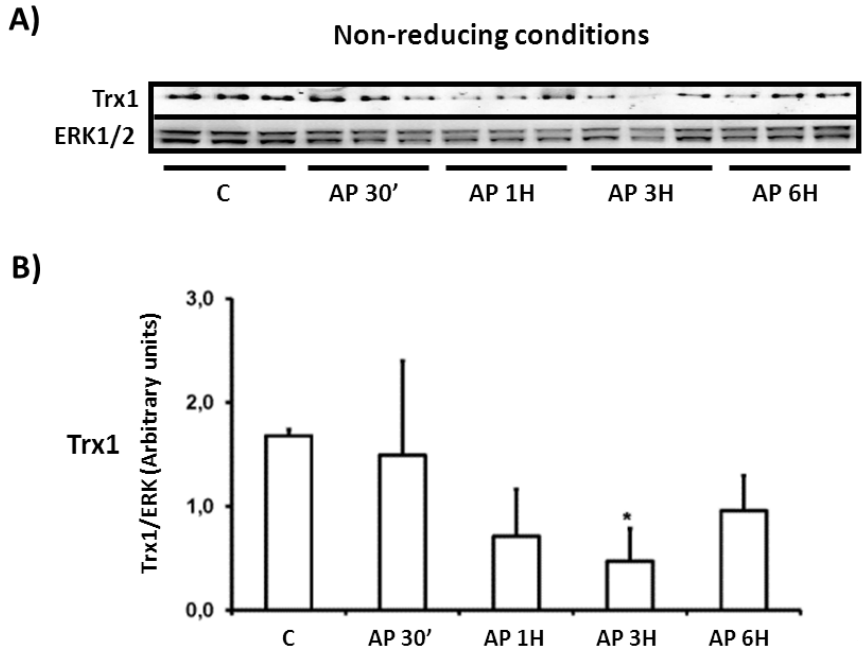


Figure 56. Protein levels of Trx1 analyzed by western blotting under non-reducing conditions in pancreas of rats with acute pancreatitis (AP). ERK1/2 was used as loading control. A) Western blotting under non-reducing conditions. The blot includes a set of three different pancreatic samples obtained at time 0, 30 min, 1, 3 and 6 hours after pancreatitis induction B) Densitometries normalized by ERK1/2. Statistical significance is expressed as follows: *p<0,05 vs control.

6.1.6. PEROXIREDOXIN 1 (Prdx1)

Peroxiredoxins participate in thiol equilibrium of proteins too because they join the thioredoxin system. Thus, it was carried out the Prdx1 western blotting under reducing and non-reducing conditions.

In figure 57, it can be observed that Prdx1 is not degraded during the course of acute pancreatitis because the western blotting under reducing conditions does not show significant changes.

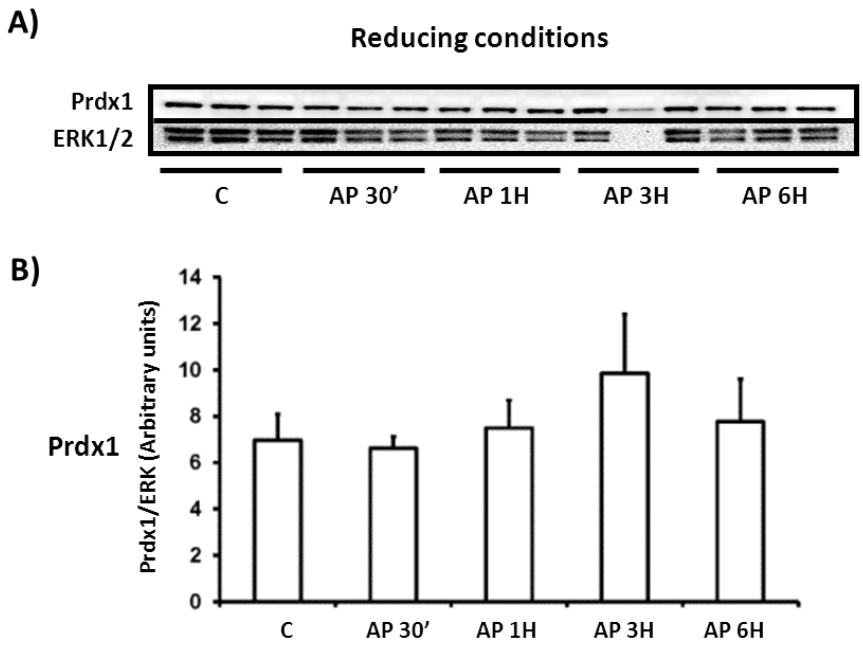


Figure 57. Protein levels of Prdx1 analyzed by western blotting under reducing conditions in pancreas of rats with acute pancreatitis (AP). ERK1/2 was used as loading control. A) Western blotting under reducing conditions. The blot includes a set of three different pancreatic samples obtained at time 0, 30 min, 1, 3 and 6 hours after pancreatitis induction B) Densitometries normalized by ERK1/2.

There were no significant changes in densitometry analysis of the western blotting of Prdx1 under non-reducing conditions (figure 58).

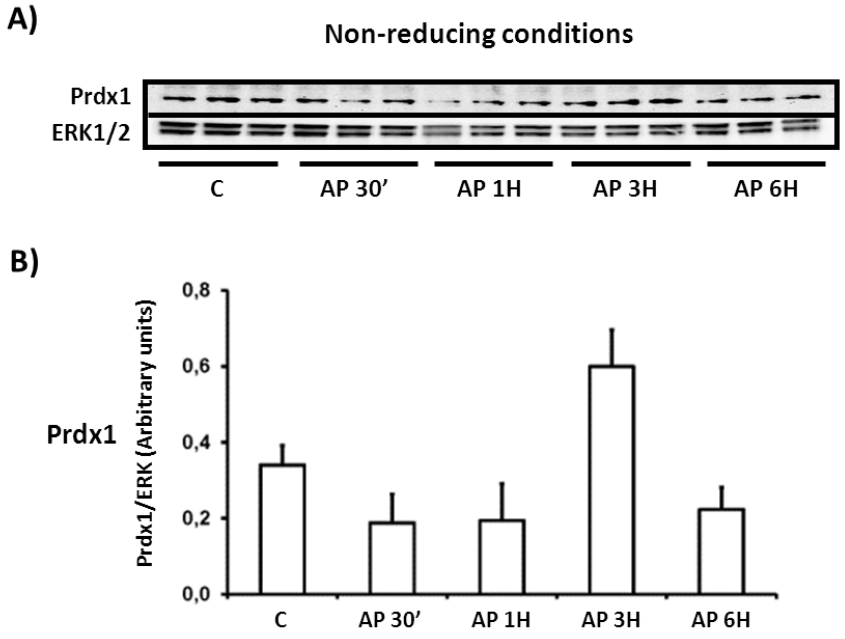


Figure 58. Protein levels of Prdx1 analyzed by western blotting under non-reducing conditions in pancreas of rats with acute pancreatitis (AP). ERK1/2 was used as loading control. A) Western blotting under non-reducing conditions. The blot includes a set of three different pancreatic samples obtained at time 0, 30 min, 1, 3 and 6 hours after pancreatitis induction B) Densitometries normalized by ERK1/2.

6.1.7. KELCH-LIKE ECH-ASSOCIATED PROTEIN-1 (KEAP1)

KEAP1 participates in the antioxidant response against oxidative stress by acting in thiol equilibrium.

It was performed the western blotting of KEAP1 under reducing conditions. It was noted that there were no changes in protein levels during the course of acute pancreatitis (figure 59).

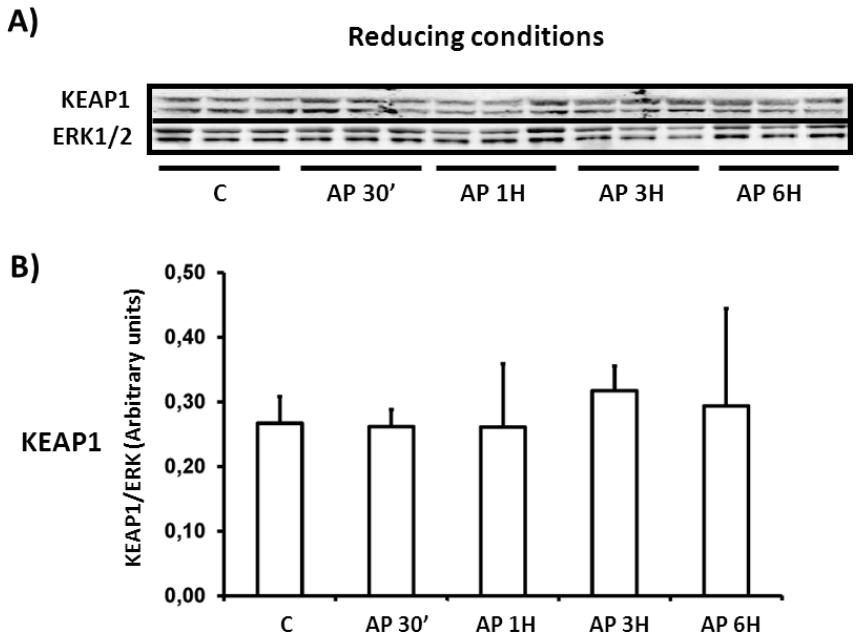


Figure 59. Protein levels of Keap1 analyzed by western blotting under reducing conditions in pancreas of rats with acute pancreatitis (AP). ERK1/2 was used as loading control. A) Western blotting under reducing conditions. The blot includes a set of three different pancreatic samples obtained at time 0, 30 min, 1, 3 and 6 hours after pancreatitis induction B) Densitometries normalized by ERK1/2.

Otherwise, when the western blotting of KEAP1 was performed under non-reducing conditions, it can be noticed that KEAP1 is rapidly oxidized in acute pancreatitis (figure 60).

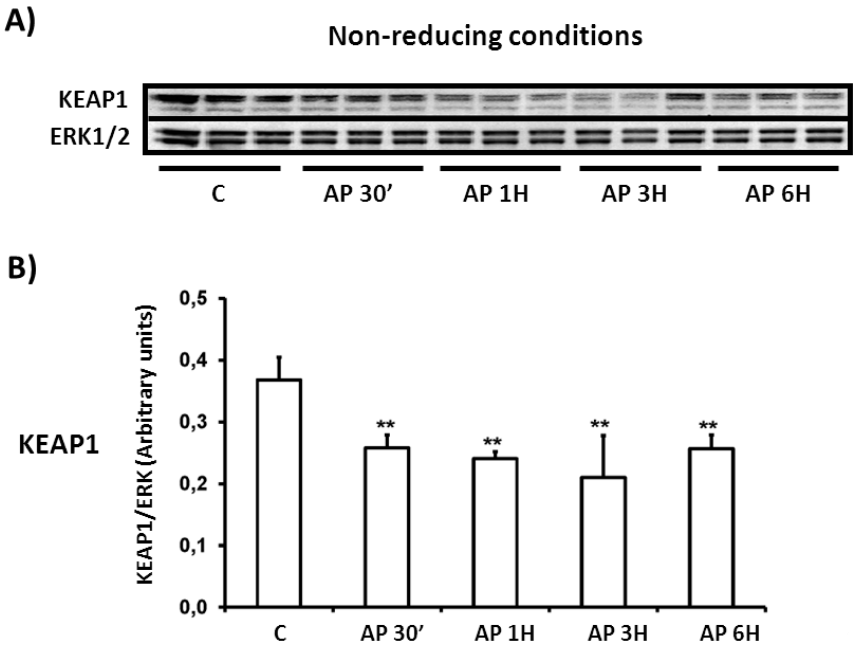


Figure 60. Protein levels of Keap1 analyzed by western blotting under non-reducing conditions in pancreas of rats with acute pancreatitis (AP). ERK1/2 was used as loading control. A) Western blotting under non-reducing conditions. The blot includes a set of three different pancreatic samples obtained at time 0, 30 min, 1, 3 and 6 hours after pancreatitis induction B) Densitometries normalized by ERK1/2. Statistical significance is expressed as follows: **p<0,01 vs control.

6.1.8. RIBONUCLEASE INHIBITOR 1 (RNH1)

Pancreatic ribonuclease is an endonuclease found in high quantity in the pancreas. It can unwind the RNA helix. For these reason, the function of its inhibitor (RNH1) in pancreas is very important to avoid RNA cleavage.

The western blotting under reducing conditions of RNH1 did not show any significant change in protein levels during acute pancreatitis development (figure 61).

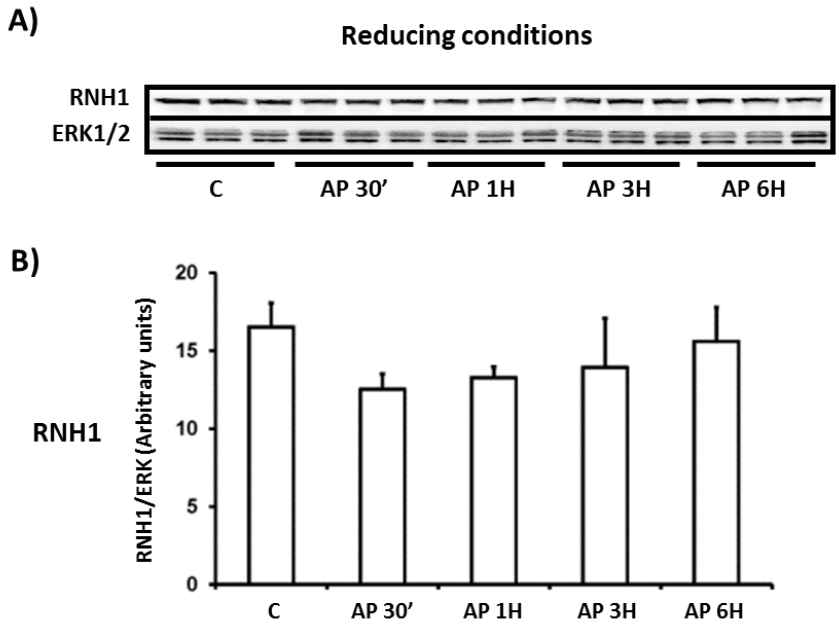


Figure 61. Protein levels of RNH1 analyzed by western blotting under reducing conditions in pancreas of rats with acute pancreatitis (AP). ERK1/2 was used as loading control. A) Western blotting under reducing conditions. The blot includes a set of three different pancreatic samples obtained at time 0, 30 min, 1, 3 and 6 hours after pancreatitis induction B) Densitometries normalized by ERK1/2.

When RNH1 levels were analysed by western blotting under non-reducing conditions, it was observed a progressive decrease from the first hour after induction of acute pancreatitis (figure 62). Specifically, this decrease was only significant at 6h of the disease (figure 62).

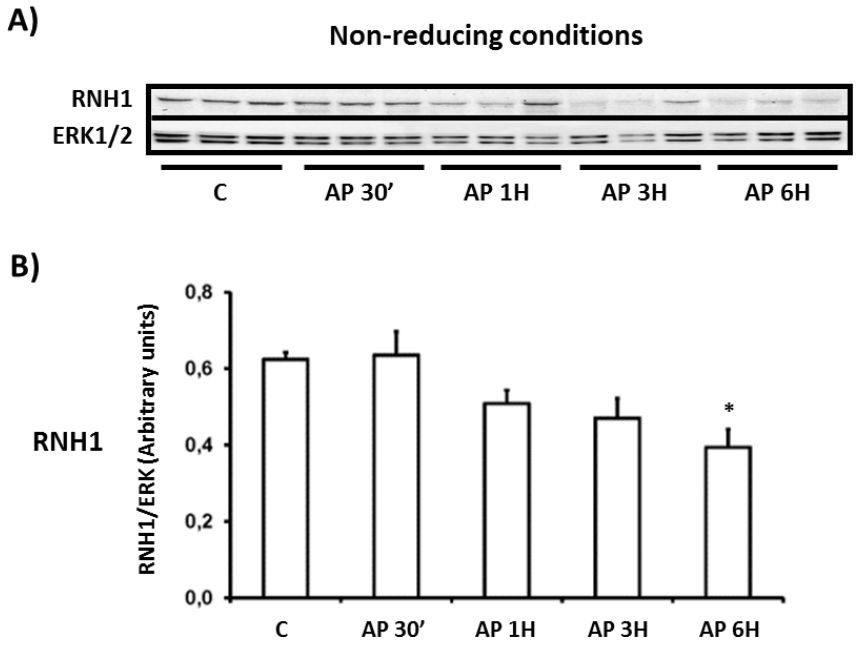


Figure 62. Protein levels of RNH1 analyzed by western blotting under non-reducing conditions in pancreas of rats with acute pancreatitis (AP). ERK1/2 was used as loading control. A) Western blotting under non-reducing conditions. The blot includes a set of three different pancreatic samples obtained at time 0, 30 min, 1, 3 and 6 hours after pancreatitis induction B) Densitometries normalized by ERK1/2. Statistical significance is expressed as follows: *p<0,05 vs control.

6.1.9. REDOX EFFECTOR FACTOR 1 (REF1)/ APURINIC/APYRIMIDINIC ENDONUCLEASE (APE1)

REF1/APE1 is a redox effector factor and also repairs DNA from damage. Like the previously studied proteins, western blotting of Ref1/Ape1 under reducing and non-reducing conditions was also performed. As it can be seen in figures 63 and 64, REF1/APE1 seems markedly degraded from the onset of acute pancreatitis. Oxidation of REF1/APE1 may additionally occur.

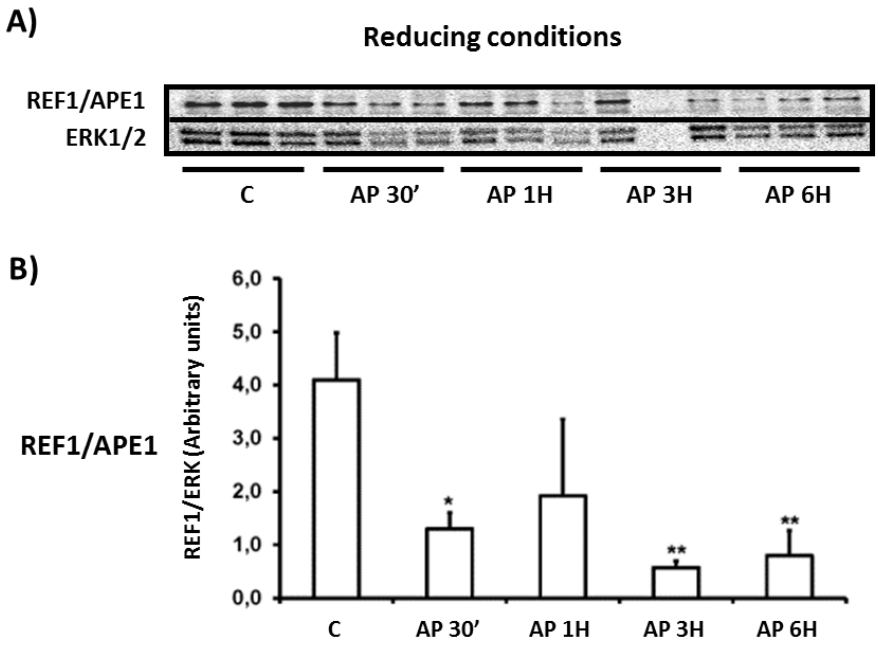


Figure 63. Protein levels of REF1/APE1 analyzed by western blotting under reducing conditions in pancreas of rats with acute pancreatitis (AP). ERK1/2 was used as loading control. A) Western blotting under reducing conditions. The blot includes a set of three different pancreatic samples obtained at time 0, 30 min, 1, 3 and 6 hours after pancreatitis induction B) Densitometries normalized by ERK1/2. Statistical significance is expressed as follows: *p<0,05 vs control; **p<0,01 vs control.

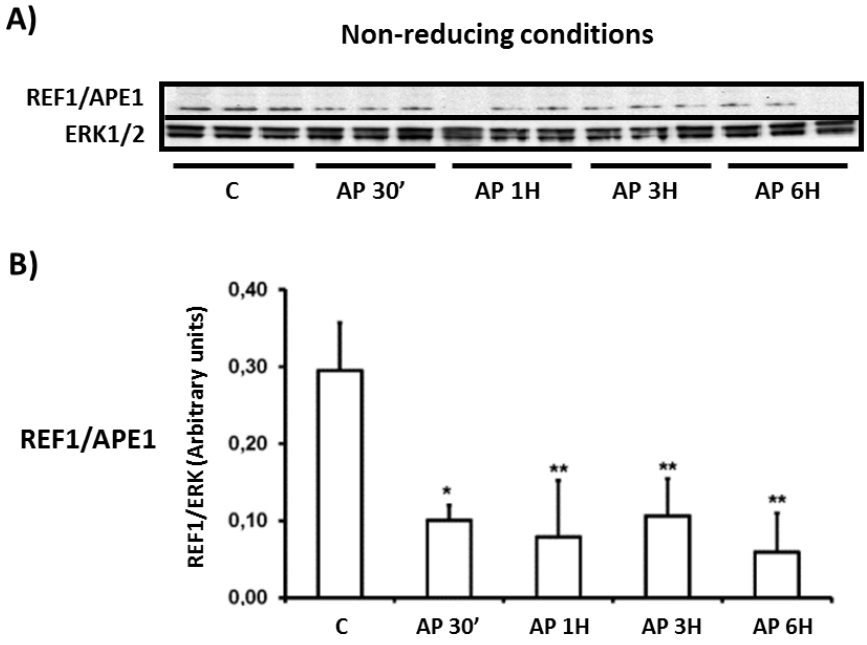


Figure 64. Protein levels of REF1/APE1 analyzed by western blotting under non-reducing conditions in pancreas of rats with acute pancreatitis (AP). ERK1/2 was used as loading control. A) Western blotting under non-reducing conditions. The blot includes a set of three different pancreatic samples obtained at time 0, 30 min, 1, 3 and 6 hours after pancreatitis induction B) Densitometries normalized by ERK1/2. Statistical significance is expressed as follows: *p<0,05 vs control; **p<0,01 vs control.

6.2. PROTEIN TARGETS OF DISULFIDE FORMATION IN PANCREAS OF RATS WITH ACUTE PANCREATITIS IDENTIFIED BY PROTEOMIC METHODS

In order to identify more targets of disulfide formation proteomic methods were performed. Diagonal electrophoresis using iodoacetamide as thiol modifier was used to separate proteins and subsequently they were identified by reverse phase-LC-MS/MS and analyzed with the computer program MASCOT.

When a protein lies above the diagonal, it indicates modification by intramolecular disulfide bridges because when the protein is reduced it decreases its migration. Whereas when a protein lies below the diagonal, it indicates the formation of intermolecular disulfide bridges because when the protein is reduced it increases its migration.

Three spots were found in all samples (from both control and treated rats) above the diagonal, that correspond to elastase, anionic trypsin 1 and cationic trypsin 3 (figure 65 and table 8). On the contrary, peroxiredoxin 4, albumin, α-amylase, and elongation factor 1α appeared under the diagonal only after pancreatitis induction (figure 65, tables 8 and 9)

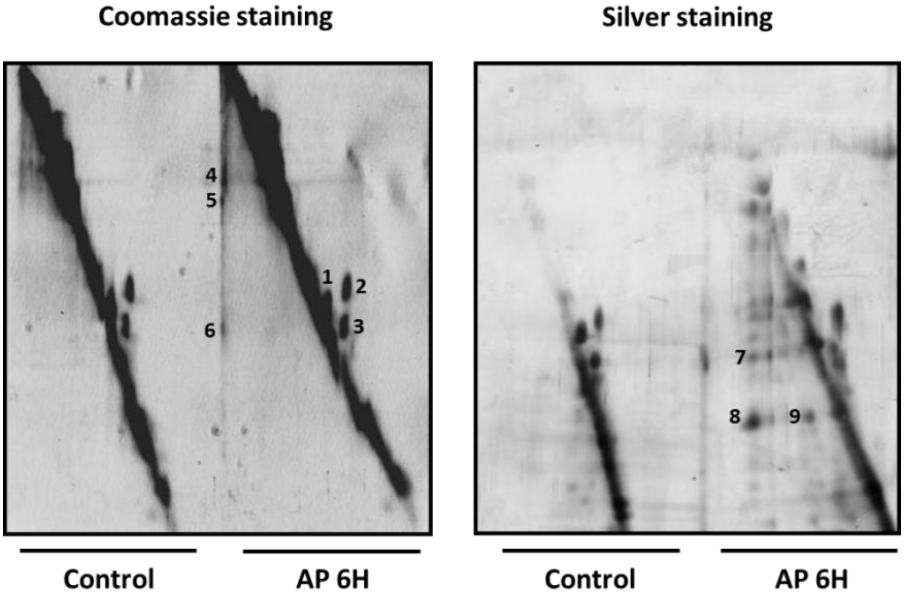


Figure 65. Identification of proteins by reverse phase-LC-MS/MS after diagonal electrophoresis and staining with comassie blue (left) or silver (right) of pancreas of rats with acute pancreatitis (AP). Proteins identified were 1: elastase, 2: anionic tripsin-1, 3: cationic tripsin-3, 4: α-amylase, 5: elongation factor 1α, 6: peroxiredoxin 4, and 7, 8, 9: albumin.

SPOT	PROTEIN NAME	Acc No	SCORE	PEPTIDE SECUENCE	POSITION
1	Chymotrypsin-like elastase family member 2A	gi|119259	240	LASPVALTSK LQTNGATPDVLQQGR VSNYIDWINSVIAK HSLSTSESGSLAVQVSK WNAQKLSNGNDIALVK LLVVDYATCSSASWWGSSVK IQTACLPPAGTILPNNYPCYVTGWGR	129-138 165-179 257-270 90-106 113-128 180-199 139-164
2	Anionic trypsin-1	gi|136409	133	VCNFVGWIQDTIAAN HPNYSSWTLNNDIMLIK LGEHNINVLEGDEQFINAAK IIKHPNYSSWTLNNDIMLIK	232-246 96-112 73-92 93-112
3	Cationic trypsin-3	gi|136417	255	LNSPATLNSR ITSNMFCLGFLEGGK HPSYNANTFDNDIMLIK LGEHNIDVVEGGEQFIDAAK CLVSGWGNTLSSGTNYPSLLQCLDAP VLSDSSCK	114-123 180-194 97-113 74-93 140-173
4	Pancreatic alpha-amylase	gi|21542378	230	GSEYFGNGR TAIVHLFEWR WADIAKECER AVLDKLHNLNTK ALVFVDNHDNQR NWGEGWGFVPTDR SGNENEFKDMVTR GHGAGGASILTFWDAR LSGLLDLALDKDYVR MAVGFMLAHPYGFTR VADYMNNLIDIGVAGFR AHFSISNSAEDPFIAIHADSK EVTINPDTTCGNDWVCEHR NVVNGQPFANWWDNGSNQVAFSR	256-264 26-35 36-45 221-232 304-315 291-303 88-100 316-331 174-188 335-349 191-207 487-507 381-399 411-433

Table 8. Proteins identified by reverse phase-LC-MS/MS after diagonal electrophoresis of pancreas of rats with acute pancreatitis. SPOTS 1, 2, 3 and 4. The table shows protein accession number (Acc. No), number of spot analyzed, name and score of the protein obtained by MASCOT search. Scores > 45 indicate identity or extensive homology ($p<0,05$). Position of the identified peptide in the protein sequence, peptide sequence and its calculated mass are also shown.

SPOT	PROTEIN NAME	Acc No	SCORE	PEPTIDE SECUENCE	POSITION
5	Elongation factor 1-alpha	gi|50402095	144	QLIVGVNK QTVAVGVIK LPLQDVYK IGGIGTVPVGR LEDGPKFLK STTTGHLIYK EHALLAYTLGVK YYVTIIDAPGHR THINIVVIGHVDSGK VETGVLKPGMVVTFAPVNVTTEVK SGDAAIVDMVPGKPMCVESFSDYPP LGR	147-154 431-439 248-255 256-266 387-395 21-30 135-146 85-96 6-20 267-290 396-423
6	Peroxiredoxin-4	gi|81917941	276	RQGGLGPIR ELKLTDYR LVQAFQYTDK QITLNDLPVGR VSVADHSLHLSK GLFIIDDKGVLR IPLLSDLNHQISK DYGVYLEDSGHTLR QITLNDLPVGRSVDETLR ENECHFYAGGQVYPGEVSR QGGLGPIRIPLLSDLNHQISK AKISKPAPYWEGTAVINGEFK HGEVCPAGWKPGSETIIPDPAGK TRENECHFYAGGQVYPGEVSR SINTEVVACSVDSQFTHLAWINTPR	167-175 102-109 233-242 215-225 69-80 203-214 176-188 189-202 215-232 50-68 168-188 81-101 243-265 48-68 142-166
7,8,9	Serum albumin	gi|124028612	306, 288, 239	SIHTLFGDK NECFLQHK LVQEVTDFAK EAHKSEIAHR FKDLGEQHFK FPNAEFAEITK HPDYSVSLLLR APQVSTPTLVEAAR RHPDYSVSLLLR LQACCDKPVLQK GLVLIAFSQYLQK DVFLGTFLYEYSR	89-97 123-130 66-75 25-34 35-44 247-257 262-372 439-452 361-372 299-310 45-57 348-360

Table 9. Proteins identified by reverse phase-LC-MS/MS after diagonal electrophoresis of pancreas of rats with acute pancreatitis. SPOTS 5,6, 7, 8 and 9. The table shows protein accession number (Acc. No), number of spot analyzed, name and score of the protein obtained by MASCOT search. Scores > 45 indicate identity or extensive homology ($p<0,05$). Position of the identified peptide in the protein sequence, peptide sequence and its calculated mass are also shown.

In order to find more proteins that suffer oxidation by disulfide bridge in acute pancreatitis, another proteomic method was used: redox switch labelling with biotinylated iodoacetamide. Free thiols of proteins were first blocked with iodoacetamide and the first dimension was performed. For the second dimension, oxidized cysteines were reduced with DTT and alkylated with biotinylated iodoacetamide. After performing diagonal electrophoresis and staining with coomasie blue, proteins with cysteines alkylated with biotinylated iodoacetamide were identified by LC/MS-MS.

It was found reversible oxidation of cysteines in mitochondrial sulfide: quinone oxidoreductase and 60S ribosomal protein L7a at 6h post-induction of acute pancreatitis (figure 66 and table 10). A majority of these modifications seems to be by low molecular weight thiols since the diagonal electrophoresis performed after the redox switch labelling developed with streptavidin-HRP revealed a marked increase in chemiluminescence along the diagonal (figure 44).

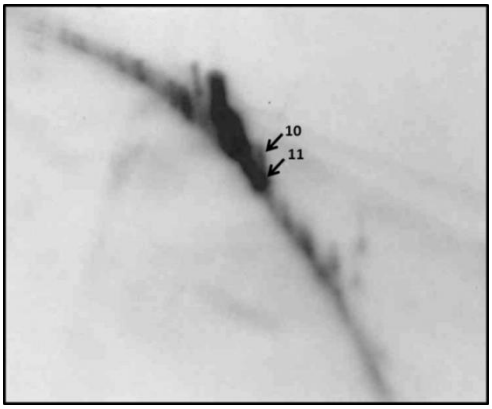


Figure 66. Identification of proteins with redox-sensitive cysteines by LC-MS/MS after diagonal electrophoresis and staining with comassie blue of pancreas of rats after 6h of acute pancreatitis. Proteins identified were: spot 10: mitochondrial sulfide: quinone oxidoreductase, and spot 11: 60S ribosomal protein L7a.

SPOT	PROTEIN NAME	Acc No	SCORE	PEPTIDE SECUENCE	POSITION	MASS (CALC)
10	sulfide: quinone oxidoreductase, mitochondrial	gi|209862751	138	VAELNPDQN**C**IR	118-129	1754,19
11	60S ribosomal protein L7a	gi|293342953	1001	MGVPY**C**IIK	177-185	1406,06

Table 10. Proteins identified by LC-MS/MS after diagonal electrophoresis in pancreas of rats after 6h of acute pancreatitis. SPOTS 10 and 11. The table shows protein accession number (Acc. No), number of spot analyzed, name and score of the protein obtained by MASCOT search. Scores > 45 indicate identity or extensive homology ($p<0,05$). Position of the identified peptide in the protein sequence, peptide sequence and its calculated mass are also shown. Calculated mass is the result of the addition of biotinylated iodoacetamiede mass to the original peptide mass (ExPASy tool). Oxidized cysteine is underlined and bold.

7. PROTEIN PHOSPHATASES ACTIVITIES IN PANCREAS OF RATS WITH ACUTE PANCREATITIS. EFFECT OF N-ACETYLCYSTEINE

In order to confirm the functional relevance of disulfide formation in acute pancreatitis, protein phophatase activities were measured.

7.1. TYROSINE PHOSPHATASE ACTIVITY IN PANCREAS OF RATS WITH ACUTE PANCREATITIS. EFFECT OF N-ACETYLCYSTEINE

Cytosolic tyrosine phosphatase activity was determined by non-radioactive ELISA-based assay in pancreas of control rats and in pancreas of rats after 1h and 6h of acute pancreatitis. Two groups of rats with acute pancreatitis were given N-acetylcysteine (NAC) at a total dose of 100 mg/kg divided in two intraperitoneal injections, the first one 1 h prior to the induction of pancreatitis and the second one immediately just after induction of

pancreatitis. Rats treated with NAC were also sacrificed at 1h and 6h after pancreatitis induction.

Cytosolic tyrosine phosphatase (PTP) activity in control rats was 3,21 ± 0,43 nmol of PO_4^{2-} released/min mg protein (figure 67). PTP activity decreased significantly at 1h after acute pancreatitis induction leading to 2,12 ± 0,61 nmol of PO_4^{2-} released/min mg protein. At this time of the disease and after treatment with NAC it was 2,22 ± 0,29 nmol of PO_4^{2-} released/min mg protein so NAC could not restore the basal activity. At 6h of acute pancreatitis, PTPs activity was maintained low. In fact, it was 1,57 ± 0,14 nmol of PO_4^{2-} released/min mg protein (i.e. 51% of the activity was lost). At 6h of acute pancreatitis, NAC was able to abrogate the loss of PTPs activity. It was 2,96 ± 1,16 nmol of PO_4^{2-} released/min mg protein and therefore NAC restored 92% of the initial activity at this time.

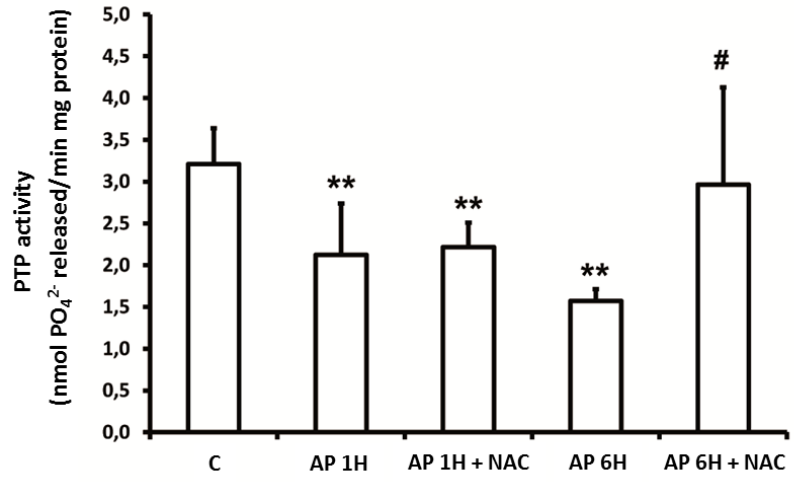


Figure 67. Tyrosine phosphatase (PTP) activity in pancreas of rats with acute pancreatitis (AP). Effect of N-acetylcysteine (NAC). The number of rats per group was 4-5. The statistical difference is indicated as follows: **P<0,01 vs. control; #P<0,05 vs. AP 6H.

7.2. ACTIVITIES OF SERINE/THREONINE PHOSPHATASES PP1, PP2A, PP2B AND PP2C IN PANCREAS OF RATS WITH ACUTE PANCREATITIS. EFFECT OF N-ACETYLCYSTEINE

Serine/threonine phosphatase activities of PP1, PP2A, PP2B and PP2C were determined by non-radioactive ELISA-based assay in pancreas of control rats and in pancreas of rats after 1h and 6h of acute pancreatitis. Two groups of rats with acute pancreatitis were given NAC at a total dose of 100 mg/kg divided in two intraperitoneal injections, the first one 1 h prior to the induction of pancreatitis and the second one immediately just after induction of pancreatitis. Rats treated with NAC were also sacrificed at 1h and 6h after pancreatitis induction.

7.2.1. SERINE/THREONINE PROTEIN PHOSPHATASE ACTIVITY OF PP1

PP1 activity did not change significantly during the course of acute pancreatitis. In addition, NAC did not modify PP1 activity (figure 68). In control rats, PP1 activity was 0,47 ± 0,13 nmol of PO_4^{2-} released/min mg protein and in rats after 1h of acute pancreatitis induction it was 0,37 ± 0,10 nmol of PO_4^{2-} released/min mg protein. At this time of the disease and after treatment with NAC it was 0,40 ± 0,12 nmol of PO_4^{2-} released/min mg protein. At 6h it was 0,41 ± 0,06 nmol of PO_4^{2-} released/min mg protein, and at this time after NAC treatment it was similar, with a value of 0,44 ± 0,20 nmol of PO_4^{2-} released/min mg protein. To sum up, PP1 activity was not affected during acute pancreatitis progression.

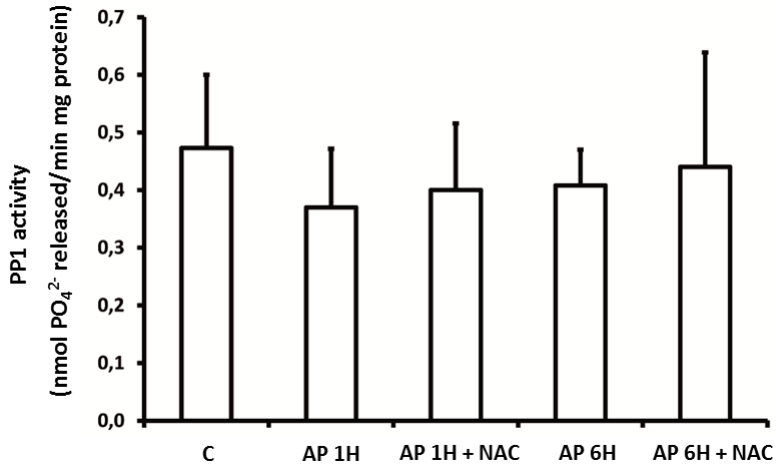


Figure 68. Serine/threonine phosphatase PP1 activity in pancreas of rats with acute pancreatitis (AP). Effect of N-acetylcysteine (NAC). The number of rats per group was 4-5.

7.2.2. SERINE/THREONINE PROTEIN PHOSPHATASE ACTIVITY OF PP2A

Figure 69 shows the results of serine/threonine phosphatase PP2A activity. In control rats it was 2,78 ± 0,54 nmol of PO_4^{2-} /min mg protein. PP2A activity decreased significantly at 1h after acute pancreatitis induction, being 1,88 ± 0,24 nmol of PO_4^{2-} released/min mg protein. It was maintained low at 6h (1,71 ± 0,07 nmol of PO_4^{2-} released/min mg protein). In both cases, NAC was able to abrogate the loss of PP2A activity. In rats with acute pancreatitis treated with NAC it was at 1h 2,49 ± 0,46 nmol of PO_4^{2-} released/min mg protein and at 6h 2,49 ± 0,34 nmol of PO_4^{2-} released/min mg protein. Therefore, NAC was able to restore up to 90% of the activity of PP2A. These results show that there is a clear loss of PP2A phosphatase activity in acute

pancreatitis, which was abrogated by N-acetylcysteine indicating that PP2A is redox sensitive.

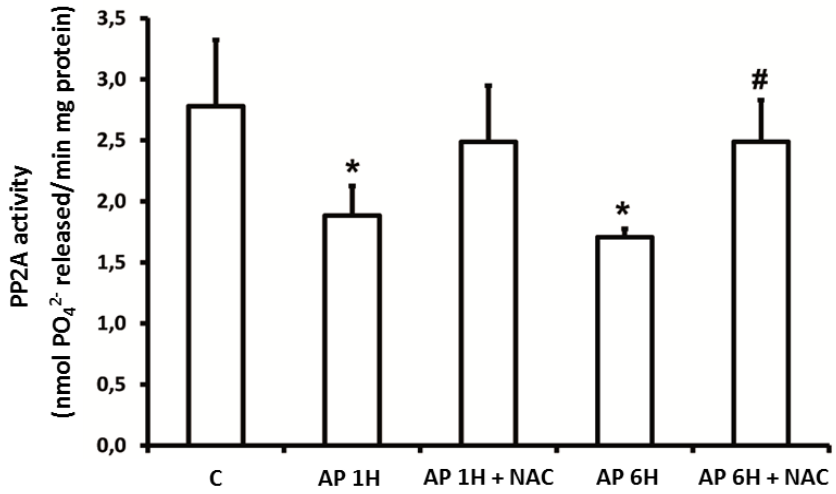


Figure 69. Serine/threonine phosphatase PP2A activity in pancreas of rats with acute pancreatitis (AP). Effect of N-acetylcysteine (NAC). The number of rats per group was 4-5. The statistical difference is indicated as follows: *P<0,05 vs. control; #P<0,05 vs. AP 6H.

7.2.3. SERINE/THREONINE PROTEIN PHOSPHATASE ACTIVITY OF PP2B

PP2B activity decreased at 6h after acute pancreatitis induction (figure 70). NAC did not modify the loss of PP2B activity (figure 70). In control rats, PP2A activity was 3,86 ± 0,49 nmol of PO_4^{2-} /min mg protein. In rats after 1h of acute pancreatitis induction it was 2,95 ± 0,44 nmol of PO_4^{2-} released/min mg protein. At this time of the disease and after the treatment with NAC, the enzyme exhibited similar activity, 3,01 ± 0,62 nmol of PO_4^{2-} released/min mg protein. At 6h it was 2,27 ± 0,14 nmol of PO_4^{2-} released/min mg protein and after NAC treatment it was 2,58 ± 0,35 nmol of PO_4^{2-} released/min mg protein.

Briefly, PP2B activity was affected during acute pancreatitis progression but NAC was not able to reverse the loss of enzyme activity.

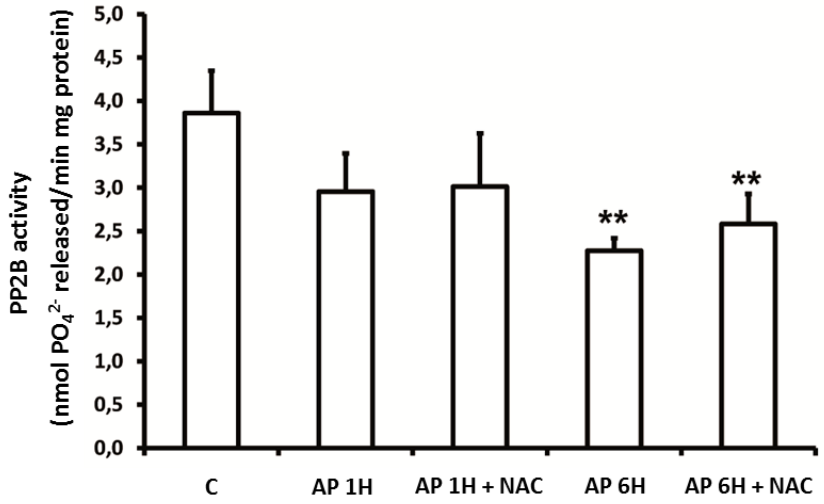


Figure 70. Serine/threonine phosphatase PP2B activity in pancreas of rats with acute pancreatitis (AP). Effect of N-acetylcysteine (NAC). The number of rats per group was 4-5. The statistical difference is indicated as follows: **P<0,01 vs. control.

7.2.4. SERINE/THREONINE PROTEIN PHOSPHATASE ACTIVITY OF PP2C

The last serine/threonine protein phosphatase activity measured was PP2C. The phosphatase activity of PP2C did not change significantly at early states of acute pancreatitis (figure 71). The basal activity of PP2C was 3,14 ± 0,57 nmol of PO_4^{2-} released/min mg protein. At 1h post-induction of acute pancreatitis, the activity of PP2C remained unchanged with value of 3,06 ± 0,79 nmol/min mg protein. At 1h, the treatment with NAC did not affect PP2C activity, which was 3,01 ± 0,30 nmol/min mg protein. On the other hand, PP2C

activity significantly diminished at 6h after the onset of acute pancreatitis (figure 71), which was 2,12 ± 0,14 nmol of PO_4^{2-} released/min mg protein. Nevertheless, NAC treatment was not able to recover PP2C activity. Indeed, it was 2,29 ± 0,23 nmol of PO_4^{2-} released/min mg protein at 6h after this treatment.

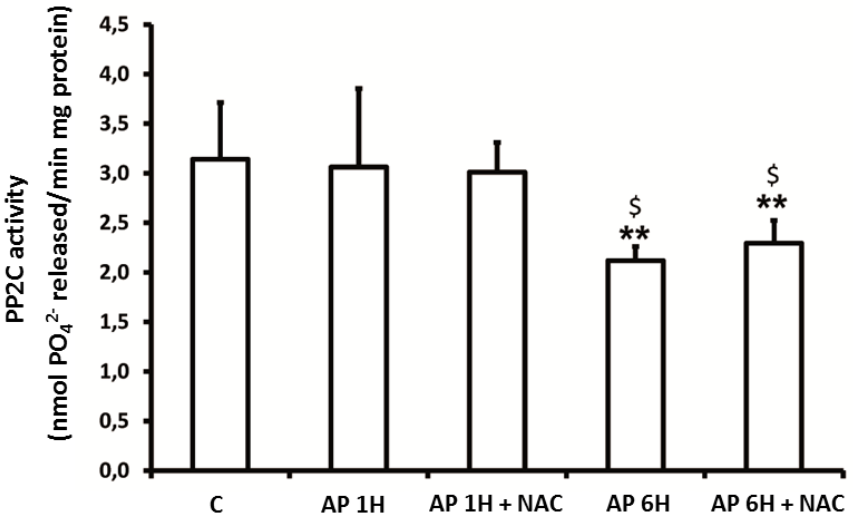


Figure 71. Serine/threonine phosphatase PP2C activity in pancreas of rats with acute pancreatitis (AP). Effect of N-acetylcysteine (NAC). The number of rats per group was 4-5. The statistical difference is indicated as follows: **P<0,01 vs. control; $^{\$}$P<0,05 vs. AP 1H.

8. PP2AC AS A TARGET OF DISULFIDE FORMATION

As shown before in figure 51, PP2Ac was identified as a target of disulfide formation in acute pancreatitis. Therefore, we decided to study oxidative modifications of PP2Ac in more depth as detailed in the following sections.

8.1. OXIDATIVE MODIFICATION OF PP2AC DURING ACUTE PANCREATITIS

Diagonal electrophoresis coupled with western blot detection was performed in pancreas of control rats and 6h after acute pancreatitis induction in order to assess the oxidative modification of PP2Ac. In control samples, PP2Ac laid in the diagonal but at 6h of the disease, the protein laid above the diagonal (figure 72) which indicates that its major modification seems to be an intramolecular disulfide bridge.

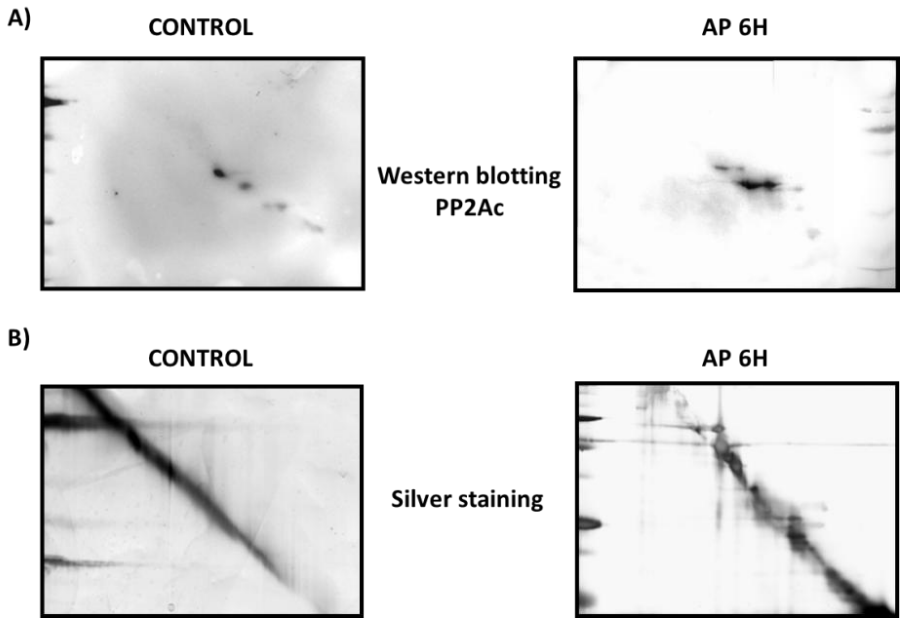


Figure 72. Oxidative modification of PP2Ac in pancreas of rats with acute pancreatitis (AP). A) Diagonal electrophoresis coupled with western blotting. B) Silver staining as loading control.

8.2. *IN VITRO* REGULATION OF THE MAPK AND INFLAMMATORY CASCADES BY PP2A

In order to study the role of PP2A inactivation in the activation of the MAPK cascade and up-regulation of inflammatory cytokines, the catalytic subunit of PP2A was silenced in pancreatic acinar 266-6 cells using lentivirus. Figure 73 shows the expression of TurboGFP which indicates that the transduction of 266-6 cells with random shRNAmir and PP2Ac shRNAmir was effective.

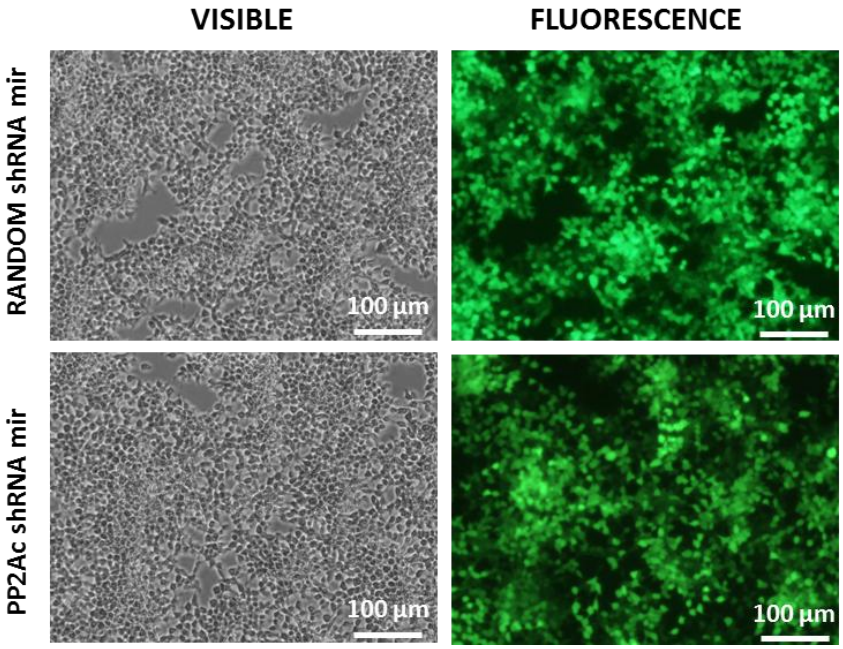


Figure 73. Transduction efficiency of pancreatic acinar 266-6 cells using lentivirus. The figure shows the expression of TurboGFP in cells transduced with random shRNAmir and PP2Ac shRNAmir. Cells were observed by fluorescence microscopy through visible and fluorescence filters.

8.2.1. REGULATION OF THE MAPK CASCADE BY PP2A

In order to assess activation of the MAPK cascade when the PP2Ac was silenced in pancreatic acinar 266-6 cells western blottings of JNK, ERK1/2, p38α and their phosphorylated forms were performed. We managed to silence PP2Ac around 65%, using tubulin as loading control. Figure 74 shows that a reduction in the expression of PP2Ac triggered a dramatic increase in phospho-ERK1/2 and phospho-p38α as an index of their activation. Therefore, PP2Ac has an important role in the MAPK cascade attenuating and controlling this pathway.

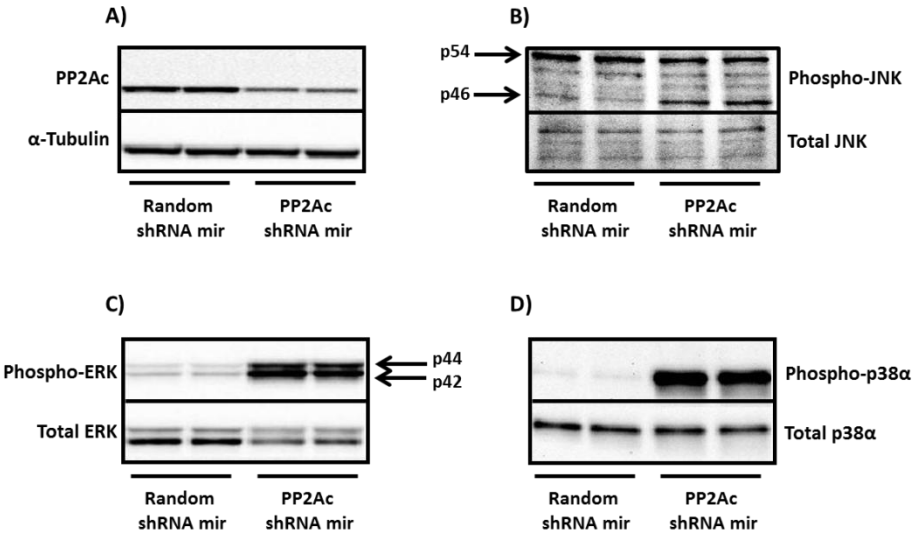


Figure 74. Modulation of the MAPK cascade by PP2Ac inactivation in pancreatic acinar 266-6 cells. The activation of the MAPKs JNK, ERK 1/2 and p38α and their phosphorylated forms was assessed by western blotting. The blots include a set of two different experiments. A) PP2Ac silencing relative to α-tubulin (loading control). B) Phospho-JNK relative to total JNK. C) Phospho-ERK1/2 relative to total ERK1/2. D) Phospho-p38α relative to total p38α.

8.2.2. REGULATION OF THE INFLAMMATORY CASCADE BY PP2A

In order to assess the role of PP2Ac in the inflammatory cascade, non-silenced and PP2Ac-silenced pancreatic acinar 266-6 cells were incubated with 10 ng/mL of TNF-α for 1h. The up-reguulation of inflammatory cytokines was studied by real time PCR (RT-PCR) of genes *il-6, cxcl1* (the equivalent in mouse to human *il-8*) and *tnf-α.*

Under basal conditions, PP2Ac silencing enhanced 8 ± 2 fold the mRNA expression of *il-6* in 266-6 cells (figure 75). There were no significant differences between control conditions and treatment with TNF-α.

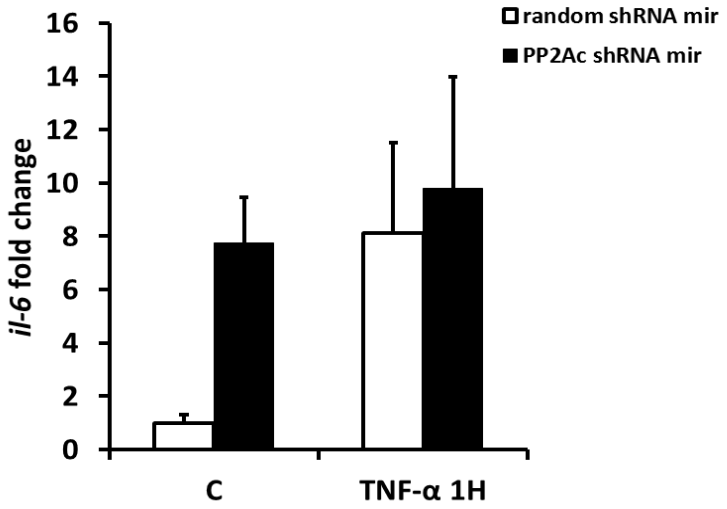


Figure 75. *il-6* RT-PCR of non-silenced and PP2Ac-silenced pancreatic acinar 266-6 cells treated with 10 ng/mL of TNF-α for 1h. *Rplp0* was used as housekeeping gene. The number of experiments performed was 3 for each condition. Values were normalized to control random shRNAmir group.

Under basal conditions, PP2Ac silencing increased 12 ± 4 fold the mRNA expression of *cxcl1* in pancreatic acinar 266-6 cells (figure 76). After incubation with 10 ng/mL TNF-α for 1h, the mRNA expression of the cytokine cxcl1 (the equivalent in mouse to human il-8) was up-regulated both in non-silenced cells (10 ± 5 fold) and when PP2Ac was silenced (80 ± 34 fold), specially in the latter where it markedly increased (figure 76).

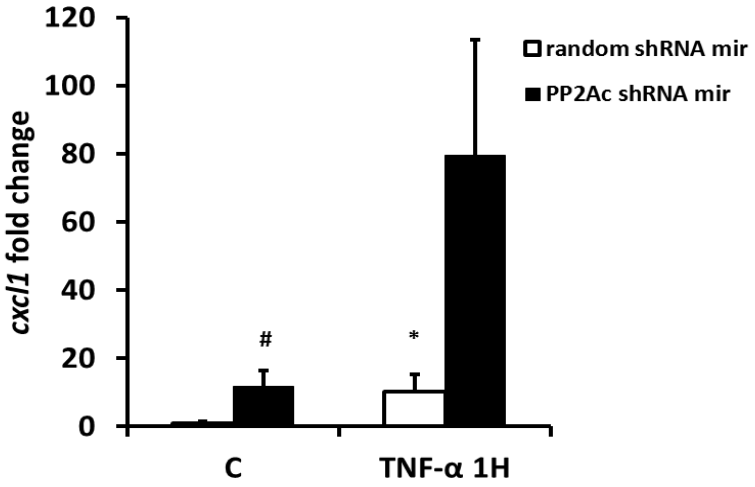


Figure 76. *cxcl1* RT-PCR of non-silenced and PP2Ac-silenced pancreatic acinar 266-6 cells treated with 10 ng/mL of TNF-α for 1h. *Rplp0* was used as housekeeping gene. The number of experiments performed was 3 for each condition. Values were normalized to control random shRNAmir group. The statistical difference is indicated as follows: *P<0,05 vs. control conditions; #P<0,05 vs. random shRNAmir

In the case of the mRNA expression of *tnf-α* under basal conditions, there were no differences between non-silenced and PP2Ac-silenced 266-6 cells (figure 77). After the pro-inflammatory stimulus with 10 ng/mL of TNF-α for 1h, the mRNA expression of *tnf-α* was significantly up-regulated both in non-silenced (7 ± 3 fold) and in PP2Ac-silenced cells (10 ± 4 fold) (figure 77).

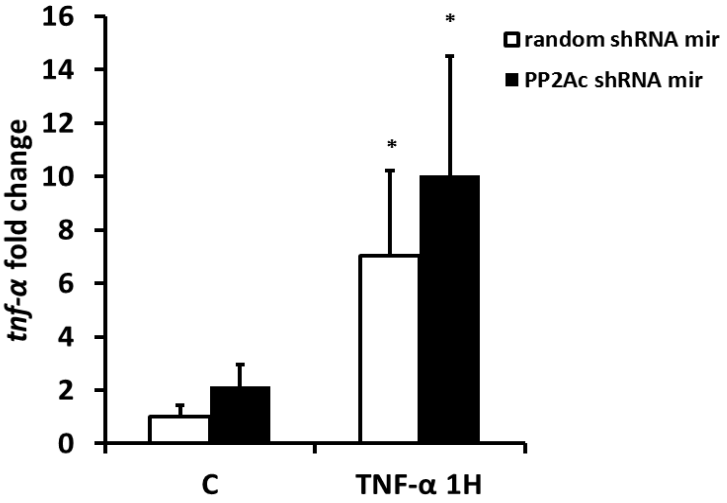


Figure 77. *tnf-α* RT-PCR of non-silenced and PP2Ac-silenced pancreatic acinar 266-6 cells treated with 10 ng/mL of TNF-α for 1h. *Rplp0* was used as housekeeping gene. The number of experiments performed was 3 for each condition. Values were normalized to control random shRNAmir group. The statistical difference is indicated as follows: *P<0,05 vs. control conditions.

To sum up, the three genes were up-regulated after the pro-inflammatory stimulus with TNF-α in 266-6 cells, and to a much greater extent *cxcl1* when the PP2Ac was silenced.

To complete the study on the role of PP2Ac in the inflammatory cascade and to simulate *in vitro* the conditions of the *in vivo* experimental model induced by sodium taurocholate, non-silenced and PP2Ac-silenced 266-6 cells were treated with 0,5% sodium taurocholate for 2h. Then, the mRNA expression of the previously studied genes (*il-6, cxcl1* and *tnf-α*) was assessed by real time PCR.

Under normal conditions, PP2Ac silencing increased 4 ± 1 fold the mRNA expression of *il-6* in 266-6 cells (figure 78). There were no significant differences between control conditions and treatment with taurocholate. Hence, a similar profile to that observed with TNF-α treatment (figure 75) was found.

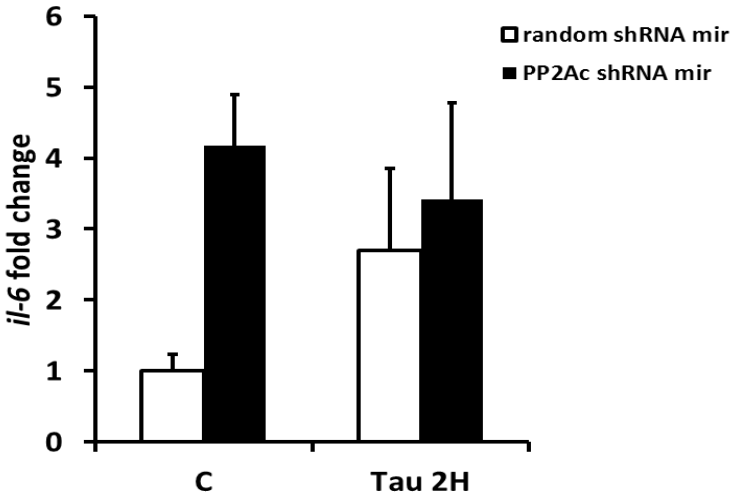


Figure 78. *il-6* RT-PCR of non-silenced and PP2Ac-silenced pancreatic acinar 266-6 cells treated with 0,5% of sodium taurocholate for 2h. *Rplp0* was used as housekeeping gene. The number of experiments performed was 3 for each condition. Values were normalized to control random shRNAmir group.

Under basal conditions, the mRNA expression of *cxcl1* did not vary between non-silenced and PP2Ac-silenced 266-6 cells (figure 79). The mRNA expression of *cxcl1* was induced 5 ± 1 fold after sodium taurocholate treatment in non-silenced 266-6 cells. It is noteworthy that *cxcl1* was markedly up-regulated up to 20 ± 8 fold in PP2Ac-silenced 266-6 cells after 2h of sodium taurocholate incubation (figure 79).

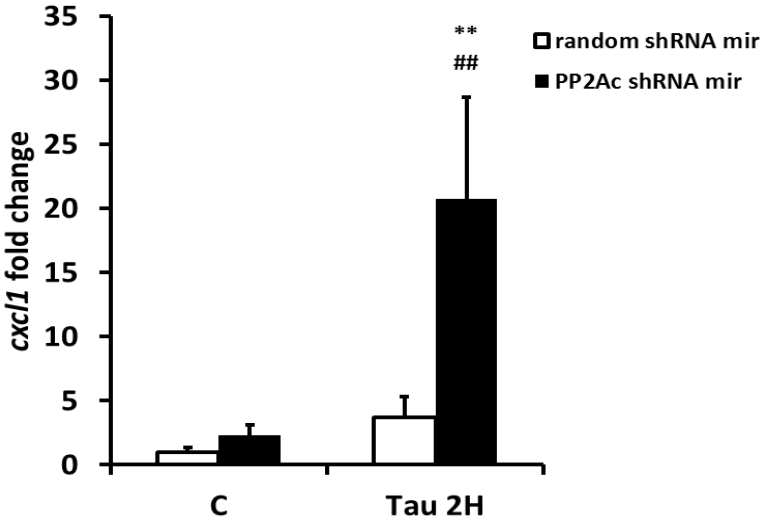


Figure 79. *cxcl1* RT-PCR of non-silenced and PP2Ac-silenced pancreatic acinar 266-6 cells treated with 0,5% of sodium taurocholate for 2h. *Rplp0* was used as housekeeping gene. The number of experiments performed was 3 for each condition. Values were normalized to control random shRNAmir group. The statistical difference is indicated as follows: **P<0,01 vs. control conditions; ##P<0,01 vs. random shRNAmir.

As in the case of *cxcl1*, there were no differences under normal conditions in the mRNA expression of *tnf-*α between non-silenced and PP2Ac-

silenced 266-6 cells (figure 80). The relative mRNA expression of *tnf-α* was 1,3 ± 0,5 after treatment with sodium taurocholate and it was 2,1 ± 0,7 fold when PP2A was silenced in 266-6 cells (figure 80). However, no significant differences were observed.

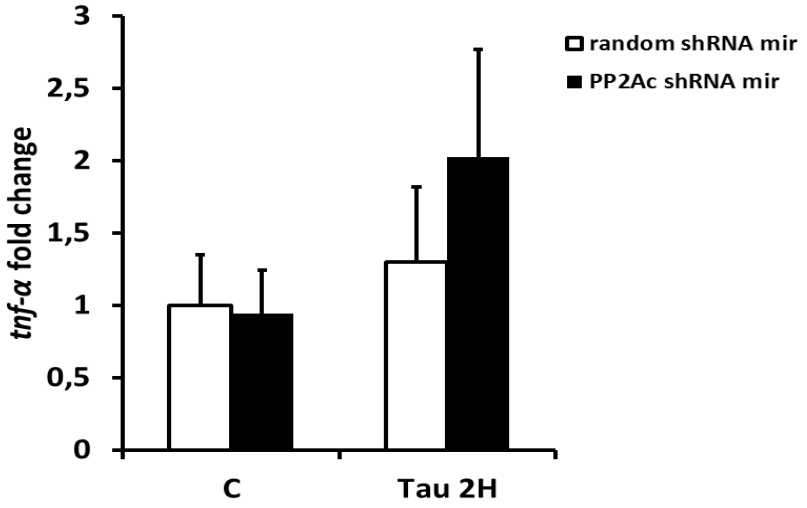


Figure 80. *tnf-α* RT-PCR of non-silenced and PP2Ac-silenced pancreatic acinar 266-6 cells treated with 0,5% of sodium taurocholate for 2h. *Rplp0* was used as housekeeping gene. The number of experiments performed was 3 for each condition. Values were normalized to random shRNAmir group.

VI. DISCUSSION

1. ACUTE PANCREATITIS: THE EXPERIMENTAL APPROACH

Nowadays, acute pancreatitis is a relatively common disease. However, the main questions that raise both doctors and researchers remain unanswered. How we could diagnose acute pancreatitis earlier? Which treatment we should give to a patient to attenuate the inflammatory process? What is the period of time that we can permit between the data of the patient is taken and the diagnosis of acute pancreatitis? In order to answer these questions, it is necessary to find out the molecular mechanisms that occur during the development of the pancreatic inflammatory response.

Fortunately, researchers have different experimental models to induce and study acute pancreatitis in animals, all characterized and well established to induce both mild and severe acute pancreatitis (Lerch and Gorelick 2013). For the development of this Thesis, it has been used a model of severe acute necrotic pancreatitis induced by retrograde infusion of sodium taurocholate in rats. This model has a high reproducibility and has been handled by our research group for several years (Pereda et al. 2008; Escobar et al. 2012). *In vivo* models enable to study the behavior of different types of cells in the animal showing more closely what would happen in humans. However, the disadvantage of *in vivo* models is the requirement of the sacrifice of a high amount of animals. In our group we also use *in vitro* cell line models of pancreatic acinar cells, such as AR42J and 266-6 that come from rats and mice respectively. *In vitro* models serve to investigate and further demonstrate the mechanisms involved. However, the observations obtained from the *in vitro* experiments not always correspond to what happens *in vivo.* Therefore, for the study of acute pancreatitis it is necessary to use both *in vitro* and *in vivo* models.

2. DISULFIDE STRESS AS A NOVEL TYPE OF OXIDATIVE STRESS IN ACUTE PANCREATITIS

Glutathione is the most abundant non-protein thiol in mammalian cells. It consists of three amino acids: glutamic acid, cysteine and glycine. Glutathione is a major cellular antioxidant and plays an important role in numerous cell functions. Thus, the redox state of glutathione (GSSG/GSH) is considered a reliable indicator of oxidative stress in cells because it reflects the intracellular balance between pro-oxidant and antioxidant species (Jones 2006).

It is known that there is an association between acute inflammation and depletion of reduced glutathione (GSH). In addition, GSH depletion in pancreas is a hallmark of the early course of acute pancreatitis (Gomez-Cambronero et al. 2000; Pereda et al. 2008; Escobar et al. 2009) and contributes to the severity of the disease (Neuschwander-Tetri et al. 1992; Alsfasser et al. 2002). One of the aims of this Thesis was to characterize the consequences of GSH depletion in acute pancreatitis at the molecular level. Therefore, we decided to measure the levels of the reduced and oxidized forms of glutathione in pancreas of control rats and pancreas of rats after 1 hour and 6 hours after the induction of acute necrotizing pancreatitis. Our results show that GSH depletion in pancreas in acute pancreatitis occurs without significant changes in GSSG levels. Indeed, GSH levels decreased after induction of pancreatitis, whereas GSSG levels were maintained without significant changes in the course of the disease. Importantly, the GSH/GSSG ratio did not change significantly after pancreatitis induction. Therefore, during acute pancreatitis GSH levels decreased, but this was not due to oxidation of glutathione.

As glutathione is a tripeptide containing the thiol amino acid cysteine, we decided to measure the levels of cysteine and cystine in control rat pancreas and in rat pancreas after induction of acute necrotizing pancreatitis to check if the decrease in GSH levels was due to degradation of GSH that would lead to an increase in the levels of its constituent amino acids. After 1 h of the beginning of the disease, cysteine levels markedly increased, coming back to the basal levels at 6h. Furthermore, cystine levels markedly increased during the course of acute pancreatitis. According to these results, we conclude that during acute pancreatitis GSH is suffering degradation and this would explain the decrease in GSH levels without glutathione oxidation as well as the increase in cysteine and cystine levels at 1h after acute pancreatitis induction. The subsequent decrease of cysteine at 6h may be due to oxidation and it would contribute to the increase in cystine levels.

Meister suggested that glutathione depletion may be due to activation of proteases, such as carboxypeptidase, that may cleave GSH (Meister 1991). Trypsinogen activation is accompanied by glutathione depletion in experimental acute pancreatitis (Luthen et al. 1998). Accordingly, the increase in cysteine and cystine levels that occurs in pancreatitis is likely to be ascribed to GSH breakdown by pancreatic proteases. In the case of glutathione, glutathione reductase and glutaredoxins contribute to maintain its reduced state (Mieyal et al. 2008). However, there are no specific cysteine reductases and the thioredoxin system exhibits limited activity to reduce cystine in mammalian cells (Mannervik et al. 1983). This would favor oxidation of the cysteine/cystine pair.

The transsulfuration pathway constitutes the major route of cysteine biosynthesis. Homocysteine is at the junction of two metabolic pathways:

remethylation to methionine and transsulfuration to cysteine. Homocysteine is an homologue of the amino acid cysteine, differing by an additional methylene bridge (-CH_2-). So that, homocysteine levels were determined in rat pancreas of control rats and after the induction of acute pancreatitis. Homocysteine levels in pancreas significantly decreased 1h after acute pancreatitis. This concurs with the moment in which cysteine levels are increased. This makes sense because the cysteine increase would produce a negative feedback in the transsulfuration pathway that would enhance the remethylation pathway and would inhibit GSH synthesis. Furthermore, the observed oxidation of homocysteine to homocystine during acute pancreatitis would also explain the decrease in homocysteine levels.

Because γ-glutamylcysteine is the immediate precursor in the synthesis of GSH, the levels of its reduced and oxidized forms were measured in order to see how they were affected during acute pancreatitis. As in the case of cysteine, γ-glutamylcysteine significantly decreased at 6h after the onset of the disease. This makes sense because cysteine is the precursor of γ-glutamylcysteine. Like the other oxidized forms of the redox couples studied above (except GSSG), the oxidized form of γ-glutamylcysteine (bis-γ-glutamylcystine), increased during the course of acute pancreatitis. This may also contribute to the decrease in γ-glutamylcysteine levels.

Protein-SH groups can form mixed disulfides with low-molecular-weight-thiols (LMWT) such as γ-glutamylcysteine, free cysteine, or glutathione (Eaton 2006). Therefore, in this work the levels of protein glutathionylation, cysteinylation and γ-glutamylcysteinylation were also determined in rat pancreas. Intracellularly, GSH is the main LMWT, making protein glutathionylation an important protein modification under normal conditions.

However, protein glutathionylation did not change significantly after pancreatitis induction. Notably, protein cysteinylation was non-detectable in pancreas under basal conditions but it increased markedly after induction of pancreatitis, particularly at 6 h when the cysteine/cystine ratio was lower. In line with this, protein γ-glutamylcysteinylation rose progressively after induction of pancreatitis and the γ-glutamylcysteine/bis-γ-glutamylcystine ratio decreased upon pancreatitis.

The increase in fluorescence after pancreatitis onset when the "redox monobromobimane switch" was performed confirmed that reversible thiol oxidations are formed in the course of the disease. The formation of protein disulfides could be a mechanism to protect proteins from further, potentially more damaging, oxidation (Hochgrafe et al. 2007). Specifically, reversible oxidation in cysteines of mitochondrial sulfide:quinone oxidoreductase and 60S ribosomal protein L7a was identified at 6h post-induction of acute pancreatitis. The majority of these modifications are likely to by low molecular weight thiols since the diagonal electrophoresis performed after the redox switch labelling revealed a marked increase in chemiluminescence along the diagonal. This would support the thiol oxidation by protein cysteinylation and γ-glutamylcysteinylation that was observed at 6h after the induction of acute pancreatitis.

All these findings show a situation of disulfide stress in acute inflammation in pancreas of mammals associated with oxidation of pairs cysteine/cystine, γ-glutamylcysteine/bis-γ-glutamylcystine, and homocysteine/homocystine, as well as protein cysteinylation and γ-glutamylcysteinylation, but without glutathione oxidation or changes in protein glutathionylation. The redox status of thiol groups in the cytosol is mainly

regulated by the systems thioredoxin/thioredoxin reductase and glutathione/glutathione reductase (Toledano et al. 2007). However, the redox status of thioredoxin and the pair GSH/GSSG seems to be regulated independently (Nkabyo et al. 2002). Moreover, Toledano and co-workers showed that the thiol redox status of yeast could be maintained when GSH was depleted (Kumar et al. 2011). Our results support all these statements and the hypothesis of Toledano and coworkers, who suggested that glutathione levels are not critical for thiol redox control in proteins. In addition, as mentioned before, the absence of a specific cysteine reductase and the limited activity of the thioredoxin system to reduce cystine in mammalian cells (Mannervik et al. 1983) would favor disulfide stress in proteins, as it occurs in the pancreas during acute pancreatitis.

The term disulfide stress was already used in bacteria referring to disulfide bonds generated by diamide (Aslund and Beckwith 1999; Zhang and Zuber 2007). However, diamide triggers typical oxidative stress and damage associated with glutathione oxidation, which is clearly different from the disulfide stress that is described here associated with a pathophysiological condition such as acute inflammation.

Given that the redox potentials of thioredoxin (from – 270 to – 300 mV) and glutathione (between - 200 and – 260 mV) in the cytoplasm are strongly reducing (Aslund and Beckwith 1999; Jones et al. 2004; Wouters et al. 2010), large drops in the cellular redox status would be required for thiol-disulfide exchanges in proteins that may cause profound effects on protein folding and activity (Aslund and Beckwith 1999). However, no such big drops would be required if the cysteine/cystine couple is involved. Indeed, it was reported that the intracellular redox potential of the cysteine/cystine couple is

between -125 and -160 mV, i.e. it is considerably more oxidized than the GSH/GSSG couple (Jones et al. 2004). Furthermore, the redox status of cysteine/cystine and GSH/GSSG are not in equilibrium and seem to be independently regulated in cells (Jones et al. 2004). Thus, the cysteine/cystine pool is more prone to oxidation and accordingly Jones and colleagues suggested that oxidation of thiol moieties by cystine as well as S-cysteinylation of thiols would be new classes of redox signaling (Jones et al. 2004).

3. TARGETS OF DISULFIDE STRESS IN ACUTE PANCREATITIS

The next step in this Thesis was to identify targets of disulfide stress that were oxidized in the course of acute pancreatitis.

Our results show that ribonuclease inhibitor, serine/threonine phosphatase PP2A, tyrosine phosphatases SHP1 and SHP2, protein disulfide isomerase (PDI), thioredoxin 1, KEAP-1, APE1/REF1, peroxiredoxin 4, albumin, α-amylase and elongation factor 1α are oxidized in acute pancreatitis marking them as first or early targets of disulfide stress. In the case of APE1/REF1, additional degradation was found in the course of pancreatitis.

On the other hand, elastase, anionic trypsin 1, and cationic trypsin 3 were found in all samples (i.e. from both control and pancreatitis) so they are not targets of disulfide stress upon the disease. These three proteins are components of the pancreatic juice. They are secreted proteins (Aslund and Beckwith 1999; Wouters et al. 2010) with constitutive disulfide bonds because they appeared above the diagonal and therefore, these disulfides are a hallmark of pancreatic tissue.

All the potential targets of disulfide stress that were studied in the present Thesis were chosen by their importance and particular relevance because of their implication in redox stability, redox signaling and the inflammatory cascade that occurs in acute pancreatitis. All the identified targets of disulfide stress could be classified into two groups: Redox buffers and redox signaling thiols. Redox buffers would include ribonuclease inhibitor and albumin.

Pancreatic ribonuclease is a nuclease that catalyzes the hydrolysis of RNA in the pancreas. It is extremely abundant and therefore, ribonuclease inhibitor (RNH) plays an important role in the protection of RNA degradation (Shapiro 2001). Pancreatic ribonuclease is nontoxic under physiological conditions due to the presence of the ribonuclease inhibitor in the cytosol of mammalian cells (Leland and Raines 2001). However, ribonucleases become cytotoxic when they escape from the inhibitor (Kim et al. 1995). The ribonuclease inhibitor has a high number of redox-sensitive cysteine residues whose oxidation causes its rapid inactivation and degradation (Ferreras et al. 1995). In addition, a remarkable increase in cytosolic ribonuclease activity is found in necrotizing pancreatitis (Pereda et al. 2008). Consequently, oxidation of RNH1 may contribute to the presence of ribonuclease activity in the cytosol in severe acute pancreatitis, leading to damage and degradation of pancreatic RNA. Furthermore, ribonuclease inhibitor plays a role in cell redox homeostasis as a redox buffer (Monti et al. 2007). Hence, regarding the cellular role of the RNH, instead of being merely an RNase inhibitor, it has been suggested that this protein, with its high content in cysteine residues, has an antioxidant function in the cell (Cui et al. 2003). Accordingly, a scavenging activity of RNH on oxygen radicals was reported (Wang and Li 2006) and RNH maintains a well buffered intracellular redox environment (Monti et al. 2007). Enzymes such as

thioredoxin are involved in the maintenance of its redox status. Thereby, oxidation of thioredoxin 1 during acute pancreatitis would exacerbate the oxidation status of RNH1 during the disease and consequently the damage to the RNA and the impairment of the redox homeostasis would be greater.

Albumin also participates in the maintenance of the homeostasis. It is involved in the regulation of oncotic pressure, the binding and transport of endogenous and exogenous compounds, and antioxidant functions (Oettl and Stauber 2007). Plasma concentrations of albumin are high. Therefore, reactive oxygen species react with albumin leading to albumin oxidation that changes its binding properties (Oettl and Stauber 2007). Albumin contains a total of 35 cysteine residues and comprises the largest thiol pool in plasma (Oettl and Stauber 2007). Because of these, it is readily conceivable that the binding properties of albumin may be altered during the development of acute pancreatitis due to thiol exchange reactions that would lead to the formation of disulfides between albumin and other molecules. The appearance of albumin under the diagonal reveals that it could take part in high molecular weight oxidized complexes in acute inflammation in agreement what has been already mentioned.

The rest of targets of disulfide stress identified in the present Thesis would be included in the group of redox signaling thiols because they contain functional redox switches as discussed below.

The reducing thiol-disulfide status of the cytosol is considered mainly regulated by the thioredoxin-thioredoxin reductase pathway and the glutathione-glutaredoxin pathway (Prinz et al. 1997; Toledano et al. 2007). Nevertheless, the GSH/GSSG and the thioredoxin 1 redox states seem to be

regulated independently (Nkabyo et al. 2002). Our results support this hypothesis since they show that oxidation of thioredoxin 1 occurs without parallel glutathione oxidation in acute pancreatitis. Thioredoxin 1 oxidation would enable and promote disulfide stress in acute inflammation. Our findings would provide a novel mechanism in the regulation of inflammation within the complexity of protein thiol-disulfide oxidoreduction that has been previously described by others (Aesif et al. 2011; Coppo and Ghezzi 2011). According to these previous reports, changes in thiol redox status mainly ascribed to protein glutathionylation as well as to thioredoxin and glutaredoxin. In addition, thioredoxin 1 is required for the activity of many enzymes that are dependent on the reduction of their disulfide bridges (Holmgren and Sengupta 2010). One of these enzymes is peroxiredoxin 1. The oxidation of peroxiredoxin 1 would decrease its detoxifying capacity against hydrogen peroxide. This loss of activity would be enhanced by the oxidation of thioredoxin 1. Hence, the oxidation of thioredoxin 1 and the decrease in the peroxiredoxin 1 antioxidant capacity would favor disulfide stress in acute pancreatitis. Thus, disulfide stress would diminish the capacity for ROS detoxification and if it is prolonged in time, it can enhance oxidative stress leading to irreversible oxidations.

The antioxidant response would also be affected during acute pancreatitis through other pathways. Adaptive response to oxidative stress includes the activation of transcription factors, such as NRF2/KEAP1 in mammals (Wouters et al. 2010; Antelmann and Helmann 2011; Dickinson and Chang 2011) through oxidation of cysteine residues to disulfide bonds (Aslund and Beckwith 1999). Reactive KEAP1 cysteines are redox sensors, and their oxidation by ROS, results in the dissociation of NRF2 from KEAP1 which allows NRF2 stabilization and translocation into the nucleus that would activate the antioxidant response (Dinkova-Kostova et al. 2002; Zhang and Hannink 2003).

The data presented in this Thesis show that the NRF2/KEAP1 pathway is activated in pancreatitis due to KEAP1 oxidation. The formation of these disulfides is transient and may be reversed by thioredoxin or glutathione (Wouters et al. 2010). Thioredoxin 1 oxidation and glutathione depletion (previously mentioned) would aggravate the oxidation status of KEAP1 due to the loss of antioxidant capacity. This would activate in a greater extent the NRF2 pathway and consequently, the antioxidant response would be up-regulated.

The cellular response to oxidative stress would also be affected by the oxidation and loss of Apurinic/apyrimidinase endonuclease 1/Redox effector factor 1 (APE1/REF1). In addition to the central role of APE1 in the base excision repair of DNA lesions generated as a consequence of oxidant-induced base damage, it functions as a redox effector factor (REF1) (Avellini et al. 2010). It means that REF1 is a protein involved in transcriptional regulation of gene expression during adaptive cellular responses to oxidative stress (Avellini et al. 2010). More concretely, REF1 up-regulates the transcription factors AP1, HIF2-α, and p53, and it is involved in post-transcriptional control of gene expression of c-myc (Bhakat et al. 2009; Luo et al. 2010). Therefore, the marked APE/REF1 loss and potential oxidation might contribute to the accumulation of mutations and might affect the regulation of cell cycle and cell proliferation to oxidative stress.

Most cellular responses require protein synthesis. The machinery of protein synthesis would also be affected during acute pancreatitis due to the oxidation of PDI, peroxiredoxin 4, and elongation factor 1α that is reported in the present Thesis.

Protein biosynthesis is the anabolic process in which proteins are formed. The process consists of two stages, the translation of messenger RNA, in which the amino acids of the polypeptide are sorted accurately from the information contained in the nucleotide sequence of DNA, and post-translational modifications that suffer polypeptides pre-formed until they reach their functional status.

The translation stage would be affected during acute pancreatitis due to the oxidation of elongation factor 1α. This protein couples the hydrolysis of GTP to GDP with the delivery of aminoacyl tRNAs to the ribosome during protein translation (Hershey 1991). It also plays a central role in nuclear export of proteins in mammalian cells (Khacho et al. 2008).

Post-translational modifications in proteins would also be affected. Disulfide formation is an essential modification that occurs within the endoplasmic reticulum (ER) of eukaryotic cells. Introduction of native disulfides is a complex process, requiring not only oxidation of cysteine residues but also reduction and isomerization of non-native disulfides (Jansens et al. 2002). In agreement with this complexity, a number of oxidoreductases have been identified, which catalyse thiol-disulfide exchange reactions within the ER (Sevier and Kaiser 2006). PDI represents one of the most extensively studied ER oxidoreductases. PDI-family proteins possess active sites containing cysteine residues within a CXXC motif. Disulfides formed between these cysteine residues can be exchanged with client proteins leading to formation of disulfides within the client protein and reduction of the PDI-family member (Tavender et al. 2010). Therefore, the overoxidation status of PDI during acute pancreatitis would introduce an excess of disulfide bridges in client proteins

that might lead to overfolding and aberrant conformations of proteins resulting result in a loss of function.

To allow continuous transfer of disulfides to client proteins, PDI must be re-oxidized. The oxidation of PDI can be catalysed by ER oxidoreductin 1 (Ero1) (Pollard et al. 1998; Frand and Kaiser 1999). Ero1 can generate disulfides *de novo* by transfer of electrons to molecular oxygen, generating hydrogen peroxide in the process (Tu and Weissman 2002; Gross et al. 2006). In higher eukaryotes, the H_2O_2 produced by Ero1 can be metabolized by peroxiredoxin 4 (Prdx4) (Tavender et al. 2008). The essential catalytic unit of Prdx4 is a dimer with a cysteine residue (Cys124) in one chain being able to reduce H_2O_2 generating water and becoming oxidized in the process. In order to maintain activity towards H_2O_2, the interchain disulfide within Prdx4 needs to be reduced. This reduction is achieved by a member thioredoxins (Kalinina et al. 2008). Thus, in addition to PDI malfunction, oxidation of Prdx4 and Trx1 would block disulfide formation in proteins entering the secretory pathway, and hence promoting the abnormal protein folding response in acute pancreatitis.

Redox signaling in the inflammatory response relies to a great extent on protein phosphatases because their activity is modulated by oxidative stress. Protein phosphatases regulate the inflammatory response by dephosphorylation of substrates, including MAPKs, generally decreasing their activation. They are key sensors of the cellular redox state because reversible oxidation of thiols in protein phosphatases leads to their inactivation (Chiarugi 2005; den Hertog et al. 2005; Chi et al. 2006).

The study of protein phosphatases in this Thesis enabled a further elucidation of the mechanisms involved in the inflammatory process during

acute pancreatitis and allowed the identification of protein phospathases as targets of disulfide stress.

PTP activity was determined at the same time points as low molecular weight thiols and their oxidized forms in order to better understand the relevance of disulfide stress. In addition, a group of rats was given NAC in order to study if disulfide stress was mediating the changes in phosphatase activity. There was a loss of tyrosine phosphatase activity in pancreatitis, which correlated with the marked cysteine oxidation observed in the two members of the family, SHP1 and SHP2. The loss of tyrosine phosphatase activity was abrogated by N-acetyl cysteine (NAC). Therefore, disulfide stress is affecting tyrosine phosphatases because the loss in the activity was prevented by NAC. This is indicating that PTPs are redox sensitive and their oxidation upon pancreatitis is reversible.

Tyrosine phosphatases play an important role in the regulation of the inflammatory cascade through NF-ƙB, MAPKs, and TNF-α (de Dios et al. 2006; Chong and Maiese 2007; Escobar et al. 2010). Therefore, the loss of PTPs activity is an example of redox signaling that would alter these pathways. The inability of PTPs to inhibit MAPK pathway would contribute to up-regulate the inflammatory cascade that occurs during acute pancreatitis.

SHP1 may down-regulate the inflammatory cascade (Chong and Maiese 2007). SHP1 and SHP2 are inactivated by oxidative stress (Lee et al. 1998). These tyrosine phosphatases are oxidized during the course of acute pancreatitis, and consequently, this would be responsible, at least in part, for the fall in tyrosine phosphatase activity. Protein tyrosine phosphatases, particularly SHP1, are critically involved in the regulation and maintenance of

cell adhesions in exocrine pancreas (Schnekenburger et al. 2005). Moreover, inhibition of tyrosine phosphatase activity was sufficient to induce dissociation of adherens junctions in pancreatic acini as a prerequisite for the development of pancreatic edema (Schnekenburger et al. 2005). In this regard, the loss of their activities may decisively contribute to formation of interstitial edema in acute pancreatitis.

When the serine/threonine phosphatase activities were studied, differences between the four enzymes (PP1, PP2A, PP2B and PP2C) comprising the family of PPPs were observed.

PP1 activity was the only serine/threonine phosphatase not affected during acute pancreatitis progression. Indeed, its activity did not change during the course of the disease.

On the other hand, PP2A, PP2B and PP2C lost their phosphatase activities at 6h post-induction of acute pancreatitis. Furthermore, PP2A already lost its activity at 1h of the time course. These three enzymes were affected in acute pancreatitis. However, when the treatment with NAC was performed, only the loss of PP2A activity was abrogated by NAC. This indicates that only PP2A is being involved in the disulfide stress of acute pancreatitis, whereas the loss of PP2B and PP2C would be due to other mechanisms that happen during the disease.

PP2A activity has been shown to be inhibited by oxidative stress (Whisler et al. 1995; Rao and Clayton 2002; Kim et al. 2003; Levinthal and Defranco 2005). Inhibition of PP2A activity by oxidative stress in neurons and in other model systems was reversed by disulfide-reducing agents (Rao and

Clayton 2002; Levinthal and Defranco 2005) in agreement with the results obtained in this Thesis. PP2Ac contains 10 cysteine residues, including a vicinal pair at positions 266 and 269 (Foley et al. 2007). Notably, this CXXC motif of PP2Ac is shared by well-established redox-active proteins including thiol-disulfide oxidoreductases of the protein disulfide isomerase family and, theoretically, it is capable of undergoing oxidation to form an intramolecular disulfide bond (Fomenko and Gladyshev 2003). Thus, it is conceivable that PP2Ac may form intermolecular and/or intramolecular disulfide bonds under oxidative stress. For this reason, it was carried out diagonal electrophoresis coupled with western blot in order to find out which was the major modification that PP2Ac suffers during acute pancreatitis. Indeed, part of the protein laid above the diagonal revealing that its major modification in acute pancreatitis seems to be an intramolecular disulfide.

Because of its relationship with oxidative stress, redox homeostasis, and its action on MAPKs, PP2A could play a key role in early phases of acute pancreatitis, particularly in the severe form of the disease, participating critically in the inflammatory cascade control. Based on this hypothesis as well as on the oxidation observed in the catalytic subunit of PP2A and its loss of activity in acute pancreatitis, we decided to silence PP2Ac in pancreatic acinar 266-6 cells in order to study the role of PP2A inactivation in the activation of the MAPK cascade and in the up-regulation of inflammatory cytokines.

PP2A may directly dephosphorylate phospho-ERK in HeLa cells (Letourneux et al. 2006) as well as phopho-p38 in T leukemia cells and other cell types (Boudreau et al. 2007). Accordingly, we have shown that PP2A silencing markedly activates both JNK and p38α in pancreatic acinar cells, as occurs *in vivo* in pancreatitis (Escobar et al. 2009). Thus, PP2A inactivation

promotes activation of two major MAPKs in 266-6 cells. The major MAPKs p38, JNK, and ERK1/2 are early activated in the course of experimental acute pancreatitis (Grady et al. 1996; Schafer et al. 1998; Wagner et al. 1999; Pereda et al. 2004). Our results would provide a mechanism to explain the activation of MAPKs in acute pancreatitis.

Our group has previously described that the inhibition of TNF-α production and xanthine oxidase led to simultaneous blockade of the three major MAPKs in the pancreas, resulting in a remarkable reduction in the inflammatory response (Pereda et al. 2004). In addition, we have previously reported that there is a cross-talk between oxidative stress and proinflammatory cytokines through MAPKs that greatly contributes to amplification of the uncontrolled inflammatory cascade and tissue injury in acute pancreatitis (Escobar et al. 2009). For these reasons, it was assessed the stimulation of the inflammatory cascade in non-silenced and PP2Ac-silenced pancreatic acinar 266-6 cells upon incubation with 10 ng/mL TNF-α for 1h.

The genes *cxcl1* (the equivalent in mouse to human *il-8*) and *tnf-α*, were up-regulated after the pro-inflammatory stimulus with TNF-α in 266-6 cells, and to a greater extent when PP2Ac was silenced. These results demonstrate that PP2A inactivation is critical in the up-regulation of the inflammatory cascade in the pancreas.

To complete the study of the role of PP2Ac in the inflammatory cascade and to simulate *in vitro* the conditions of the *in vivo* experimental model induced by sodium taurocholate, non-silenced and PP2Ac-silenced 266-6 cells were treated with 0,5% sodium taurocholate for 2h. Again, it was observed that PP2A has an important function in the up-regulation of CXCL1

during acute pancreatitis. The increase in CXCL1 expression would contribute to neutrophil chemotaxis, activation, and degranulation (Leser et al. 1991; Kusske et al. 1996) that occurs during acute pancreatitis. It is worth noting that CXCL1 is used as a marker of severity in acute pancreatitis (Leser et al. 1991; Norman et al. 1995; Norman 1998).

4. CONCLUDING REMARKS

In this Thesis it has been described disulfide stress as a specific type of oxidative stress in acute pancreatitis. Disulfide stress would be a novel mechanism of redox signaling independent of glutathione redox status involved in inflammation. The disulfide stress reported in this Thesis would provide a new molecular mechanism that occurs during the early development of the pancreatic inflammatory response. All these findings would help to better understand the molecular basis of the pancreatic disease and the main targets of oxidative stress that are affected during acute pancreatitis. Knowledge of the molecular biology of a disease is important because, ultimately, has its expression in a macromolecular level. The targets identified and the study of the antioxidant and inflammatory response also may contribute to improve the therapeutic treatment of acute pancreatitis. On the other hand, the new mechanism that is involved in acute inflammation in mammals would provide a new tool for the study of other diseases in which inflammation is present.

Any progress means a step forward in Biomedicine, but much remains to be done. Experimental approaches to the pancreatic disease have restrictions and the laboratory thecniques are not easy. Although targets of disulfide stress have been identified, our group has as a future prospect the identification of cysteinylated proteins by the *in vivo* administration of

biotinylated cysteine. The approach is not easy and we are working on it, but with effort and perseverance we will advance in the study of acute pancreatitis in order to improve patients' therapy and health.

VII.CONCLUSIONS/CONCLUSIONES

• CONCLUSIONS

1. Disulfide stress should be considered a novel specific type of oxidative stress involved in redox signaling in pancreas during acute pancreatitis.

2. Disulfide stress occurs in acute pancreatitis independently of the glutathione system. It is associated with oxidation of pairs cysteine/cystine, γ-glutamylcysteine/bis-γ-glutamylcystine and homocysteine/homocystine, as well as protein cysteinylation, and γ-glutamylcysteinylation, but without glutathione oxidation or changes in protein glutathionylation.

3. Two types of targets of disulfide stress were identified: Redox buffers, such as ribonuclease inhibitor or albumin; and redox signaling thiols that include thioredoxin 1, REF1/APE1, KEAP1, protein disulfide isomerase and tyrosine and serine/threonine phosphatases.

4. PP2A silencing in pancreatic acinar cells, *in vitro* leads to activation of p38 and JNK MAPKs and up-regulation of cytokines CXCL-1, IL-6 and TNF-α.

• CONCLUSIONES

1. El estrés por disulfuro debe considerarse un tipo específico y novedoso de estrés oxidativo implicado en la señalización redox en el páncreas durante la pancreatitis aguda.

2. El estrés por disulfuro tiene lugar en la pancreatitis aguda de forma independiente del sistema del glutatión. Se asocia con la oxidación de los pares cisteína/cistina, γ-glutamilcisteína/bis-γ-glutamilcistina y homocisteína/ homocistina, así como con la cisteinilación y γ-glutamilcisteinilación de proteínas, pero en ausencia de oxidación del glutatión o cambios en glutationilación de proteínas.

3. Dos tipos de dianas de estrés oxidativo han sido identificadas: Los tampones redox, tales como el inhibidor de la ribonucleasa o la albúmina, y los tioles de señalización redox que incluyen la tiorredoxina 1, REF1/APE1, KEAP1, la proteína disulfuro isomerasa y las tirosín y serín/treonín fosfatasas.

4. El silenciamiento de la PP2A en células acinares pancreáticas *in vitro* produce la activación de las MAPKs p38 y JNK así como la inducción de las citoquinas CXCL-1, IL-6 y TNF-α.

VIII. BIBLIOGRAPHY

Aesif, S. W., I. Kuipers, J. van der Velden, J. E. Tully, A. S. Guala, et al. (2011). "Activation of the glutaredoxin-1 gene by nuclear factor kappaB enhances signaling." Free Radic Biol Med **51**(6): 1249-1257.

Aho, H. J., S. M. Koskensalo and T. J. Nevalainen (1980). "Experimental pancreatitis in the rat. Sodium taurocholate-induced acute haemorrhagic pancreatitis." Scand J Gastroenterol **15**(4): 411-416.

Akhtar, M. W., C. R. Sunico, T. Nakamura and S. A. Lipton (2012). "Redox Regulation of Protein Function via Cysteine S-Nitrosylation and Its Relevance to Neurodegenerative Diseases." Int J Cell Biol **2012**: 463756.

Alonso, A., J. Sasin, N. Bottini, I. Friedberg, A. Osterman, et al. (2004). "Protein tyrosine phosphatases in the human genome." Cell **117**(6): 699-711.

Alsfasser, G., M. Gock, L. Herzog, M. M. Gebhard, C. Herfarth, et al. (2002). "Glutathione depletion with L-buthionine-(S,R)-sulfoximine demonstrates deleterious effects in acute pancreatitis of the rat." Dig Dis Sci **47**(8): 1793-1799.

Ames, B. N. (1983). "Dietary carcinogens and anticarcinogens. Oxygen radicals and degenerative diseases." Science **221**(4617): 1256-1264.

Antelmann, H. and J. D. Helmann (2011). "Thiol-based redox switches and gene regulation." Antioxid Redox Signal **14**(6): 1049-1063.

Arner, E. S. and A. Holmgren (2000). "Physiological functions of thioredoxin and thioredoxin reductase." Eur J Biochem **267**(20): 6102-6109.

Arthur, J. R. (2000). "The glutathione peroxidases." Cell Mol Life Sci **57**(13-14): 1825-1835.

Aruoma, O. I., B. Halliwell, B. M. Hoey and J. Butler (1989). "The antioxidant action of N-acetylcysteine: its reaction with hydrogen peroxide, hydroxyl radical, superoxide, and hypochlorous acid." Free Radic Biol Med **6**(6): 593-597.

Ascenzi, P., M. Colasanti, T. Persichini, M. Muolo, F. Polticelli, et al. (2000). "Re-evaluation of amino acid sequence and structural consensus rules for cysteine-nitric oxide reactivity." Biol Chem **381**(7): 623-627.

Ashino, S., K. Takeda, H. Li, V. Taylor, A. Joetham, et al. (2013). "Janus kinase 1/3 signaling pathways are key initiators of T2 differentiation and lung allergic responses." J Allergy Clin Immunol.

Aslund, F. and J. Beckwith (1999). "Bridge over troubled waters: sensing stress by disulfide bond formation." Cell **96**(6): 751-753.

Austin, J. L., C. Roberts, M. J. Rosenholtz and H. A. Reber (1980). "Effects of partial duct obstruction and drainage of pancreatic function." J Surg Res **28**(5): 426-433.

Avellini, C., M. Orsaria, U. Baccarani, G. L. Adani, D. Lorenzin, et al. (2010). "Apurinic apyrimidinic endonuclease/redox effector factor 1 immunoreactivity and grading in hepatocellular carcinoma risk of relapse after liver transplantation." Transplant Proc **42**(4): 1204-1208.

Avery, S. V. (2011). "Molecular targets of oxidative stress." Biochem J **434**(2): 201-210.

Baird, L. and A. T. Dinkova-Kostova (2011). "The cytoprotective role of the Keap1-Nrf2 pathway." Arch Toxicol **85**(4): 241-272.

Banmeyer, I., C. Marchand, C. Verhaeghe, B. Vucic, J. F. Rees, et al. (2004). "Overexpression of human peroxiredoxin 5 in subcellular compartments of Chinese hamster ovary cells: effects on cytotoxicity and DNA damage caused by peroxides." Free Radic Biol Med **36**(1): 65-77.

Bauer, G. (2002). "Signaling and proapoptotic functions of transformed cell-derived reactive oxygen species." Prostaglandins Leukot Essent Fatty Acids **66**(1): 41-56.

Beer, S. M., E. R. Taylor, S. E. Brown, C. C. Dahm, N. J. Costa, et al. (2004). "Glutaredoxin 2 catalyzes the reversible oxidation and glutathionylation of mitochondrial membrane thiol proteins: implications for mitochondrial redox regulation and antioxidant DEFENSE." J Biol Chem **279**(46): 47939-47951.

Bellomo, G., S. A. Jewell and S. Orrenius (1982). "The metabolism of menadione impairs the ability of rat liver mitochondria to take up and retain calcium." J Biol Chem **257**(19): 11558-11562.

Ben-Levy, R., S. Hooper, R. Wilson, H. F. Paterson and C. J. Marshall (1998). "Nuclear export of the stress-activated protein kinase p38 mediated by its substrate MAPKAP kinase-2." Curr Biol **8**(19): 1049-1057.

Benham, A. M. (2012). "The protein disulfide isomerase family: key players in health and disease." Antioxid Redox Signal **16**(8): 781-789.

Benhar, M., M. T. Forrester and J. S. Stamler (2009). "Protein denitrosylation: enzymatic mechanisms and cellular functions." Nat Rev Mol Cell Biol **10**(10): 721-732.

Berndt, C., C. H. Lillig and A. Holmgren (2007). "Thiol-based mechanisms of the thioredoxin and glutaredoxin systems: implications for diseases in the cardiovascular system." Am J Physiol Heart Circ Physiol **292**(3): H1227-1236.

Bhakat, K. K., A. K. Mantha and S. Mitra (2009). "Transcriptional regulatory functions of mammalian AP-endonuclease (APE1/Ref-1), an essential multifunctional protein." Antioxid Redox Signal **11**(3): 621-638.

Blinman, T. A., I. Gukovsky, M. Mouria, V. Zaninovic, E. Livingston, et al. (2000). "Activation of pancreatic acinar cells on isolation from tissue: cytokine upregulation via p38 MAP kinase." Am J Physiol Cell Physiol **279**(6): C1993-2003.

Bogoyevitch, M. A., K. R. Ngoei, T. T. Zhao, Y. Y. Yeap and D. C. Ng (2010). "c-Jun N-terminal kinase (JNK) signaling: recent advances and challenges." Biochim Biophys Acta **1804**(3): 463-475.

Bone, R. C. (1996). "Immunologic dissonance: a continuing evolution in our understanding of the systemic inflammatory response syndrome (SIRS) and the multiple organ dysfunction syndrome (MODS)." Ann Intern Med **125**(8): 680-687.

Bone, R. C. (1996). "Sir Isaac Newton, sepsis, SIRS, and CARS." Crit Care Med **24**(7): 1125-1128.

Boudreau, R. T., D. M. Conrad and D. W. Hoskin (2007). "Apoptosis induced by protein phosphatase 2A (PP2A) inhibition in T leukemia cells is negatively regulated by PP2A-associated p38 mitogen-activated protein kinase." Cell Signal **19**(1): 139-151.

Boulton, T. G., G. D. Yancopoulos, J. S. Gregory, C. Slaughter, C. Moomaw, et al. (1990). "An insulin-stimulated protein kinase similar to yeast kinases involved in cell cycle control." Science **249**(4964): 64-67.

Boveris, A., A. O. Stoppani, R. Docampo and F. S. Cruz (1978). "Superoxide anion production and trypanocidal action of naphthoquinones on Trypanosoma cruzi." Comp Biochem Physiol C **61 C**(2): 327-329.

Bradford, M. M. (1976). "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding." Anal Biochem **72**: 248-254.

Brasier, A. R. (2006). "The NF-kappaB regulatory network." Cardiovasc Toxicol **6**(2): 111-130.

Brigelius-Flohe, R. and M. Maiorino (2013). "Glutathione peroxidases." Biochim Biophys Acta **1830**(5): 3289-3303.

Brot, N. and H. Weissbach (1983). "Biochemistry and physiological role of methionine sulfoxide residues in proteins." Arch Biochem Biophys **223**(1): 271-281.

Bulleid, N. J. and R. B. Freedman (1988). "Defective co-translational formation of disulphide bonds in protein disulphide-isomerase-deficient microsomes." Nature **335**(6191): 649-651.

Burke, T. R., Jr. and Z. Y. Zhang (1998). "Protein-tyrosine phosphatases: structure, mechanism, and inhibitor discovery." Biopolymers **47**(3): 225-241.

Cai, Z. and L. J. Yan (2013). "Protein Oxidative Modifications: Beneficial Roles in Disease and Health." J Biochem Pharmacol Res **1**(1): 15-26.

Campos, M., P. Fadden, G. Alms, Z. Qian and T. A. Haystead (1996). "Identification of protein phosphatase-1-binding proteins by microcystin-biotin affinity chromatography." J Biol Chem **271**(45): 28478-28484.

Camps, M., A. Nichols and S. Arkinstall (2000). "Dual specificity phosphatases: a gene family for control of MAP kinase function." FASEB J **14**(1): 6-16.

Carballal, S., B. Alvarez, L. Turell, H. Botti, B. A. Freeman, et al. (2007). "Sulfenic acid in human serum albumin." Amino Acids **32**(4): 543-551.

Carballal, S., R. Radi, M. C. Kirk, S. Barnes, B. A. Freeman, et al. (2003). "Sulfenic acid formation in human serum albumin by hydrogen peroxide and peroxynitrite." Biochemistry **42**(33): 9906-9914.

Cargnello, M. and P. P. Roux (2011). "Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases." Microbiol Mol Biol Rev **75**(1): 50-83.

Casanova, J. L., S. M. Holland and L. D. Notarangelo (2012). "Inborn errors of human JAKs and STATs." Immunity **36**(4): 515-528.

Catalan, R. E., L. Gargiulo, A. M. Martinez, M. C. Calcerrada and A. Liras (1997). "Protein tyrosine phosphatase activity modulation by endothelin-1 in rabbit platelets." FEBS Lett **400**(3): 280-284.

Ceulemans, H. and M. Bollen (2004). "Functional diversity of protein phosphatase-1, a cellular economizer and reset button." Physiol Rev **84**(1): 1-39.

Circu, M. L. and T. Y. Aw (2010). "Reactive oxygen species, cellular redox systems, and apoptosis." Free Radic Biol Med **48**(6): 749-762.

Closa, D., M. Bardaji, G. Hotter, N. Prats, E. Gelpi, et al. (1996). "Hepatic involvement in pancreatitis-induced lung damage." Am J Physiol **270**(1 Pt 1): G6-13.

Closa, D., L. Sabater, L. Fernandez-Cruz, N. Prats, E. Gelpi, et al. (1999). "Activation of alveolar macrophages in lung injury associated with experimental acute pancreatitis is mediated by the liver." Ann Surg **229**(2): 230-236.

Cohen, P. (1989). "The structure and regulation of protein phosphatases." Annu Rev Biochem **58**: 453-508.

Cohen, P. T. (2002). "Protein phosphatase 1--targeted in many directions." J Cell Sci **115**(Pt 2): 241-256.

Cooper, J. A., D. F. Bowen-Pope, E. Raines, R. Ross and T. Hunter (1982). "Similar effects of platelet-derived growth factor and epidermal growth factor on the phosphorylation of tyrosine in cellular proteins." Cell **31**(1): 263-273.

Coppo, L. and P. Ghezzi (2011). "Thiol regulation of pro-inflammatory cytokines and innate immunity: protein S-thiolation as a novel molecular mechanism." Biochem Soc Trans **39**(5): 1268-1272.

Cortot, A., J. P. Armand and J. C. Soria (2006). "[PI3K-AKT-mTOR pathway inhibitors]." Bull Cancer **93**(1): 19-26.

Cotgreave, I. A. (1997). "N-acetylcysteine: pharmacological considerations and experimental and clinical applications." Adv Pharmacol **38**: 205-227.

Cotgreave, I. A. and R. G. Gerdes (1998). "Recent trends in glutathione biochemistry--glutathione-protein interactions: a molecular link between oxidative stress and cell proliferation?" Biochem Biophys Res Commun **242**(1): 1-9.

Cuadrado, A. and A. R. Nebreda (2010). "Mechanisms and functions of p38 MAPK signalling." Biochem J **429**(3): 403-417.

Cui, X. Y., P. F. Fu, D. N. Pan, Y. Zhao, J. Zhao, et al. (2003). "The antioxidant effects of ribonuclease inhibitor." Free Radic Res **37**(10): 1079-1085.

Cumming, R. C. and D. Schubert (2005). "Amyloid-beta induces disulfide bonding and aggregation of GAPDH in Alzheimer's disease." FASEB J **19**(14): 2060-2062.

Curran, F. J., N. Sattar, D. Talwar, J. N. Baxter and C. W. Imrie (2000). "Relationship of carotenoid and vitamins A and E with the acute inflammatory response in acute pancreatitis." Br J Surg **87**(3): 301-305.

Chai, Y. C., S. Hendrich and J. A. Thomas (1994). "Protein S-thiolation in hepatocytes stimulated by t-butyl hydroperoxide, menadione, and neutrophils." Arch Biochem Biophys **310**(1): 264-272.

Chance, B., B. Schoener, R. Oshino, F. Itshak and Y. Nakase (1979). "Oxidation-reduction ratio studies of mitochondria in freeze-trapped samples. NADH and flavoprotein fluorescence signals." J Biol Chem **254**(11): 4764-4771.

Chen, R. H., C. Sarnecki and J. Blenis (1992). "Nuclear localization and regulation of erk- and rsk-encoded protein kinases." Mol Cell Biol **12**(3): 915-927.

Chen, Z., T. B. Gibson, F. Robinson, L. Silvestro, G. Pearson, et al. (2001). "MAP kinases." Chem Rev **101**(8): 2449-2476.

Chevallet, M., S. Luche and T. Rabilloud (2006). "Silver staining of proteins in polyacrylamide gels." Nat Protoc **1**(4): 1852-1858.

Chi, H., S. P. Barry, R. J. Roth, J. J. Wu, E. A. Jones, et al. (2006). "Dynamic regulation of pro- and anti-inflammatory cytokines by MAPK phosphatase 1 (MKP-1) in innate immune responses." Proc Natl Acad Sci U S A **103**(7): 2274-2279.

Chiari, H. (1896). "Uber selbstverdaung des Menschlichen Pancreas." Zeitschrift für Heilkunde **17**: 22.

Chiarugi, P. (2005). "PTPs versus PTKs: the redox side of the coin." Free Radic Res **39**(4): 353-364.

Choi, Y. B., L. Tenneti, D. A. Le, J. Ortiz, G. Bai, et al. (2000). "Molecular basis of NMDA receptor-coupled ion channel modulation by S-nitrosylation." Nat Neurosci **3**(1): 15-21.

Chong, Z. Z. and K. Maiese (2007). "The Src homology 2 domain tyrosine phosphatases SHP-1 and SHP-2: diversified control of cell growth, inflammation, and injury." Histol Histopathol **22**(11): 1251-1267.

Dalle-Donne, I., R. Rossi, R. Colombo, D. Giustarini and A. Milzani (2006). "Biomarkers of oxidative damage in human disease." Clin Chem **52**(4): 601-623.

Dalle-Donne, I., R. Rossi, D. Giustarini, A. Milzani and R. Colombo (2003). "Protein carbonyl groups as biomarkers of oxidative stress." Clin Chim Acta **329**(1-2): 23-38.

Davies, M. G. and P. O. Hagen (1997). "Systemic inflammatory response syndrome." Br J Surg **84**(7): 920-935.

Davies, M. J., S. Fu, H. Wang and R. T. Dean (1999). "Stable markers of oxidant damage to proteins and their application in the study of human disease." Free Radic Biol Med **27**(11-12): 1151-1163.

De Campos, T., J. Deree and R. Coimbra (2007). "From acute pancreatitis to end-organ injury: mechanisms of acute lung injury." Surg Infect (Larchmt) **8**(1): 107-120.

De Dios, I., L. Ramudo, J. R. Alonso, J. S. Recio, A. C. Garcia-Montero, et al. (2005). "CD45 expression on rat acinar cells: involvement in pro-inflammatory cytokine production." FEBS Lett **579**(28): 6355-6360.

de Dios, I., L. Ramudo, A. C. Garcia-Montero and M. A. Manso (2006). "Redox-sensitive modulation of CD45 expression in pancreatic acinar cells during acute pancreatitis." J Pathol **210**(2): 234-239.

Deleault, K. M., S. J. Skinner and S. A. Brooks (2008). "Tristetraprolin regulates TNF TNF-alpha mRNA stability via a proteasome dependent mechanism involving the combined action of the ERK and p38 pathways." Mol Immunol **45**(1): 13-24.

Demols, A., J. L. Van Laethem, E. Quertinmont, F. Legros, H. Louis, et al. (2000). "N-acetylcysteine decreases severity of acute pancreatitis in mice." Pancreas **20**(2): 161-169.

Demple, B., T. Herman and D. S. Chen (1991). "Cloning and expression of APE, the cDNA encoding the major human apurinic endonuclease: definition of a family of DNA repair enzymes." Proc Natl Acad Sci U S A **88**(24): 11450-11454.

den Hertog, J., A. Groen and T. van der Wijk (2005). "Redox regulation of protein-tyrosine phosphatases." Arch Biochem Biophys **434**(1): 11-15.

Deplancke, B. and H. R. Gaskins (2002). "Redox control of the transsulfuration and glutathione biosynthesis pathways." Curr Opin Clin Nutr Metab Care **5**(1): 85-92.

Derijard, B., J. Raingeaud, T. Barrett, I. H. Wu, J. Han, et al. (1995). "Independent human MAP-kinase signal transduction pathways defined by MEK and MKK isoforms." Science **267**(5198): 682-685.

Desnues, B., C. Cuny, G. Gregori, S. Dukan, H. Aguilaniu, et al. (2003). "Differential oxidative damage and expression of stress defence regulons in culturable and non-culturable Escherichia coli cells." EMBO Rep **4**(4): 400-404.

Dhanasekaran, D. N. and E. P. Reddy (2008). "JNK signaling in apoptosis." Oncogene **27**(48): 6245-6251.

Diaz, D., C. M. Krejsa and T. J. Kavanagh (2002). "Expression of glutamate-cysteine ligase during mouse development." Mol Reprod Dev **62**(1): 83-91.

Dickinson, B. C. and C. J. Chang (2011). "Chemistry and biology of reactive oxygen species in signaling or stress responses." Nat Chem Biol **7**(8): 504-511.

Dinarello, C. A. (2000). "Proinflammatory cytokines." Chest **118**(2): 503-508.

Dinkova-Kostova, A. T., W. D. Holtzclaw, R. N. Cole, K. Itoh, N. Wakabayashi, et al. (2002). "Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants." Proc Natl Acad Sci U S A **99**(18): 11908-11913.

Doroshow, J. H. and K. J. Davies (1983). "Comparative cardiac oxygen radical metabolism by anthracycline antibiotics, mitoxantrone, bisantrene, 4'-(9-acridinylamino)-methanesulfon-m-anisidide, and neocarzinostatin." Biochem Pharmacol **32**(19): 2935-2939.

Drazic, A. and J. Winter (2014). "The physiological role of reversible methionine oxidation." Biochim Biophys Acta.

Dubuisson, M., D. Vander Stricht, A. Clippe, F. Etienne, T. Nauser, et al. (2004). "Human peroxiredoxin 5 is a peroxynitrite reductase." FEBS Lett **571**(1-3): 161-165.

Eaton, P. (2006). "Protein thiol oxidation in health and disease: techniques for measuring disulfides and related modifications in complex protein mixtures." Free Radic Biol Med **40**(11): 1889-1899.

Eaton, P., N. Wright, D. J. Hearse and M. J. Shattock (2002). "Glyceraldehyde phosphate dehydrogenase oxidation during cardiac ischemia and reperfusion." J Mol Cell Cardiol **34**(11): 1549-1560.

Ecker, J. (2012). "Profiling eicosanoids and phospholipids using LC-MS/MS: principles and recent applications." J Sep Sci. **35**: 9.

Edman, J. C., L. Ellis, R. W. Blacher, R. A. Roth and W. J. Rutter (1985). "Sequence of protein disulphide isomerase and implications of its relationship to thioredoxin." Nature **317**(6034): 267-270.

Engstrom, N. E., A. Holmgren, A. Larsson and S. Soderhall (1974). "Isolation and characterization of calf liver thioredoxin." J Biol Chem **249**(1): 205-210.

Escobar, J., J. Pereda, A. Arduini, J. Sandoval, M. L. Moreno, et al. (2012). "Oxidative and nitrosative stress in acute pancreatitis. Modulation by pentoxifylline and oxypurinol." Biochem Pharmacol **83**(1): 122-130.

Escobar, J., J. Pereda, A. Arduini, J. Sandoval, L. Sabater, et al. (2009). "Cross-talk between oxidative stress and pro-inflammatory cytokines in acute pancreatitis: a key role for protein phosphatases." Curr Pharm Des **15**(26): 3027-3042.

Escobar, J., J. Pereda, A. Arduini, J. Sandoval, L. Sabater, et al. (2010). "Role of redox signaling, protein phosphatases and histone acetylation in the inflammatory cascade in acute pancreatitis. Therapeutic implications." Inflamm Allergy Drug Targets **9**(2): 97-108.

Escobar, J., J. Pereda, G. Lopez-Rodas and J. Sastre (2012). "Redox signaling and histone acetylation in acute pancreatitis." Free Radic Biol Med **52**(5): 819-837.

Estrela, J. M., A. Ortega and E. Obrador (2006). "Glutathione in cancer biology and therapy." Crit Rev Clin Lab Sci **43**(2): 143-181.

Feddersen, C. O., P. Barth, A. Puchner and P. von Wichert (1993). "[N-acetylcysteine decreases functional and structural, ARDS-typical lung changes in endotoxin-treated rats]." Med Klin (Munich) **88**(4): 197-206.

Fenton (1894). "Oxidation of tartaric acid in the presence of iron." Oxidation of tartaric acid in the presence of iron **65**.

Ferreras, M., J. G. Gavilanes, C. Lopez-Otin and J. M. Garcia-Segura (1995). "Thiol-disulfide exchange of ribonuclease inhibitor bound to ribonuclease A. Evidence of active inhibitor-bound ribonuclease." J Biol Chem **270**(48): 28570-28578.

Finkel, T. (1998). "Oxygen radicals and signaling." Curr Opin Cell Biol **10**(2): 248-253.

Foley, T. D., L. A. Petro, C. M. Stredny and T. M. Coppa (2007). "Oxidative inhibition of protein phosphatase 2A activity: role of catalytic subunit disulfides." Neurochem Res **32**(11): 1957-1964.

Fomenko, D. E. and V. N. Gladyshev (2003). "Identity and functions of CxxC-derived motifs." Biochemistry **42**(38): 11214-11225.

Forman, H. J., M. Maiorino and F. Ursini (2010). "Signaling functions of reactive oxygen species." Biochemistry **49**(5): 835-842.

Foster, M. W., D. T. Hess and J. S. Stamler (2009). "Protein S-nitrosylation in health and disease: a current perspective." Trends Mol Med **15**(9): 391-404.

Foster, M. W., T. J. McMahon and J. S. Stamler (2003). "S-nitrosylation in health and disease." Trends Mol Med **9**(4): 160-168.

Foulkes, J. G. and P. Cohen (1980). "The regulation of glycogen metabolism. Purification and properties of protein phosphatase inhibitor-2 from rabbit skeletal muscle." Eur J Biochem **105**(1): 195-203.

Frand, A. R. and C. A. Kaiser (1999). "Ero1p oxidizes protein disulfide isomerase in a pathway for disulfide bond formation in the endoplasmic reticulum." Mol Cell **4**(4): 469-477.

Frangioni, J. V., P. H. Beahm, V. Shifrin, C. A. Jost and B. G. Neel (1992). "The nontransmembrane tyrosine phosphatase PTP-1B localizes to the endoplasmic reticulum via its 35 amino acid C-terminal sequence." Cell **68**(3): 545-560.

Freeman, B. A. and J. D. Crapo (1982). "Biology of disease: free radicals and tissue injury." Lab Invest **47**(5): 412-426.

Frei, B. (1994). "Reactive oxygen species and antioxidant vitamins: mechanisms of action." Am J Med **97**(3A): 5S-13S; discussion 22S-28S.

Furukawa, M. and Y. Xiong (2005). "BTB protein Keap1 targets antioxidant transcription factor Nrf2 for ubiquitination by the Cullin 3-Roc1 ligase." Mol Cell Biol **25**(1): 162-171.

Garcia-Hernandez, V., N. Sarmiento, C. Sanchez-Bernal, L. Matellan, J. J. Calvo, et al. (2013). "Modulation in the expression of SHP-1, SHP-2 and PTP1B due to the inhibition of MAPKs, cAMP and neutrophils early on in the development of cerulein-induced acute pancreatitis in rats." Biochim Biophys Acta.

Garg, P. K. (2012). "Chronic pancreatitis in India and Asia." Curr Gastroenterol Rep **14**(2): 118-124.

Genereux, J. C., A. K. Boal and J. K. Barton (2010). "DNA-mediated charge transport in redox sensing and signaling." J Am Chem Soc **132**(3): 891-905.

Ghoreschi, K., A. Laurence and J. J. O'Shea (2009). "Janus kinases in immune cell signaling." Immunol Rev **228**(1): 273-287.

Gilbert, H. F. (1984). "Redox control of enzyme activities by thiol/disulfide exchange." Methods Enzymol **107**: 330-351.

Gilbert, H. F. (1995). "Thiol/disulfide exchange equilibria and disulfide bond stability." Methods Enzymol **251**: 8-28.

Gilmore, T. D. (1999). "The Rel/NF-kappaB signal transduction pathway: introduction." Oncogene **18**(49): 6842-6844.

Gilmore, T. D. (2006). "Introduction to NF-kappaB: players, pathways, perspectives." Oncogene **25**(51): 6680-6684.

Giustarini, D., I. Dalle-Donne, A. Milzani and R. Rossi (2009). "Oxidative stress induces a reversible flux of cysteine from tissues to blood in vivo in the rat." FEBS J **276**(17): 4946-4958.

Giustarini, D., A. Milzani, G. Aldini, M. Carini, R. Rossi, et al. (2005). "S-nitrosation versus S-glutathionylation of protein sulfhydryl groups by S-nitrosoglutathione." Antioxid Redox Signal **7**(7-8): 930-939.

Go, Y. M. and D. P. Jones (2008). "Redox compartmentalization in eukaryotic cells." Biochim Biophys Acta **1780**(11): 1273-1290.

Goldstein, S., D. Meyerstein and G. Czapski (1993). "The Fenton reagents." Free Radic Biol Med **15**(4): 435-445.

Gomez-Cambronero, L., B. Camps, J. G. de La Asuncion, M. Cerda, A. Pellin, et al. (2000). "Pentoxifylline ameliorates cerulein-induced pancreatitis in rats: role of glutathione and nitric oxide." J Pharmacol Exp Ther **293**(2): 670-676.

Grady, T., A. Saluja, A. Kaiser and M. Steer (1996). "Edema and intrapancreatic trypsinogen activation precede glutathione depletion during caerulein pancreatitis." Am J Physiol **271**(1 Pt 1): G20-26.

Groen, A., S. Lemeer, T. van der Wijk, J. Overvoorde, A. J. Heck, et al. (2005). "Differential oxidation of protein-tyrosine phosphatases." J Biol Chem **280**(11): 10298-10304.

Gross, E., C. S. Sevier, N. Heldman, E. Vitu, M. Bentzur, et al. (2006). "Generating disulfides enzymatically: reaction products and electron acceptors of the endoplasmic reticulum thiol oxidase Ero1p." Proc Natl Acad Sci U S A **103**(2): 299-304.

Gueraud, F., M. Atalay, N. Bresgen, A. Cipak, P. M. Eckl, et al. (2010). "Chemistry and biochemistry of lipid peroxidation products." Free Radic Res **44**(10): 1098-1124.

Gukovskaya, A. S., I. Gukovsky, Y. Jung, M. Mouria and S. J. Pandol (2002). "Cholecystokinin induces caspase activation and mitochondrial dysfunction in pancreatic acinar cells. Roles in cell injury processes of pancreatitis." J Biol Chem **277**(25): 22595-22604.

Gullberg, D. (2008). "Re-programmable tumour cells: cytokines from the immune system assume new roles with the help of stem cells." Scand J Immunol **67**(6): 632-633.

Haber, F. and J. Weiss (1934). "The catalytic compensation of hydrogen peroxide by iron salts." Proc R. Soc. London **147**: 21.

Halangk, W., M. M. Lerch, B. Brandt-Nedelev, W. Roth, M. Ruthenbuerger, et al. (2000). "Role of cathepsin B in intracellular trypsinogen activation and the onset of acute pancreatitis." J Clin Invest **106**(6): 773-781.

Haldar, S. M. and J. S. Stamler (2013). "S-nitrosylation: integrator of cardiovascular performance and oxygen delivery." J Clin Invest **123**(1): 101-110.

Halliwell, B. (1991). "Reactive oxygen species in living systems: source, biochemistry, and role in human disease." Am J Med **91**(3C): 14S-22S.

Halliwell, B. (1996). "Oxidative stress, nutrition and health. Experimental strategies for optimization of nutritional antioxidant intake in humans." Free Radic Res **25**(1): 57-74.

Halliwell, B., R. Aeschbach, J. Loliger and O. I. Aruoma (1995). "The characterization of antioxidants." Food Chem Toxicol **33**(7): 601-617.

Halliwell, B. and J. M. Gutteridge (1990). "Role of free radicals and catalytic metal ions in human disease: an overview." Methods Enzymol **186**: 1-85.

Han, J., J. D. Lee, L. Bibbs and R. J. Ulevitch (1994). "A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells." Science **265**(5173): 808-811.

Han, J., J. D. Lee, Y. Jiang, Z. Li, L. Feng, et al. (1996). "Characterization of the structure and function of a novel MAP kinase kinase (MKK6)." J Biol Chem **271**(6): 2886-2891.

Hashimoto, D., M. Ohmuraya, M. Hirota, A. Yamamoto, K. Suyama, et al. (2008). "Involvement of autophagy in trypsinogen activation within the pancreatic acinar cells." J Cell Biol **181**(7): 1065-1072.

Hawkins, B. J., M. Madesh, C. J. Kirkpatrick and A. B. Fisher (2007). "Superoxide flux in endothelial cells via the chloride channel-3 mediates intracellular signaling." Mol Biol Cell **18**(6): 2002-2012.

Hernansanz-Agustin, P., A. Izquierdo-Alvarez, A. Garcia-Ortiz, S. Ibiza, J. M. Serrador, et al. (2013). "Nitrosothiols in the immune system: signaling and protection." Antioxid Redox Signal **18**(3): 288-308.

Hershey, J. W. (1991). "Translational control in mammalian cells." Annu Rev Biochem **60**: 717-755.

Hess, D. T., A. Matsumoto, S. O. Kim, H. E. Marshall and J. S. Stamler (2005). "Protein S-nitrosylation: purview and parameters." Nat Rev Mol Cell Biol **6**(2): 150-166.

Hirota, K., M. Murata, Y. Sachi, H. Nakamura, J. Takeuchi, et al. (1999). "Distinct roles of thioredoxin in the cytoplasm and in the nucleus. A two-step mechanism of redox regulation of transcription factor NF-kappaB." J Biol Chem **274**(39): 27891-27897.

Hochgrafe, F., J. Mostertz, D. C. Pother, D. Becher, J. D. Helmann, et al. (2007). "S-cysteinylation is a general mechanism for thiol protection of Bacillus subtilis proteins after oxidative stress." J Biol Chem **282**(36): 25981-25985.

Holmgren, A. (1989). "Thioredoxin and glutaredoxin systems." J Biol Chem **264**(24): 13963-13966.

Holmgren, A. (2000). "Antioxidant function of thioredoxin and glutaredoxin systems." Antioxid Redox Signal **2**(4): 811-820.

Holmgren, A. and R. Sengupta (2010). "The use of thiols by ribonucleotide reductase." Free Radic Biol Med **49**(11): 1617-1628.

Holland, S., E. Lodwig, T. Sideri, T. Reader, I. Clarke, et al. (2007). "Application of the comprehensive set of heterozygous yeast deletion mutants to elucidate the molecular basis of cellular chromium toxicity." Genome Biol **8**(12): R268.

Hunter, T. (1989). "Protein-tyrosine phosphatases: the other side of the coin." Cell **58**(6): 1013-1016.

Hunter, T. (2000). "Signaling--2000 and beyond." Cell **100**(1): 113-127.

Itoh, K., N. Wakabayashi, Y. Katoh, T. Ishii, K. Igarashi, et al. (1999). "Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain." Genes Dev **13**(1): 76-86.

Jaffrey, S. R., H. Erdjument-Bromage, C. D. Ferris, P. Tempst and S. H. Snyder (2001). "Protein S-nitrosylation: a physiological signal for neuronal nitric oxide." Nat Cell Biol **3**(2): 193-197.

Jansens, A., E. van Duijn and I. Braakman (2002). "Coordinated nonvectorial folding in a newly synthesized multidomain protein." Science **298**(5602): 2401-2403.

Jarvis, L. A., S. J. Toering, M. A. Simon, M. A. Krasnow and R. K. Smith-Bolton (2006). "Sprouty proteins are in vivo targets of Corkscrew/SHP-2 tyrosine phosphatases." Development **133**(6): 1133-1142.

Jha, R. K., Q. Ma, H. Sha and M. Palikhe (2009). "Acute pancreatitis: a literature review." Med Sci Monit **15**(7): RA147-156.

Jiang, Y., C. Chen, Z. Li, W. Guo, J. A. Gegner, et al. (1996). "Characterization of the structure and function of a new mitogen-activated protein kinase (p38beta)." J Biol Chem **271**(30): 17920-17926.

Jin, Y. H., A. B. Clark, R. J. Slebos, H. Al-Refai, J. A. Taylor, et al. (2003). "Cadmium is a mutagen that acts by inhibiting mismatch repair." Nat Genet **34**(3): 326-329.

Johnson, S. A. and T. Hunter (2005). "Kinomics: methods for deciphering the kinome." Nat Methods **2**(1): 17-25.

Jones, D. P. (2006). "Redefining oxidative stress." Antioxid Redox Signal **8**(9-10): 1865-1879.

Jones, D. P., J. L. Carlson, V. C. Mody, J. Cai, M. J. Lynn, et al. (2000). "Redox state of glutathione in human plasma." Free Radic Biol Med **28**(4): 625-635.

Jones, D. P., Y. M. Go, C. L. Anderson, T. R. Ziegler, J. M. Kinkade, Jr., et al. (2004). "Cysteine/cystine couple is a newly recognized node in the circuitry for biologic redox signaling and control." FASEB J **18**(11): 1246-1248.

Ju, K. D., J. H. Yu, H. Kim and K. H. Kim (2006). "Role of mitogen-activated protein kinases, NF-kappaB, and AP-1 on cerulein-induced IL-8 expression in pancreatic acinar cells." Ann N Y Acad Sci **1090**: 368-374.

Kalinina, E. V., N. N. Chernov and A. N. Saprin (2008). "Involvement of thio-, peroxi-, and glutaredoxins in cellular redox-dependent processes." Biochemistry (Mosc) **73**(13): 1493-1510.

Karin, M. (2006). "Nuclear factor-kappaB in cancer development and progression." Nature **441**(7092): 431-436.

Kazlauskas, A. and J. A. Cooper (1988). "Protein kinase C mediates platelet-derived growth factor-induced tyrosine phosphorylation of p42." J Cell Biol **106**(4): 1395-1402.

Kensler, T. W., N. Wakabayashi and S. Biswal (2007). "Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway." Annu Rev Pharmacol Toxicol **47**: 89-116.

Kettenhofen, N. J. and M. J. Wood (2010). "Formation, reactivity, and detection of protein sulfenic acids." Chem Res Toxicol **23**(11): 1633-1646.

Khacho, M., K. Mekhail, K. Pilon-Larose, A. Pause, J. Cote, et al. (2008). "eEF1A is a novel component of the mammalian nuclear protein export machinery." Mol Biol Cell **19**(12): 5296-5308.

Kim, G., S. J. Weiss and R. L. Levine (2014). "Methionine oxidation and reduction in proteins." Biochim Biophys Acta **1840**(2): 901-905.

Kim, H., J. Y. Seo, K. H. Roh, J. W. Lim and K. H. Kim (2000). "Suppression of NF-kappaB activation and cytokine production by N-acetylcysteine in pancreatic acinar cells." Free Radic Biol Med **29**(7): 674-683.

Kim, H. S., M. C. Song, I. H. Kwak, T. J. Park and I. K. Lim (2003). "Constitutive induction of p-Erk1/2 accompanied by reduced activities of protein phosphatases 1 and 2A and MKP3 due to reactive oxygen species during cellular senescence." J Biol Chem **278**(39): 37497-37510.

Kim, J. S., J. Soucek, J. Matousek and R. T. Raines (1995). "Mechanism of ribonuclease cytotoxicity." J Biol Chem **270**(52): 31097-31102.

Knoops, B., A. Clippe, C. Bogard, K. Arsalane, R. Wattiez, et al. (1999). "Cloning and characterization of AOEB166, a novel mammalian antioxidant enzyme of the peroxiredoxin family." J Biol Chem **274**(43): 30451-30458.

Koike, H., M. L. Steer and J. Meldolesi (1982). "Pancreatic effects of ethionine: blockade of exocytosis and appearance of crinophagy and autophagy precede cellular necrosis." Am J Physiol **242**(4): G297-307.

Konorev, E. A., B. Kalyanaraman and N. Hogg (2000). "Modification of creatine kinase by S-nitrosothiols: S-nitrosation vs. S-thiolation." Free Radic Biol Med **28**(11): 1671-1678.

Koren, S. and I. G. Fantus (2007). "Inhibition of the protein tyrosine phosphatase PTP1B: potential therapy for obesity, insulin resistance and type-2 diabetes mellitus." Best Pract Res Clin Endocrinol Metab **21**(4): 621-640.

Kulinskii, V. I. and L. S. Kolesnichenko (2009). "[Glutathione system. I. Synthesis, transport, glutathione transferases, glutathione peroxidases]." Biomed Khim **55**(3): 255-277.

Kumar, C., A. Igbaria, B. D'Autreaux, A. G. Planson, C. Junot, et al. (2011). "Glutathione revisited: a vital function in iron metabolism and ancillary role in thiol-redox control." EMBO J **30**(10): 2044-2056.

Kusske, A. M., A. J. Rongione and H. A. Reber (1996). "Cytokines and acute pancreatitis." Gastroenterology **110**(2): 639-642.

Kyriakis, J. M. and J. Avruch (2001). "Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation." Physiol Rev **81**(2): 807-869.

Lammers, T. and S. Lavi (2007). "Role of type 2C protein phosphatases in growth regulation and in cellular stress signaling." Crit Rev Biochem Mol Biol **42**(6): 437-461.

Lander, E. S., L. M. Linton, B. Birren, C. Nusbaum, M. C. Zody, et al. (2001). "Initial sequencing and analysis of the human genome." Nature **409**(6822): 860-921.

Lankisch, P. G., A. B. Lowenfels and P. Maisonneuve (2002). "What is the risk of alcoholic pancreatitis in heavy drinkers?" Pancreas **25**(4): 411-412.

Lawler, S., Y. Fleming, M. Goedert and P. Cohen (1998). "Synergistic activation of SAPK1/JNK1 by two MAP kinase kinases in vitro." Curr Biol **8**(25): 1387-1390.

Lee, J. C., J. T. Laydon, P. C. McDonnell, T. F. Gallagher, S. Kumar, et al. (1994). "A protein kinase involved in the regulation of inflammatory cytokine biosynthesis." Nature **372**(6508): 739-746.

Lee, S. R., K. S. Kwon, S. R. Kim and S. G. Rhee (1998). "Reversible inactivation of protein-tyrosine phosphatase 1B in A431 cells stimulated with epidermal growth factor." J Biol Chem **273**(25): 15366-15372.

Leland, P. A. and R. T. Raines (2001). "Cancer chemotherapy--ribonucleases to the rescue." Chem Biol **8**(5): 405-413.

Lenormand, P., C. Sardet, G. Pages, G. L'Allemain, A. Brunet, et al. (1993). "Growth factors induce nuclear translocation of MAP kinases (p42mapk and

p44mapk) but not of their activator MAP kinase kinase (p45mapkk) in fibroblasts." J Cell Biol **122**(5): 1079-1088.

Lerch, M. M. and F. S. Gorelick (2013). "Models of acute and chronic pancreatitis." Gastroenterology **144**(6): 1180-1193.

Leser, H. G., V. Gross, C. Scheibenbogen, A. Heinisch, R. Salm, et al. (1991). "Elevation of serum interleukin-6 concentration precedes acute-phase response and reflects severity in acute pancreatitis." Gastroenterology **101**(3): 782-785.

Letourneux, C., G. Rocher and F. Porteu (2006). "B56-containing PP2A dephosphorylate ERK and their activity is controlled by the early gene IEX-1 and ERK." EMBO J **25**(4): 727-738.

Leung, P. S. and Y. C. Chan (2009). "Role of oxidative stress in pancreatic inflammation." Antioxid Redox Signal **11**(1): 135-165.

Levine, R. L. and E. R. Stadtman (2001). "Oxidative modification of proteins during aging." Exp Gerontol **36**(9): 1495-1502.

Levinthal, D. J. and D. B. Defranco (2005). "Reversible oxidation of ERK-directed protein phosphatases drives oxidative toxicity in neurons." J Biol Chem **280**(7): 5875-5883.

Levonen, A. L., A. Landar, A. Ramachandran, E. K. Ceaser, D. A. Dickinson, et al. (2004). "Cellular mechanisms of redox cell signalling: role of cysteine modification in controlling antioxidant defences in response to electrophilic lipid oxidation products." Biochem J **378**(Pt 2): 373-382.

Levy, D. E. and J. E. Darnell, Jr. (2002). "Stats: transcriptional control and biological impact." Nat Rev Mol Cell Biol **3**(9): 651-662.

Li, N. and M. Karin (1999). "Is NF-kappaB the sensor of oxidative stress?" FASEB J **13**(10): 1137-1143.

Lin, E., S. E. Calvano and S. F. Lowry (2000). "Inflammatory cytokines and cell response in surgery." Surgery **127**(2): 117-126.

Livak, K. J. and T. D. Schmittgen (2001). "Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method." Methods **25**(4): 402-408.

LoPiccolo, J., G. M. Blumenthal, W. B. Bernstein and P. A. Dennis (2008). "Targeting the PI3K/Akt/mTOR pathway: effective combinations and clinical considerations." Drug Resist Updat **11**(1-2): 32-50.

Lu, G. and Y. Wang (2008). "Functional diversity of mammalian type 2C protein phosphatase isoforms: new tales from an old family." Clin Exp Pharmacol Physiol **35**(2): 107-112.

Lubec, G., J. A. Widness, M. Hayde, D. Menzel and A. Pollak (1997). "Hydroxyl radical generation in oxygen-treated infants." Pediatrics **100**(4): 700-704.

Luo, M., H. He, M. R. Kelley and M. M. Georgiadis (2010). "Redox regulation of DNA repair: implications for human health and cancer therapeutic development." Antioxid Redox Signal **12**(11): 1247-1269.

Luthen, R., J. H. Grendell, C. Niederau and D. Haussinger (1998). "Trypsinogen activation and glutathione content are linked to pancreatic injury in models of biliary acute pancreatitis." Int J Pancreatol **24**(3): 193-202.

Luthen, R., C. Niederau, M. Niederau, L. D. Ferrell and J. H. Grendell (1995). "Influence of ductal pressure and infusates on activity and subcellular distribution of lysosomal enzymes in the rat pancreas." Gastroenterology **109**(2): 573-581.

Makhija, R. and A. N. Kingsnorth (2002). "Cytokine storm in acute pancreatitis." J Hepatobiliary Pancreat Surg **9**(4): 401-410.

Mannervik, B., K. Axelsson, A. C. Sundewall and A. Holmgren (1983). "Relative contributions of thioltransferase-and thioredoxin-dependent systems in reduction of low-molecular-mass and protein disulphides." Biochem J **213**(2): 519-523.

Martin, J. L. (1995). "Thioredoxin--a fold for all reasons." Structure **3**(3): 245-250.

Martinez-Ruiz, A. and S. Lamas (2004). "Detection and proteomic identification of S-nitrosylated proteins in endothelial cells." Arch Biochem Biophys **423**(1): 192-199.

Martinez-Ruiz, A. and S. Lamas (2004). "S-nitrosylation: a potential new paradigm in signal transduction." Cardiovasc Res **62**(1): 43-52.

Martinez-Ruiz, A. and S. Lamas (2007). "Signalling by NO-induced protein S-nitrosylation and S-glutathionylation: convergences and divergences." Cardiovasc Res **75**(2): 220-228.

Matayatsuk, C., A. Poljak, S. Bustamante, G. A. Smythe, R. W. Kalpravidh, et al. (2007). "Quantitative determination of ortho- and meta-tyrosine as biomarkers of protein oxidative damage in beta-thalassemia." Redox Rep **12**(5): 219-228.

Maulik, N. (2002). "Redox signaling of angiogenesis." Antioxid Redox Signal **4**(5): 805-815.

Mayer, J., B. Rau, F. Gansauge and H. G. Beger (2000). "Inflammatory mediators in human acute pancreatitis: clinical and pathophysiological implications." Gut **47**(4): 546-552.

McBean, J. (2012). "The transsulfuration pathway: a source of cysteine for glutathione in astrocytes." Amino Acids **42**(1): 7.

McCutcheon, A. D. and D. Race (1962). "Experimental Pancreatitis A Possible Etiology of Postoperative Pancreatitis." Ann Surg **155**(4): 523-531.

Medicherla, B. and A. L. Goldberg (2008). "Heat shock and oxygen radicals stimulate ubiquitin-dependent degradation mainly of newly synthesized proteins." J Cell Biol **182**(4): 663-673.

Medzhitov, R. (2008). "Origin and physiological roles of inflammation." Nature **454**(7203): 428-435.

Meier, R., J. Rouse, A. Cuenda, A. R. Nebreda and P. Cohen (1996). "Cellular stresses and cytokines activate multiple mitogen-activated-protein kinase kinase homologues in PC12 and KB cells." Eur J Biochem **236**(3): 796-805.

Meister, A. (1988). "Glutathione metabolism and its selective modification." J Biol Chem **263**(33): 17205-17208.

Meister, A. (1991). "Glutathione deficiency produced by inhibition of its synthesis, and its reversal; applications in research and therapy." Pharmacol Ther **51**(2): 155-194.

Meister, A. and M. E. Anderson (1983). "Glutathione." Annu Rev Biochem **52**: 711-760.

Meloche, S. and J. Pouyssegur (2007). "The ERK1/2 mitogen-activated protein kinase pathway as a master regulator of the G1- to S-phase transition." Oncogene **26**(22): 3227-3239.

Mieyal, J. J., M. M. Gallogly, S. Qanungo, E. A. Sabens and M. D. Shelton (2008). "Molecular mechanisms and clinical implications of reversible protein S-glutathionylation." Antioxid Redox Signal **10**(11): 1941-1988.

Mitsuhashi, S., N. Matsuura, M. Ubukata, H. Oikawa, H. Shima, et al. (2001). "Tautomycetin is a novel and specific inhibitor of serine/threonine protein phosphatase type 1, PP1." Biochem Biophys Res Commun **287**(2): 328-331.

Mitsuhashi, S., H. Shima, N. Tanuma, N. Matsuura, M. Takekawa, et al. (2003). "Usage of tautomycetin, a novel inhibitor of protein phosphatase 1 (PP1), reveals that PP1 is a positive regulator of Raf-1 in vivo." J Biol Chem **278**(1): 82-88.

Mizukami, Y., K. Yoshioka, S. Morimoto and K. Yoshida (1997). "A novel mechanism of JNK1 activation. Nuclear translocation and activation of JNK1 during ischemia and reperfusion." J Biol Chem **272**(26): 16657-16662.

Mohler, E. R., M. T. Franklin and L. P. Adam (1996). "Intracellular signaling by 8-epi-prostaglandin F2 alpha is mediated by thromboxane A2/prostaglandin endoperoxide receptors in porcine carotid arteries." Biochem Biophys Res Commun **225**(3): 915-923.

Montero-Julian, F. A. (2001). "The soluble IL-6 receptors: serum levels and biological function." Cell Mol Biol (Noisy-le-grand) **47**(4): 583-597.

Monti, D. M., N. Montesano Gesualdi, J. Matousek, F. Esposito and G. D'Alessio (2007). "The cytosolic ribonuclease inhibitor contributes to intracellular redox homeostasis." FEBS Lett **581**(5): 930-934.

Muhl, H. and J. Pfeilschifter (2003). "Endothelial nitric oxide synthase: a determinant of TNFalpha production by human monocytes/macrophages." Biochem Biophys Res Commun **310**(3): 677-680.

Murphy, E., M. Kohr, J. Sun, T. Nguyen and C. Steenbergen (2012). "S-nitrosylation: a radical way to protect the heart." J Mol Cell Cardiol **52**(3): 568-577.

Mustacich, D. and G. Powis (2000). "Thioredoxin reductase." Biochem J **346 Pt 1**: 1-8.

Naik, E. and V. M. Dixit (2011). "Mitochondrial reactive oxygen species drive proinflammatory cytokine production." J Exp Med **208**(3): 417-420.

Nakae, Y., S. Naruse, M. Kitagawa, S. Hirao, R. Yamamoto, et al. (1995). "Activation of trypsinogen in experimental models of acute pancreatitis in rats." Pancreas **10**(3): 306-313.

Nakajima, T., T. Ueda, Y. Takeyama, T. Yasuda, M. Shinzeki, et al. (2007). "Protective effects of vascular endothelial growth factor on intestinal epithelial apoptosis and bacterial translocation in experimental severe acute pancreatitis." Pancreas **34**(4): 410-416.

Narayan, M. (2012). "Disulfide bonds: protein folding and subcellular protein trafficking." FEBS J **279**(13): 2272-2282.

Neuschwander-Tetri, B. A., L. D. Ferrell, R. J. Sukhabote and J. H. Grendell (1992). "Glutathione monoethyl ester ameliorates caerulein-induced pancreatitis in the mouse." J Clin Invest **89**(1): 109-116.

Niki, E. and M. G. Traber (2012). "A history of vitamin E." Ann Nutr Metab **61**(3): 207-212.

Nimmo, G. A. and P. Cohen (1978). "The regulation of glycogen metabolism. Phosphorylation of inhibitor-1 from rabbit skeletal muscle, and its

interaction with protein phosphatases-III and -II." Eur J Biochem **87**(2): 353-365.

Nishida, M., Y. Maruyama, R. Tanaka, K. Kontani, T. Nagao, et al. (2000). "G alpha(i) and G alpha(o) are target proteins of reactive oxygen species." Nature **408**(6811): 492-495.

Nkabyo, Y. S., T. R. Ziegler, L. H. Gu, W. H. Watson and D. P. Jones (2002). "Glutathione and thioredoxin redox during differentiation in human colon epithelial (Caco-2) cells." Am J Physiol Gastrointest Liver Physiol **283**(6): G1352-1359.

Norman, J. (1998). "The role of cytokines in the pathogenesis of acute pancreatitis." Am J Surg **175**(1): 76-83.

Norman, J. G., G. W. Fink, W. Denham, J. Yang, G. Carter, et al. (1997). "Tissue-specific cytokine production during experimental acute pancreatitis. A probable mechanism for distant organ dysfunction." Dig Dis Sci **42**(8): 1783-1788.

Norman, J. G., M. G. Franz, G. S. Fink, J. Messina, P. J. Fabri, et al. (1995). "Decreased mortality of severe acute pancreatitis after proximal cytokine blockade." Ann Surg **221**(6): 625-631; discussion 631-624.

Oettl, K. and R. E. Stauber (2007). "Physiological and pathological changes in the redox state of human serum albumin critically influence its binding properties." Br J Pharmacol **151**(5): 580-590.

Oliver, C. J. and S. Shenolikar (1998). "Physiologic importance of protein phosphatase inhibitors." Front Biosci **3**: D961-972.

Orhan, H., N. P. Vermeulen, C. Tump, H. Zappey and J. H. Meerman (2004). "Simultaneous determination of tyrosine, phenylalanine and deoxyguanosine oxidation products by liquid chromatography-tandem mass spectrometry as non-invasive biomarkers for oxidative damage." J Chromatogr B Analyt Technol Biomed Life Sci **799**(2): 245-254.

Pajares, M. A., C. Duran, F. Corrales, M. M. Pliego and J. M. Mato (1992). "Modulation of rat liver S-adenosylmethionine synthetase activity by glutathione." J Biol Chem **267**(25): 17598-17605.

Pandol, S. J., A. K. Saluja, C. W. Imrie and P. A. Banks (2007). "Acute pancreatitis: bench to the bedside." Gastroenterology **132**(3): 1127-1151.

Pani, G., B. Bedogni, R. Colavitti, R. Anzevino, S. Borrello, et al. (2001). "Cell compartmentalization in redox signaling." IUBMB Life **52**(1-2): 7-16.

Papachristou, G. I. (2008). "Prediction of severe acute pancreatitis: current knowledge and novel insights." World J Gastroenterol **14**(41): 6273-6275.

Pawloski, J. R., D. T. Hess and J. S. Stamler (2001). "Export by red blood cells of nitric oxide bioactivity." Nature **409**(6820): 622-626.

Pearson, G., F. Robinson, T. Beers Gibson, B. E. Xu, M. Karandikar, et al. (2001). "Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions." Endocr Rev **22**(2): 153-183.

Pereda, J., J. Escobar, J. Sandoval, J. L. Rodriguez, L. Sabater, et al. (2008). "Glutamate cysteine ligase up-regulation fails in necrotizing pancreatitis." Free Radic Biol Med **44**(8): 1599-1609.

Pereda, J., L. Sabater, L. Aparisi, J. Escobar, J. Sandoval, et al. (2006). "Interaction between cytokines and oxidative stress in acute pancreatitis." Curr Med Chem **13**(23): 2775-2787.

Pereda, J., L. Sabater, N. Cassinello, L. Gomez-Cambronero, D. Closa, et al. (2004). "Effect of simultaneous inhibition of TNF-alpha production and xanthine oxidase in experimental acute pancreatitis: the role of mitogen activated protein kinases." Ann Surg **240**(1): 108-116.

Perkins, N. D. (2007). "Integrating cell-signalling pathways with NF-kappaB and IKK function." Nat Rev Mol Cell Biol **8**(1): 49-62.

Pollard, M. G., K. J. Travers and J. S. Weissman (1998). "Ero1p: a novel and ubiquitous protein with an essential role in oxidative protein folding in the endoplasmic reticulum." Mol Cell **1**(2): 171-182.

Poole, L. B., P. A. Karplus and A. Claiborne (2004). "Protein sulfenic acids in redox signaling." Annu Rev Pharmacol Toxicol **44**: 325-347.

Presnell, C. E., G. Bhatti, L. S. Numan, M. Lerche, S. K. Alkhateeb, et al. (2013). "Computational insights into the role of glutathione in oxidative stress." Curr Neurovasc Res **10**(2): 185-194.

Prinz, W. A., F. Aslund, A. Holmgren and J. Beckwith (1997). "The role of the thioredoxin and glutaredoxin pathways in reducing protein disulfide bonds in the Escherichia coli cytoplasm." J Biol Chem **272**(25): 15661-15667.

Pryor, W. A., G. L. Squadrito and M. Friedman (1995). "A new mechanism for the toxicity of ozone." Toxicol Lett **82-83**: 287-293.

Rahman, S. H., K. Ibrahim, M. Larvin, A. Kingsnorth and M. J. McMahon (2004). "Association of antioxidant enzyme gene polymorphisms and glutathione status with severe acute pancreatitis." Gastroenterology **126**(5): 1312-1322.

Raingeaud, J., S. Gupta, J. S. Rogers, M. Dickens, J. Han, et al. (1995). "Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine." J Biol Chem **270**(13): 7420-7426.

Raman, M., W. Chen and M. H. Cobb (2007). "Differential regulation and properties of MAPKs." Oncogene **26**(22): 3100-3112.

Ramudo, L., M. A. Manso, S. Sevillano and I. de Dios (2005). "Kinetic study of TNF-alpha production and its regulatory mechanisms in acinar cells during acute pancreatitis induced by bile-pancreatic duct obstruction." J Pathol **206**(1): 9-16.

Ramudo, L., S. Yubero, M. A. Manso, S. Vicente and I. De Dios (2009). "Signal transduction of MCP-1 expression induced by pancreatitis-associated ascitic fluid in pancreatic acinar cells." J Cell Mol Med **13**(7): 1314-1320.

Rao, R. K. and L. W. Clayton (2002). "Regulation of protein phosphatase 2A by hydrogen peroxide and glutathionylation." Biochem Biophys Res Commun **293**(1): 610-616.

Ravichandran, V., T. Seres, T. Moriguchi, J. A. Thomas and R. B. Johnston, Jr. (1994). "S-thiolation of glyceraldehyde-3-phosphate dehydrogenase induced by the phagocytosis-associated respiratory burst in blood monocytes." J Biol Chem **269**(40): 25010-25015.

Ray, L. B. and T. W. Sturgill (1988). "Insulin-stimulated microtubule-associated protein kinase is phosphorylated on tyrosine and threonine in vivo." Proc Natl Acad Sci U S A **85**(11): 3753-3757.

Regner, S., J. Manjer, S. Appelros, C. Hjalmarsson, J. Sadic, et al. (2008). "Protease activation, pancreatic leakage, and inflammation in acute pancreatitis: differences between mild and severe cases and changes over the first three days." Pancreatology **8**(6): 600-607.

Requena, J. R., R. L. Levine and E. R. Stadtman (2003). "Recent advances in the analysis of oxidized proteins." Amino Acids **25**(3-4): 221-226.

Rhee, S. G., H. Z. Chae and K. Kim (2005). "Peroxiredoxins: a historical overview and speculative preview of novel mechanisms and emerging concepts in cell signaling." Free Radic Biol Med **38**(12): 1543-1552.

Richman, P. G. and A. Meister (1975). "Regulation of gamma-glutamyl-cysteine synthetase by nonallosteric feedback inhibition by glutathione." J Biol Chem **250**(4): 1422-1426.

Rinalducci, S., L. Murgiano and L. Zolla (2008). "Redox proteomics: basic principles and future perspectives for the detection of protein oxidation in plants." J Exp Bot **59**(14): 3781-3801.

Rinna, A. and H. J. Forman (2008). "SHP-1 inhibition by 4-hydroxynonenal activates Jun N-terminal kinase and glutamate cysteine ligase." Am J Respir Cell Mol Biol **39**(1): 97-104.

Rock, C. L., R. A. Jacob and P. E. Bowen (1996). "Update on the biological characteristics of the antioxidant micronutrients: vitamin C, vitamin E, and the carotenoids." J Am Diet Assoc **96**(7): 693-702; quiz 703-694.

Rogers, L. K., B. L. Leinweber and C. V. Smith (2006). "Detection of reversible protein thiol modifications in tissues." Anal Biochem **358**(2): 171-184.

Rojkind, M., J. A. Dominguez-Rosales, N. Nieto and P. Greenwel (2002). "Role of hydrogen peroxide and oxidative stress in healing responses." Cell Mol Life Sci **59**(11): 1872-1891.

Roos, G. and J. Messens (2011). "Protein sulfenic acid formation: from cellular damage to redox regulation." Free Radic Biol Med **51**(2): 314-326.

Rouault, T. A. and R. D. Klausner (1996). "The impact of oxidative stress on eukaryotic iron metabolism." EXS **77**: 183-197.

Rusnak, F. and P. Mertz (2000). "Calcineurin: form and function." Physiol Rev **80**(4): 1483-1521.

Sajewicz, W., S. Milnerowicz and S. Nabzdyk (2006). "Blood plasma antioxidant defense in patients with pancreatitis." Pancreas **32**(2): 139-144.

Salmeen, A. and D. Barford (2005). "Functions and mechanisms of redox regulation of cysteine-based phosphatases." Antioxid Redox Signal **7**(5-6): 560-577.

Salsbury, F. R., Jr., S. T. Knutson, L. B. Poole and J. S. Fetrow (2008). "Functional site profiling and electrostatic analysis of cysteines modifiable to cysteine sulfenic acid." Protein Sci **17**(2): 299-312.

Sampathkumar, R., M. Balasubramanyam, S. Sudarslal, M. Rema, V. Mohan, et al. (2005). "Increased glutathionylated hemoglobin (HbSSG) in type 2 diabetes subjects with microangiopathy." Clin Biochem **38**(10): 892-899.

Sandoval, J., J. Escobar, J. Pereda, N. Sacilotto, J. L. Rodriguez, et al. (2009). "Pentoxifylline prevents loss of PP2A phosphatase activity and recruitment of histone acetyltransferases to proinflammatory genes in acute pancreatitis." J Pharmacol Exp Ther **331**(2): 609-617.

Sandri, G., E. Panfili and L. Ernster (1990). "Hydrogen peroxide production by monoamine oxidase in isolated rat-brain mitochondria: its effect on glutathione levels and Ca2+ efflux." Biochim Biophys Acta **1035**(3): 300-305.

Saravanabhavan, G., E. Blais, R. Vincent and P. Kumarathasan (2010). "A high performance liquid chromatography-electrochemical array method for the measurement of oxidative/nitrative changes in human urine." J Chromatogr A **1217**(19): 3269-3274.

Schafer, C., S. E. Ross, M. J. Bragado, G. E. Groblewski, S. A. Ernst, et al. (1998). "A role for the p38 mitogen-activated protein kinase/Hsp 27 pathway in cholecystokinin-induced changes in the actin cytoskeleton in rat pancreatic acini." J Biol Chem **273**(37): 24173-24180.

Schnekenburger, J., J. Mayerle, B. Kruger, I. Buchwalow, F. U. Weiss, et al. (2005). "Protein tyrosine phosphatase kappa and SHP-1 are involved in the regulation of cell-cell contacts at adherens junctions in the exocrine pancreas." Gut **54**(10): 1445-1455.

Schoenberg, M. H., M. Buchler and H. G. Beger (1992). "The role of oxygen radicals in experimental acute pancreatitis." Free Radic Biol Med **12**(6): 515-522.

Schoneich, C., K. D. Asmus, U. Dillinger and F. von Bruchhausen (1989). "Thiyl radical attack on polyunsaturated fatty acids: a possible route to lipid peroxidation." Biochem Biophys Res Commun **161**(1): 113-120.

Schuppe-Koistinen, I., R. Gerdes, P. Moldeus and I. A. Cotgreave (1994). "Studies on the reversibility of protein S-thiolation in human endothelial cells." Arch Biochem Biophys **315**(2): 226-234.

Selhub, J. (1999). "Homocysteine metabolism." Annu Rev Nutr **19**: 217-246.

Serero, A., J. Lopes, A. Nicolas and S. Boiteux (2008). "Yeast genes involved in cadmium tolerance: Identification of DNA replication as a target of cadmium toxicity." DNA Repair (Amst) **7**(8): 1262-1275.

Sevier, C. S. and C. A. Kaiser (2006). "Conservation and diversity of the cellular disulfide bond formation pathways." Antioxid Redox Signal **8**(5-6): 797-811.

Sevillano, S., A. M. De la Mano, I. De Dios, L. Ramudo and M. A. Manso (2003). "Major pathological mechanisms of acute pancreatitis are prevented by N-acetylcysteine." Digestion **68**(1): 34-40.

Sevillano, S., A. M. de la Mano, M. A. Manso, A. Orfao and I. De Dios (2003). "N-acetylcysteine prevents intra-acinar oxygen free radical production in pancreatic duct obstruction-induced acute pancreatitis." Biochim Biophys Acta **1639**(3): 177-184.

Shacter, E. (2000). "Quantification and significance of protein oxidation in biological samples." Drug Metab Rev **32**(3-4): 307-326.

Shapiro, R. (2001). "Cytoplasmic ribonuclease inhibitor." Methods Enzymol **341**: 611-628.

Shaul, Y. D. and R. Seger (2007). "The MEK/ERK cascade: from signaling specificity to diverse functions." Biochim Biophys Acta **1773**(8): 1213-1226.

Sheffner, A. L. (1963). "The reduction in vitro in viscosity of mucoprotein solutions by a new mucolytic agent, N-acetyl-L-cysteine." Ann N Y Acad Sci **106**: 298-310.

Shi, Y. (2009). "Serine/threonine phosphatases: mechanism through structure." Cell **139**(3): 468-484.

Shimizu, Y. and L. M. Hendershot (2009). "Oxidative folding: cellular strategies for dealing with the resultant equimolar production of reactive oxygen species." Antioxid Redox Signal **11**(9): 2317-2331.

Sies, H. (1986). "Biochemistry of Oxidative Stress." Angewandte Chemie International Edition in English **25**(12): 14.

Sies, H. (1991). "Oxidative stress: from basic research to clinical application." Am J Med **91**(3C): 31S-38S.

Sitia, R. and S. N. Molteni (2004). "Stress, protein (mis)folding, and signaling: the redox connection." Sci STKE **2004**(239): pe27.

Skulachev, V. P. (1996). "Role of uncoupled and non-coupled oxidations in maintenance of safely low levels of oxygen and its one-electron reductants." Q Rev Biophys **29**(2): 169-202.

Sohal, R. S. (2002). "Role of oxidative stress and protein oxidation in the aging process." Free Radic Biol Med **33**(1): 37-44.

Spadaro, D., B. W. Yun, S. H. Spoel, C. Chu, Y. Q. Wang, et al. (2010). "The redox switch: dynamic regulation of protein function by cysteine modifications." Physiol Plant **138**(4): 360-371.

Spickett, C. M., I. Wiswedel, W. Siems, K. Zarkovic and N. Zarkovic (2010). "Advances in methods for the determination of biologically relevant lipid peroxidation products." Free Radic Res **44**(10): 1172-1202.

Spink, J. and J. Cohen (1997). "Synergy and specificity in induction of gene activity by proinflammatory cytokines: potential therapeutic targets." Shock **7**(6): 405-412.

Stadtman, E. R. and R. L. Levine (2003). "Free radical-mediated oxidation of free amino acids and amino acid residues in proteins." Amino Acids **25**(3-4): 207-218.

Starkov, A. A., G. Fiskum, C. Chinopoulos, B. J. Lorenzo, S. E. Browne, et al. (2004). "Mitochondrial alpha-ketoglutarate dehydrogenase complex generates reactive oxygen species." J Neurosci **24**(36): 7779-7788.

Steer, M. (2002). "Pancreatitis severity: who calls the shots?" Gastroenterology **122**(4): 1168-1172.

Stone, J. R. and S. Yang (2006). "Hydrogen peroxide: a signaling messenger." Antioxid Redox Signal **8**(3-4): 243-270.

Sun, J., M. Morgan, R. F. Shen, C. Steenbergen and E. Murphy (2007). "Preconditioning results in S-nitrosylation of proteins involved in

regulation of mitochondrial energetics and calcium transport." Circ Res **101**(11): 1155-1163.

Swingle, M., L. Ni and R. E. Honkanen (2007). "Small-molecule inhibitors of ser/thr protein phosphatases: specificity, use and common forms of abuse." Methods Mol Biol **365**: 23-38.

Tao, L. and A. M. English (2004). "Protein S-glutathiolation triggered by decomposed S-nitrosoglutathione." Biochemistry **43**(13): 4028-4038.

Tateishi, N., T. Higashi, A. Naruse, K. Nakashima and H. Shiozaki (1977). "Rat liver glutathione: possible role as a reservoir of cysteine." J Nutr **107**(1): 51-60.

Tateishi, N., T. Higashi, S. Shinya, A. Naruse and Y. Sakamoto (1974). "Studies on the regulation of glutathione level in rat liver." J Biochem (Tokyo) **75**(1): 93-103.

Tateishi, N., T. Higashi, S. Shinya, A. Naruse and Y. Sakamoto (1974). "Studies on the regulation of glutathione level in rat liver." J Biochem **75**(1): 93-103.

Tavender, T. J., A. M. Sheppard and N. J. Bulleid (2008). "Peroxiredoxin IV is an endoplasmic reticulum-localized enzyme forming oligomeric complexes in human cells." Biochem J **411**(1): 191-199.

Tavender, T. J., J. J. Springate and N. J. Bulleid (2010). "Recycling of peroxiredoxin IV provides a novel pathway for disulphide formation in the endoplasmic reticulum." EMBO J **29**(24): 4185-4197.

Tell, G., F. Quadrifoglio, C. Tiribelli and M. R. Kelley (2009). "The many functions of APE1/Ref-1: not only a DNA repair enzyme." Antioxid Redox Signal **11**(3): 601-620.

Terradez, P., M. Asensi, M. C. Lasso de la Vega, I. R. Puertes, J. Vina, et al. (1993). "Depletion of tumour glutathione in vivo by buthionine sulphoximine: modulation by the rate of cellular proliferation and inhibition of cancer growth." Biochem J **292 (Pt 2)**: 477-483.

Therond, P., D. Bonnefont-Rousselot, A. Davit-Spraul, M. Conti and A. Legrand (2000). "Biomarkers of oxidative stress: an analytical approach." Curr Opin Clin Nutr Metab Care **3**(5): 373-384.

Thomas, J. A., B. Poland and R. Honzatko (1995). "Protein sulfhydryls and their role in the antioxidant function of protein S-thiolation." Arch Biochem Biophys **319**(1): 1-9.

Thorpe, G. W., C. S. Fong, N. Alic, V. J. Higgins and I. W. Dawes (2004). "Cells have distinct mechanisms to maintain protection against different reactive oxygen species: oxidative-stress-response genes." Proc Natl Acad Sci U S A **101**(17): 6564-6569.

Tiidus, P. M., J. Pushkarenko and M. E. Houston (1996). "Lack of antioxidant adaptation to short-term aerobic training in human muscle." Am J Physiol **271**(4 Pt 2): R832-836.

Tolbert, N. E. and E. Essner (1981). "Microbodies: peroxisomes and glyoxysomes." J Cell Biol **91**(3 Pt 2): 271s-283s.

Toledano, M. B., C. Kumar, N. Le Moan, D. Spector and F. Tacnet (2007). "The system biology of thiol redox system in Escherichia coli and yeast: differential functions in oxidative stress, iron metabolism and DNA synthesis." FEBS Lett **581**(19): 3598-3607.

Tonks, N. K. (2006). "Protein tyrosine phosphatases: from genes, to function, to disease." Nat Rev Mol Cell Biol **7**(11): 833-846.

Tretter, L. and V. Adam-Vizi (2004). "Generation of reactive oxygen species in the reaction catalyzed by alpha-ketoglutarate dehydrogenase." J Neurosci **24**(36): 7771-7778.

Trush, M. A., E. G. Mimnaugh and T. E. Gram (1982). "Activation of pharmacologic agents to radical intermediates. Implications for the role of free radicals in drug action and toxicity." Biochem Pharmacol **31**(21): 3335-3346.

Tu, B. P. and J. S. Weissman (2002). "The FAD- and O(2)-dependent reaction cycle of Ero1-mediated oxidative protein folding in the endoplasmic reticulum." Mol Cell **10**(5): 983-994.

Turrens, J. F. (2003). "Mitochondrial formation of reactive oxygen species." J Physiol **552**(Pt 2): 335-344.

Uehara, T., T. Nakamura, D. Yao, Z. Q. Shi, Z. Gu, et al. (2006). "S-nitrosylated protein-disulphide isomerase links protein misfolding to neurodegeneration." Nature **441**(7092): 513-517.

Valavanidis, A., T. Vlachogianni and C. Fiotakis (2009). "8-hydroxy-2' -deoxyguanosine (8-OHdG): A critical biomarker of oxidative stress and carcinogenesis." J Environ Sci Health C Environ Carcinog Ecotoxicol Rev **27**(2): 120-139.

Venter, J. C., M. D. Adams, E. W. Myers, P. W. Li, R. J. Mural, et al. (2001). "The sequence of the human genome." Science **291**(5507): 1304-1351.

Viedma, J. A., M. Perez-Mateo, J. E. Dominguez and F. Carballo (1992). "Role of interleukin-6 in acute pancreatitis. Comparison with C-reactive protein and phospholipase A." Gut **33**(9): 1264-1267.

Villeneuve, N. F., A. Lau and D. D. Zhang (2010). "Regulation of the Nrf2-Keap1 antioxidant response by the ubiquitin proteasome system: an insight into cullin-ring ubiquitin ligases." Antioxid Redox Signal **13**(11): 1699-1712.

Vina, J., J. Sastre, F. Pallardo and C. Borras (2003). "Mitochondrial theory of aging: importance to explain why females live longer than males." Antioxid Redox Signal **5**(5): 8.

Vina, J. R., M. Palacin, I. R. Puertes, R. Hernandez and J. Vina (1989). "Role of the gamma-glutamyl cycle in the regulation of amino acid translocation." Am J Physiol **257**(6 Pt 1): E916-922.

von Sonntag, C. (1994). "Radiation chemistry in the 1990s: pressing questions relating to the areas of radiation biology and environmental research." Int J Radiat Biol **65**(1): 19-26.

Wagner, A. C., W. Metzler, T. Hofken, H. Weber and B. Goke (1999). "p38 map kinase is expressed in the pancreas and is immediately activated following cerulein hyperstimulation." Digestion **60**(1): 41-47.

Wagner, E. F. and A. R. Nebreda (2009). "Signal integration by JNK and p38 MAPK pathways in cancer development." Nat Rev Cancer **9**(8): 537-549.

Wait, R., S. Begum, D. Brambilla, A. M. Carabelli, F. Conserva, et al. (2005). "Redox options in two-dimensional electrophoresis." Amino Acids **28**(3): 239-272.

Wakabayashi, N., A. T. Dinkova-Kostova, W. D. Holtzclaw, M. I. Kang, A. Kobayashi, et al. (2004). "Protection against electrophile and oxidant stress by induction of the phase 2 response: fate of cysteines of the Keap1 sensor modified by inducers." Proc Natl Acad Sci U S A **101**(7): 2040-2045.

Walker, L. J., C. N. Robson, E. Black, D. Gillespie and I. D. Hickson (1993). "Identification of residues in the human DNA repair enzyme HAP1 (Ref-1) that are essential for redox regulation of Jun DNA binding." Mol Cell Biol **13**(9): 5370-5376.

Wang, B., P. Zhang and Q. Wei (2008). "Recent progress on the structure of Ser/Thr protein phosphatases." Sci China C Life Sci **51**(6): 487-494.

Wang, G., Y. Hong, M. K. Johnson and R. J. Maier (2006). "Lipid peroxidation as a source of oxidative damage in Helicobacter pylori: protective roles of peroxiredoxins." Biochim Biophys Acta **1760**(11): 1596-1603.

Wang, S. and H. Li (2006). "Radical scavenging activity of ribonuclease inhibitor from cow placenta." Biochemistry (Mosc) **71**(5): 520-524.

Wang, Y., B. W. Yun, E. Kwon, J. K. Hong, J. Yoon, et al. (2006). "S-nitrosylation: an emerging redox-based post-translational modification in plants." J Exp Bot **57**(8): 1777-1784.

Watabe, S., H. Kohno, H. Kouyama, T. Hiroi, N. Yago, et al. (1994). "Purification and characterization of a substrate protein for mitochondrial ATP-dependent protease in bovine adrenal cortex." J Biochem **115**(4): 648-654.

Watanabe, O., F. M. Baccino, M. L. Steer and J. Meldolesi (1984). "Supramaximal caerulein stimulation and ultrastructure of rat pancreatic acinar cell: early morphological changes during development of experimental pancreatitis." Am J Physiol **246**(4 Pt 1): G457-467.

Wessely-Szponder, J. (2008). "The influence of TNFalpha and IL-8 on secretory action of neutrophils isolated from heifers in the course of bovine respiratory disease." Acta Vet Hung **56**(2): 187-196.

Weston, C. R. and R. J. Davis (2002). "The JNK signal transduction pathway." Curr Opin Genet Dev **12**(1): 14-21.

Whisler, R. L., M. A. Goyette, I. S. Grants and Y. G. Newhouse (1995). "Sublethal levels of oxidant stress stimulate multiple serine/threonine kinases and suppress protein phosphatases in Jurkat T cells." Arch Biochem Biophys **319**(1): 23-35.

Widmann, C., S. Gibson, M. B. Jarpe and G. L. Johnson (1999). "Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human." Physiol Rev **79**(1): 143-180.

Wouters, M. A., S. W. Fan and N. L. Haworth (2010). "Disulfides as redox switches: from molecular mechanisms to functional significance." Antioxid Redox Signal **12**(1): 53-91.

Wu, M., Q. Bian, Y. Liu, A. F. Fernandes, A. Taylor, et al. (2009). "Sustained oxidative stress inhibits NF-kappaB activation partially via inactivating the proteasome." Free Radic Biol Med **46**(1): 62-69.

Xiao, R., B. Wilkinson, A. Solovyov, J. R. Winther, A. Holmgren, et al. (2004). "The contributions of protein disulfide isomerase and its homologues to oxidative protein folding in the yeast endoplasmic reticulum." J Biol Chem **279**(48): 49780-49786.

Xu, F., M. J. Xu, R. Zhao, A. Guerrah, F. Zeng, et al. (2002). "Tyrosine phosphatases SHP-1 and SHP-2 are associated with distinct tyrosine-phosphorylated proteins." Exp Cell Res **272**(1): 75-83.

Yadav, D. and A. B. Lowenfels (2013). "The epidemiology of pancreatitis and pancreatic cancer." Gastroenterology **144**(6): 1252-1261.

Yan, L. J., L. Liu and M. J. Forster (2012). "Reversible inactivation of dihydrolipoamide dehydrogenase by Angeli's salt." Sheng Wu Wu Li Hsueh Bao **28**(4): 341-350.

Yap, T. A., M. D. Garrett, M. I. Walton, F. Raynaud, J. S. de Bono, et al. (2008). "Targeting the PI3K-AKT-mTOR pathway: progress, pitfalls, and promises." Curr Opin Pharmacol **8**(4): 393-412.

Yoon, S. and R. Seger (2006). "The extracellular signal-regulated kinase: multiple substrates regulate diverse cellular functions." Growth Factors **24**(1): 21-44.

Yoshitake, S., H. Nanri, M. R. Fernando and S. Minakami (1994). "Possible differences in the regenerative roles played by thioltransferase and thioredoxin for oxidatively damaged proteins." J Biochem **116**(1): 42-46.

Youn, C. K., S. H. Kim, D. Y. Lee, S. H. Song, I. Y. Chang, et al. (2005). "Cadmium down-regulates human OGG1 through suppression of Sp1 activity." J Biol Chem **280**(26): 25185-25195.

Yu, J. H., J. W. Lim, W. Namkung, H. Kim and K. H. Kim (2002). "Suppression of cerulein-induced cytokine expression by antioxidants in pancreatic acinar cells." Lab Invest **82**(10): 1359-1368.

Yubero, S., L. Ramudo, M. A. Manso and I. De Dios (2009). "The role of redox status on chemokine expression in acute pancreatitis." Biochim Biophys Acta **1792**(2): 148-154.

Zelko, I. N., T. J. Mariani and R. J. Folz (2002). "Superoxide dismutase multigene family: a comparison of the CuZn-SOD (SOD1), Mn-SOD (SOD2), and EC-SOD (SOD3) gene structures, evolution, and expression." Free Radic Biol Med **33**(3): 337-349.

Zhang, D. D. and M. Hannink (2003). "Distinct cysteine residues in Keap1 are required for Keap1-dependent ubiquitination of Nrf2 and for stabilization

of Nrf2 by chemopreventive agents and oxidative stress." Mol Cell Biol **23**(22): 8137-8151.

Zhang, H., Y. Luo, W. Zhang, Y. He, S. Dai, et al. (2007). "Endothelial-specific expression of mitochondrial thioredoxin improves endothelial cell function and reduces atherosclerotic lesions." Am J Pathol **170**(3): 1108-1120.

Zhang, W., B. D. Ames and C. T. Walsh (2011). "Identification of phenylalanine 3-hydroxylase for meta-tyrosine biosynthesis." Biochemistry **50**(24): 5401-5403.

Zhang, X. H. and H. Weissbach (2008). "Origin and evolution of the protein-repairing enzymes methionine sulphoxide reductases." Biol Rev Camb Philos Soc **83**(3): 249-257.

Zhang, X. P., L. Wang and Y. F. Zhou (2008). "The pathogenic mechanism of severe acute pancreatitis complicated with renal injury: a review of current knowledge." Dig Dis Sci **53**(2): 297-306.

Zhang, Y. and P. Zuber (2007). "Requirement of the zinc-binding domain of ClpX for Spx proteolysis in Bacillus subtilis and effects of disulfide stress on ClpXP activity." J Bacteriol **189**(21): 7669-7680.

Zhang, Z. Y. (2002). "Protein tyrosine phosphatases: structure and function, substrate specificity, and inhibitor development." Annu Rev Pharmacol Toxicol **42**: 209-234.

Ziegler, D. M. (1985). "Role of reversible oxidation-reduction of enzyme thiols-disulfides in metabolic regulation." Annu Rev Biochem **54**: 305-329.

Zipper, L. M. and R. T. Mulcahy (2000). "Inhibition of ERK and p38 MAP kinases inhibits binding of Nrf2 and induction of GCS genes." Biochem Biophys Res Commun **278**(2): 484-492.

IX. ANNEX/ANEXO

ARTICLE IN PRESS

Free Radical Biology and Medicine ■ (■■■■) ■■■–■■■

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

Disulfide stress: a novel type of oxidative stress in acute pancreatitis

Mari-Luz Moreno [a], Javier Escobar [a,b], Alicia Izquierdo-Álvarez [c], Anabel Gil [a], Salvador Pérez [a], Javier Pereda [a], Inés Zapico [c,d], Máximo Vento [b], Luis Sabater [e], Anabel Marina [d], Antonio Martínez-Ruiz [c], Juan Sastre [a,*]

[a] Department of Physiology, School of Pharmacy, University of Valencia, 46100 Burjasot (Valencia), Spain
[b] Division of Neonatology, University Hospital Materno-Infantil La Fe, 46026 Valencia, Spain
[c] Servicio de Inmunología, Hospital Universitario de La Princesa, Instituto de Investigación Sanitaria Princesa (IP), Madrid, Spain
[d] Centro de Biología Molecular Severo Ochoa, CSIC–Universidad Autónoma de Madrid, Madrid, Spain
[e] Department of Surgery, University Clinic Hospital, University of Valencia, 46010 Valencia, Spain

ARTICLE INFO

Article history:
Received 17 October 2013
Received in revised form
26 December 2013
Accepted 7 January 2014

Keywords:
Thiol oxidation
Glutathione
Cysteine
Protein disulfides
Protein phosphatases
Free radicals

ABSTRACT

Glutathione oxidation and protein glutathionylation are considered hallmarks of oxidative stress in cells because they reflect thiol redox status in proteins. Our aims were to analyze the redox status of thiols and to identify mixed disulfides and targets of redox signaling in pancreas in experimental acute pancreatitis as a model of acute inflammation associated with glutathione depletion. Glutathione depletion in pancreas in acute pancreatitis is not associated with any increase in oxidized glutathione levels or protein glutathionylation. Cystine and homocystine levels as well as protein cysteinylation and γ-glutamyl cysteinylation markedly rose in pancreas after induction of pancreatitis. Protein cysteinylation was undetectable in pancreas under basal conditions. Targets of disulfide stress were identified by Western blotting, diagonal electrophoresis, and proteomic methods. Cysteinylated albumin was detected. Redox-sensitive PP2A and tyrosine protein phosphatase activities diminished in pancreatitis and this loss was abrogated by *N*-acetylcysteine. According to our findings, disulfide stress may be considered a specific type of oxidative stress in acute inflammation associated with protein cysteinylation and γ-glutamylcysteinylation and oxidation of the pair cysteine/cystine, but without glutathione oxidation or changes in protein glutathionylation. Two types of targets of disulfide stress were identified: redox buffers, such as ribonuclease inhibitor or albumin, and redox-signaling thiols, which include thioredoxin 1, APE1/Ref1, Keap1, tyrosine and serine/threonine phosphatases, and protein disulfide isomerase. These targets exhibit great relevance in DNA repair, cell proliferation, apoptosis, endoplasmic reticulum stress, and inflammatory response. Disulfide stress would be a specific mechanism of redox signaling independent of glutathione redox status involved in inflammation.

The human proteome comprises 214,000 cysteine (Cys) residues including thiols, disulfides, and zinc fingers [1]. The thiol–disulfide proteome can be divided into two groups, an inert structural group and a redox-sensitive regulatory group [2], which includes thioredoxins, peroxiredoxins, disulfide oxidoreductases, tyrosine phosphatases, zinc-binding proteins, cytokine receptors, ribonucleotide reductase, and retinol-binding protein, among others [3]. Most of the redox-active Cys regulate cell functions in response to redox changes acting as redox-sensing thiols, whereas only a small subset is essential for cell signaling comprising redox-signaling thiols [4]. The number of oxidative Cys modifications (glutathionylation, protein disulfides, nitrosylation, and formation of sulfenic, sulfinic, or sulfonic acid and other derivatives), the complexity of multiple modifications within the same protein, and the large spectrum of target Cys residues in different proteins make a comprehensive view of the role of Cys modifications in redox signaling very difficult [1].

Disulfides are formed in the cytosol during oxidative stress [3,5,6]. Thiol–disulfide redox potentials in proteins vary from −95 to −470 mV[2] and reaction rate constants of protein thiols toward H_2O_2 vary by 7 orders of magnitude [7]. This variation in the reactivity of protein thiols is due to the microenvironments created in the protein structure. Thus, the pK_a of the catalytic

Abbreviations: AP1, activator protein 1; APE1, apurinic/apyrimidinase endonuclease 1; BIAM, biotinylated iodoacetamide; GSH, reduced glutathione; GSSG, oxidized glutathione; *ho-1*, heme oxygenase-1; IAM, iodoacetamide; Keap1, Kelch-like ECH-associated protein 1; MBrB, monobromobimane; NAC, *N*-acetylcysteine; NEM, *N*-ethylmaleimide; *nqo1*, NADPH quinone oxidoreductase 1; p53, protein 53 or tumor protein 53; PDI, protein disulfide isomerase; PP1, protein phosphatase 1; PP2A, protein phosphatase 2 A; PP2Ac, protein phosphatase 2 A, catalytic subunit; PP2B, protein phosphatase 2B or calcineurin; PP2C, protein phosphatase 2C; PRDX1, peroxiredoxin 1; PTP, protein tyrosine phosphatases; Ref1, redox effector factor 1; RNH1, ribonuclease inhibitor 1; SHP1, Src homology phosphatase-1; SHP2, Src homology phosphatase-2; TRX1, thioredoxin 1

* Corresponding author. Fax: +34 96 3543395.
E-mail address: juan.sastre@uv.es (J. Sastre).

0891-5849/$ - see front matter © 2014 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.freeradbiomed.2014.01.009

Please cite this article as: Moreno, M.L; et al. Disulfide stress: a novel type of oxidative stress in acute pancreatitis. *Free Radic. Biol. Med.* (2014), http://dx.doi.org/10.1016/j.freeradbiomed.2014.01.009

cysteine together with the internal amino acid sequence between cysteines and conformational changes determine their reactivity and redox potential [3,8].

Protein glutathionylation is considered a hallmark of oxidative stress associated with disulfide formation. So far the reduced-to-oxidized glutathione (GSH/GSSG)[1] ratio has been considered a reliable indicator of oxidative stress because it reflects the balance between antioxidant status and pro-oxidant reactions and the protein thiol redox status in cells [9,10]. However, recent studies in yeast have shown that GSH levels seem to be critical for iron–sulfur cluster maturation but not for thiol redox control, which would primarily be exerted by other redox systems such as the thioredoxin pathway [11].

On the other hand, the pair cysteine/cystine influences the redox potential extracellularly, particularly in plasma [12,13]. Plasma cysteine/cystine redox potential may regulate the intracellular oxidation redox status because oxidation of cysteine to cystine in plasma caused oxidation of proteins linked to cell structure and cell death in endothelial cells [12].

Glutathione depletion is often associated with acute inflammation and it is a hallmark of the early course of acute pancreatitis [14–16]. However, the early pancreatic GSH depletion is not accompanied by any increase in GSSG levels in experimental models of acute pancreatitis [15,17]. The aims of this work were to analyze the redox status of intracellular low-molecular-weight free thiols and to identify mixed disulfides and targets of redox signaling in pancreas in experimental acute pancreatitis as a model of acute inflammation associated with glutathione depletion.

Materials and methods

Materials

N-ethylmaleimide (NEM), iodoacetamide (IAM), dithiothreitol (DTT), urea, Tris, SDS, EDTA, glycerol, Igepal, neocuproine, Lucy-565, and sodium taurocholate were purchased from Sigma–Aldrich (St. Louis, MO, USA). Monobromobimane (MBrB) was purchased from Calbiochem–Merck (Darmstadt, Germany). Fluorescein-5-maleimide was purchased from Anaspec (Fremont, CA, USA). All electrophoresis supplies were purchased from either Bio-Rad (Hercules, CA, USA) or Invitrogen (San Diego, CA,, USA).

Animals

Male Wistar rats were used. These animal studies were performed in accordance with protocols approved by the University of Valencia. All animals received human care according to the criteria outlined in the *Guide for the Care and Use of Laboratory Animals* prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH Publication 86-23 revised 1985). Wistar rats were placed under deep anesthesia with isoflurane before being treated with a solution of 3.5% sodium taurocholate in 0.9% sodium chloride. Acute pancreatitis was induced by a retrograde infusion of the solution described before. At 30 min, 1 h, 3 h, and 6 h after the induction of acute pancreatitis, the rats were anesthetized again and the pancreas were harvested and immediately snap-frozen in liquid N_2. NAC was given at a total dose of 100 mg/kg of rat divided in two ip injections, the first one 1 h before the induction of pancreatitis and the second one immediately just after induction of pancreatitis as in [18]. Lipase activity was measured in plasma and histological analysis was performed to confirm the appropriate induction of pancreatitis. Histological analysis is shown in Supplementary Fig. 1.

RT-PCR

Quantitative RT-PCR for heme oxygenase (*ho-1*), NADPH:quinone oxidoreductase 1 (*nqo1*), and *rplp0* as the corresponding reference was performed using the following primers: *ho-1,* forward, 5′-TTGAGCTGTTTGAGGAGCTG-3′, reverse, 5′-TGCTGATCTGGGATTT-TCCT-3′; *nqo1,* forward, 5′-ATCAGCGCTTGACACTACGA-3′, reverse, 5′-ACCACCTCCCATCCTTTCTT-3′; *rplp0,* forward, 5′-CAGCAGGTGTTT-GACAATGG-3′, reverse 5′-CCCTCTAGGAAGCGAGTGTG-3′.

Sample processing for the determination of the reduced and oxidized forms of low-molecular-weight thiols (LMWSH)

One hundred milligrams of fresh pancreas was homogenized in 400 μl of phosphate-buffered saline (PBS) containing 10 mM NEM and 10 μM acivicin. NEM, a cell-permeative thiol modifier, was added at a neutral pH to achieve optimum rapid blocking of free thiol groups, following the recommendation raised by Giustarini et al. [19]. Samples were deproteinized with 4% v/v perchloric acid (PCA) and centrifuged at 11,000 rpm for 15 min at 4 °C. Five microliters of a standard solution of 25 nM thiosalicylic acid, 10 mM NEM was added to 100 μl of supernatant and these samples were subjected to ultraperformance liquid chromatography coupled to tandem mass spectrometry (UPLC–MS/MS) analysis.

Sample processing for quantification of mixed disulfides

Fresh pancreas was homogenized in 10% trichloroacetic acid (TCA) in a relationship of 100 μl of buffer per 100 mg of tissue. Homogenates were centrifuged for 2 min over 10,000*g*. The pellet was washed with 10% TCA. The pellet was resuspended in 50 mM Hepes (pH 8), 2% SDS. Sodium bicarbonate (powder) was added until saturation. When the pellet was completely resuspended, an aliquot was taken for measuring proteins by BCA assay. DTT was added to the samples to reach a final concentration of 2.5 mM. Samples were incubated for 1 h at 40 °C. At this stage the pH was under 9–10. NEM was added to a final concentration of 10 mM with 4% PCA. The samples were centrifuged for 10 min, 10,000*g* at 4 °C. The supernatant was ready for measuring LMWSH by mass spectrometry.

LC–MS/MS method for quantification of LMWSH and mixed disulfides

GSH and GSSG, cysteine, cystine, γ-glutamylcysteine, bis-γ-glutamylcystine, homocysteine, and homocystine were determined by UPLC–MS/MS as described in Quintana-Cabrera et al. [20], with minor modifications.

Samples were subjected to UPLC–MS/MS analysis at the Central Service for the Support to Experimental Research of the University of Valencia, using an Acquity UPLC coupled to a TQD-Acquity mass spectrometer detector (Waters, Manchester, UK). Analytical separation was performed using a core shell C18 Acquity UPLC BEH column (2.1 × 50 mm, 1.7 μm, Waters) using an injection volume of 5 μl. The solvent gradient consisted of solvent mixture A, water:formic acid (100:0.5 v/v), and mixture B, isopropanol: acetonitrile:formic acid (50:50:0.1 v/v/v). The gradient elution program was as follows: 0–2.52 min, 0% B; 2.52–4.4 min, up to 65% B; 4.4–6 min, 65% B; 6 to 6.1 min, down to 0% B; 6.1–11 min, 0% B. The flow rate of the mobile phase was set at 0.35 ml/min and the column at room temperature (30 ± 3 °C).

Positive-ion electrospray tandem mass spectra were recorded using the following conditions: capillary voltage 3.5 kV, source temperature 120 °C, nebulization and cone gases were set at 690 and 25 L/h, respectively. Cone and collision voltages optimized for each analyte are summarized in Table 1. Linear calibration curves

Please cite this article as: Moreno, M.L; et al. Disulfide stress: a novel type of oxidative stress in acute pancreatitis. *Free Radic. Biol. Med.* (2014), http://dx.doi.org/10.1016/j.freeradbiomed.2014.01.009

Table 1
Transitions and retention times for analytes determined by LC–MS/MS.

Analyte	Cone (V)	Collision (eV)	Transition (*m/z*)	Retention time (min)
GS-NEM	30	15	433 > 304	4.32
GSSG	30	25	613 > 355	1.46
Cys-NEM	30	20	247 > 158	1.79
Cystine	15	15	241 > 152	0.42
γ-GluCys-NEM	40	20	376 > 314	4.33
γ-GluCysS	20	18	499 > 241	1.13
Homocys-NEM	30	15	261 > 215	4.26
Homocystine	35	10	269 > 136	0.58
Thiosal-NEM	25	10	278 > 153	5.03

in the 0.4–50,000 nM (GSH, GSSG, cysteine, homocysteine, cystine) and 0.1–12,500 nM (γ-glutamylcysteine, bis-γ-glutamylcystine, homocysteine, homocystine) concentration range were obtained using peak area values. The samples were analyzed twice undiluted and diluted 1:50 to get GSH into the calibration range. Transitions (*m/z*) and retention times for each analyte are summarized in Table 1.

Tissue preparation for redox switches, diagonal electrophoresis, and Western blotting

Frozen pancreas were weighed and placed immediately in chilled homogenizer with homogenization buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Igepal, and 10 μl/ml protease inhibitor buffer). NEM (50 mM) or IAM (100 mM) was added to the homogenization buffer depending on the experiment.

Redox monobromobimane switch

Pancreatic homogenates prepared with NEM as indicated above were acidified by addition of a solution of TCA (10% w/v). After separation of proteins by centrifugation at 12,000g for 2 min at 4 °C, the pellet was resuspended in a solution of PBS and 50 mM DTT and brought to a pH of 7–7.5.Samples were incubated for 1 h at 42 °C. Samples were precipitated again as indicated above to get rid of the excess of DTT. At this stage, the samples were resuspended using urea buffer (PBS, 8 M urea). Five microliters of 18.4 mM monobromobimane was added to 90 μl of sample. A 12% polyacrylamide resolving gel with 4% stacking gel was used. Sixteen microliters of reducing sample buffer containing 100 μg of protein of each sample was added to each well. SDS–PAGE was carried out until the dye front reached the bottom of the gel. Protein disulfides were detected by placing the gel under UV radiation.

Redox fluorescence switch

For each sample 50 μg of NEM-treated protein extract was blocked at 0.5 g/L in TEN buffer (50 mM Tris, pH 7.5, 1 mM EDTA, 100 μM neocuproine) with 2% SDS and 50 mM NEM at 37 °C for 30 min. Samples were precipitated with acetone and resuspended in 100 μl of TENS buffer (50 mM Tris, pH 7.5, 1 mM EDTA, 100 μM neocuproine, 1% SDS) with 2.5 mM DTT and incubated for 10 min at room temperature. Samples were precipitated with acetone and resuspended in 100 μl of TENS buffer with 40 μM fluorescein-5-maleimide (Anaspec) and incubated for 30 min at 37 °C. The fluorophore reaction was stopped by adding 2.5 mM DTT. After another precipitation with acetone, samples were resuspended in loading buffer and separated by SDS–PAGE. For total protein analysis Lucy-565 (Sigma–Aldrich) was used following the manufacturer's instructions. Images of different fluorophores were obtained using a Kodak Image Station 4000 MM with excitation/emission filters centered respectively in 470/535 nm for fluorescein and 550/600 nm for Lucy-565.

Diagonal electrophoresis

Diagonal electrophoresis is a two-dimensional electrophoresis that allows assessment of reversible protein oxidation by formation of inter- and/or intramolecular disulfide bridges [21–23]. The only differences between the first and the second dimension are the redox conditions of the electrophoresis. First dimension is performed under nonreducing conditions and second dimension under reducing conditions. When a protein lies above the diagonal, i.e., decreasing its migration when it is reduced, it indicates modification by intramolecular disulfides, whereas when it lies below the diagonal, i.e., increasing its migration when it is reduced, it indicates the formation of intermolecular disulfides [22–25].

A 12% polyacrylamide resolving gel with a 4% stacking gel was used first. Twelve microliters of nonreducing sample buffer containing 100 μg of protein of each sample was added to each well. Alternative wells were used in the nonreducing first dimension to easily excise the lanes. One-dimensional electrophoresis was carried out at 90 V until the dye reached the end of the gel. The entire lane was excised using a sharp fine scalpel. The entire lane was soaked for 30 min in equilibration buffer (6 M urea, 0.375 M Tris–HCl, pH 8.8, 2% SDS, 20% glycerol) containing 2% DTT at 42 °C on a rocker, followed by 30 min in equilibration buffer containing 2.5% IAM at the same temperature. The gel lane was rinsed in running buffer and placed horizontally on a 12% resolving gel. The slot excised lane was carefully put onto the slab gel using the blunt end of a small spatula. Five microliters of protein standard mixture was also migrated alongside the diagonal. Warm agarose containing bromophenol blue was layered on top of the slab gel. Electrophoresis was performed until the dye front reached the bottom of the gel. Proteins were detected by silver staining.

The same procedure was carried out as described before but the alkylation agent was changed. Biotinylated IAM (BIAM) was used at a concentration of 98 mM and the gel lane was incubated at 42 °C for 2 h. Proteins were detected by Coomassie staining.

In the case of PP2Ac, after the diagonal electrophoresis was performed, a Western blot was carried out using its specific antibody from Millipore.

Proteome in-gel digestion and reverse-phase–liquid chromatography (RP–LC–MS/MS) analysis of oxidative modifications

NEM-treated protein extracts from samples of animals subjected to 6 h of acute pancreatitis were suspended in a volume up to 120 μl of sample buffer and then applied onto 1.2-cm-wide wells of a conventional SDS–PAGE gel (0.75 mm thick, 4% stacking, and 10% resolving). The run was stopped as soon as the front entered 3 mm into the resolving gel, so that the whole proteome became concentrated in the stacking/resolving gel interface. The unseparated protein bands were visualized by Coomassie staining, excised, cut into cubes (2 × 2 mm), and placed into 0.5-ml microcentrifuge tubes [26]. The gel pieces were destained in acetonitrile:water (ACN:H_2O, 1:1) and digested in situ with sequencing-grade trypsin (Promega, Madison, WI, USA). The gel pieces were shrunk by removing all liquid using sufficient ACN. Acetonitrile was pipetted out and the gel pieces were dried in a SpeedVac. The dried gel pieces were reswollen in 50 mM ammonium bicarbonate with 60 ng/μl trypsin at 5/1 protein/trypsin (w/w) ratio in 50 mM ammonium bicarbonate, pH 8.8. The tubes were kept in ice for 2 h and incubated at 37 °C for 12 h. Digestion was stopped by the addition of 1% trifluoroacetic acid (TFA). Whole

Please cite this article as: Moreno, M.L; et al. Disulfide stress: a novel type of oxidative stress in acute pancreatitis. *Free Radic. Biol. Med.* (2014), http://dx.doi.org/10.1016/j.freeradbiomed.2014.01.009

supernatants were dried down and then desalted onto ZipTip C18 tips (Millipore) until the mass spectrometric analysis.

The protein digest was dried, resuspended in 10 μl of 0.1% formic acid, and analyzed by RP–LC–MS/MS in an Easy-nLC II system coupled to an ion trap LTQ-Orbitrap-Velos-Pro mass spectrometer (Thermo Scientific, Waltham, MA, USA). The peptides were concentrated (online) by reverse-phase chromatography using a 0.1 × 20-mm C18 RP precolumn (Proxeon) and then separated using a 0.075 × 100-mm C18 RP column (Proxeon) operating at 0.3 μl/min. Peptides were eluted using a 90-min gradient from 5 to 40% solvent B (solvent A, 0.1% formic acid in water; solvent B, 0.1% formic acid, 80% acetonitrile in water). Electrospray ionization was done using a nano-bore stainless steel emitter i.d. 30 μm (Proxeon) interface. The Orbitrap resolution was set at 30.000.

Peptides were detected in survey scans from 400 to 1600 amu (1 μscan), followed by one-data-dependent MS/MS scans, using an isolation width of 2 u (in mass-to-charge ratio units), normalized collision energy of 35%, and dynamic exclusion.

Peptide identification from raw data was carried out using the Sequest algorithm (Proteome Discoverer 1.3, Thermo Scientific). Database search was performed against UniProt-rattus.fasta. The following constraints were used for the searches: tryptic cleavage after Arg and Lys, up to two missed cleavage sites, and tolerances of 10 ppm for precursor ions and 0.8 Da for MS/MS fragment ions, and the searches were performed allowing optional Met oxidation and Cys cysteinylation. The search against decoy database (integrated decoy approach) used a false discovery rate of < 0.01.

The MS/MS spectra from the peptide were analyzed by assigning the fragments to the candidate sequence, after calculating the series of theoretical fragmentations.

Identification of proteins by LC–MS/MS

In-gel proteins were digested with sequencing-grade trypsin (Promega) and subjected to LC–MS/MS analysis.

The final peptide solution was concentrated by a speed vacuum concentrator at a final volume of 9 μl. Only the samples treated with BIAM were purified before LC–MS/MS analysis with Dynabeads MyOne Streptavidin T1 (Invitrogen). Five microliters of each sample was loaded onto a trap column (NanoLC column, 3 μm C18-CL, 75 μm × 15 cm; Eksigen) and desalted with 0.1% TFA at 2 μl/min for 10 min. The peptides were then loaded onto an analytical column (LC column, 3 μm C18-CL, 75 μm × 25 cm; Eksigen) equilibrated in 5% acetonitrile 0.1% FA (formic acid). Elution was carried out with a linear gradient of 5–40% solution B (solution A, 0.1% FA; solution B, ACN, 0.1% FA) at a flow rate of 300 nl/min. Peptides were analyzed in a mass spectrometer (NanoESI qQTOF, 5600 TripleTOF; AB Sciex). The TripleTOF was operated in information-dependent acquisition mode, in which a 0.25-s TOF MS scan from 350 to 1250 *m/z* was performed, followed by 0.05-s product ion scans from 100 to 1500 *m/z* on the 50 most intense 2–5 charged ions. Protein identification was performed using ProteinPilot version 4.0.8085 (AB Sciex) or Mascot version 2.2 (Matrix Science) search engines. ProteinPilot default parameters were used to generate a peak list directly from 5600 TripleTOF wiff files. The Paragon algorithm of ProteinPilot was used to search the Expasy protein database (515,203 sequences; 181,334,896 residues) with the following parameters: trypsin specificity, Cys alkylation, no taxonomy restriction, and the search effort set to thorough. To avoid using the same spectral evidence in more than one protein, the identified proteins were grouped based on MS/MS spectra by the ProteinPilot Progroup algorithm. Thus, proteins sharing MS/MS spectra are grouped, regardless of the peptide sequence assigned. The protein within each group that can explain more spectral data with confidence is shown as the primary protein of the group. Only the proteins of the group for which there is individual evidence (unique peptides with enough confidence) are listed, usually toward the end of the protein list.

For Mascot searches, the peak lists were generated directly from QSTAR wiff files by Mascot Daemon version 2.2.2 (Matrix Science) with Sciex Analyst import filter options using the default parameters. Database search was done in the Expasy protein database (515,203 sequences; 181,334,896 residues). The search parameters were set to tryptic specificity, Cys alkylation, no taxonomy restriction, two missed cleavages, and a tolerance in the mass measurement of 50 ppm in MS mode and 0.5 Da for MS/MS ions, Met oxidation, and Asn/Gln deamination.

Western blot

Protein concentration was measured in pancreatic homogenates by the BCA assay (Thermo Scientific). Forty micrograms of protein of each sample was added to either nonreducing or reducing sample buffer and separated by 12% SDS–PAGE.

When electrophoresis was performed under reducing conditions, the following sample buffer was used: 130 mM Tris–HCl, pH 6.8, 10% glycerol, 0.05% bromophenol blue, 2% SDS, 100 mM DTT. When electrophoresis was performed under nonreducing conditions, the sample buffer had the same composition but without DTT.

The following primary antibodies were used: extracellular signal-regulated kinase 1/2 (ERK1/2; 1:1000, 4695 S, Cell Signaling), Keap1 (1:1000, 4617 S, Cell Signaling), protein disulfide isomerase (PDI; 1:1000, 2446 S, Cell Signaling), p-ERK (1:1000, 4370 S, Cell Signaling), PP2Ac (1:1000, 4695 S, Cell Signaling), p38α (1:1000, sc-535, Santa Cruz Biotechnology), p-p38α (1:1000, 4511 S, Cell Signaling), PRDX1 (1:1000, 8732 S, Cell Signaling), Ref1/apurinic/apyrimidinase endonuclease 1 (APE1) (1:1000, 4128 S, Cell Signaling), RNH1 (1:1000, Ab86443, Abcam), SHP1 (1:1000, Ab18708, Abcam), SHP2 (1:1000, 3752 S, Cell Signaling), TRX1 (1:1000, 2429 S, Cell Signaling), and tubulin (1:1000, sc-8035, Santa Cruz Biotechnology). Immunodetection was performed with a chemiluminescence detection kit (Santa Cruz Biotechnology). Light emission was measured by a CCD camera. Gray values of bands were quantified with Image Lab-Bio-Rad processing software and normalized against ERK1/2 or tubulin.

Phosphatase activity

The activities of serine/threonine phosphatase PP2A and tyrosine phosphatases were measured using an assay kit manufactured by Promega (Reference Nos. V2460, V2471). Pancreatic samples were homogenized in homogenization buffer containing 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.1% Triton X-100, and a mixture of proteases inhibitors (10 μl/ml). Then the samples were purified with columns supplied in the kit to get rid of phosphates. The following specific buffers were used for each phosphatase activity: PP2A (buffer 5 ×), 250 mM imidazole (pH 7.2), 1 mM EDTA, 5 mg/ml bovine serum albumin, 50 nM tautomycetin; PTPs (buffer 5 ×) [54], Tris–HCl, 100 mM, pH 6.8, EDTA 5 mM, EGTA 5 mM, NaF 125 mM.

Statistical analysis

Results are expressed as the mean ± standard deviation with the number of experiments given in parentheses. Statistical analysis was performed in two steps. One-way analysis of variance was performed first. When the overall comparison of groups was significant, differences between individual groups were investigated using the Scheffé test. Differences were considered to be significant at $p < 0.05$.

Please cite this article as: Moreno, M.L; et al. Disulfide stress: a novel type of oxidative stress in acute pancreatitis. *Free Radic. Biol. Med.* (2014), http://dx.doi.org/10.1016/j.freeradbiomed.2014.01.009

Results

Our results confirm that GSH depletion in pancreas in acute pancreatitis occurs without significant changes in GSSG levels (Figs. 1A and B). Indeed, GSH levels decreased around 50% at 1 and 6 h after induction of pancreatitis, whereas GSSG levels were maintained without significant changes in the course of the disease. Importantly, the GSH/GSSG ratio and protein glutathionylation did not change significantly after pancreatitis induction (Fig. 2A). GSH/GSSG ratio was 59 ± 18 in pancreas from control rats and 51 ± 20 in pancreas from rats 6 h after induction of acute pancreatitis.

We measured GSH and GSSG levels in plasma after induction of pancreatitis in rats to ascertain whether there is export of GSH or GSSG toward the blood. Supplementary Fig. 2 shows that neither GSH nor GSSG levels increased in plasma during pancreatitis, suggesting that the loss of glutathione is not attributable to export from cells toward the blood.

Cystine and homocystine levels markedly increased in pancreas after induction of pancreatitis, the latter being undetectable under basal conditions (Figs. 1D and H). Importantly, protein cysteinylation was undetectable in pancreas under basal conditions but increased markedly after induction of pancreatitis (Fig. 2A), particularly at 6 h when the cysteine/cystine ratio was lower (5621 ± 2437 in controls vs 320 ± 240 in rats at 6 h postinduction; $p < 0.01$). In line with this, protein γ-glutamylcysteinylation rose progressively after induction of pancreatitis (Fig. 2A) and the

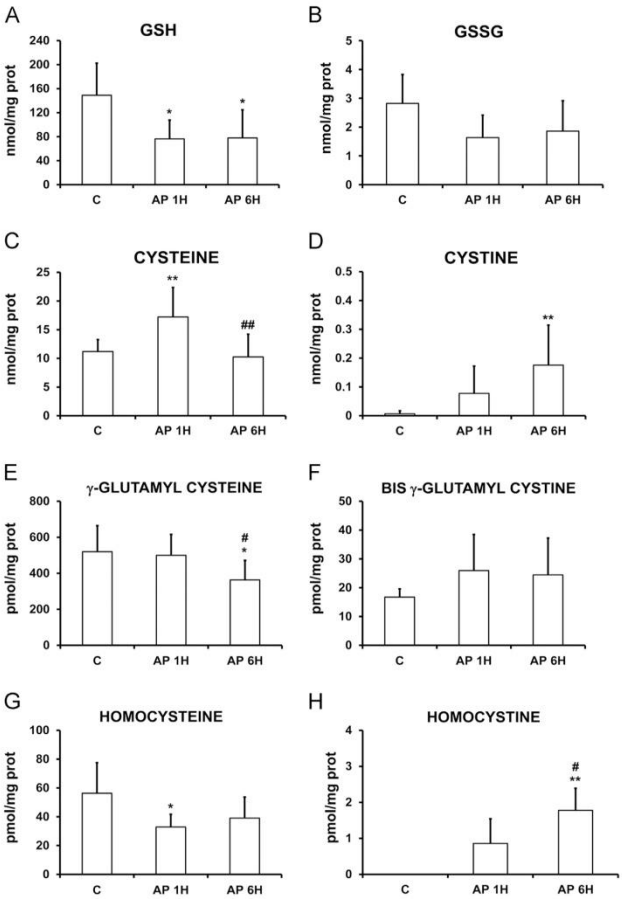


Fig. 1. Levels of free low-molecular-weight thiols and disulfides in pancreas of rats with acute pancreatitis. Levels of reduced and oxidized forms of low-molecular-weight thiols were determined by LC–MS/MS in their free state. (A) Reduced glutathione (GSH), (B) oxidized glutathione (GSSG), (C) L-cysteine, (D) cystine, (E) γ-glutamylcysteine, (F) bis-γ-glutamylcystine, (G) homocysteine, and (H) homocystine. Abbreviations used: C, control rats; AP 1H, 1 h after induction of acute pancreatitis; AP 6 H, 6 h after induction of acute pancreatitis. The number of rats per group was 6–8. The statistical difference is indicated as follows: $^{*}p < 0.05$ vs C; $^{**}p < 0.01$ vs C; $^{\#}p < 0.05$ vs AP 1 H; $^{\#\#}p < 0.01$ vs AP 1 H.

Please cite this article as: Moreno, M.L; et al. Disulfide stress: a novel type of oxidative stress in acute pancreatitis. *Free Radic. Biol. Med.* (2014), http://dx.doi.org/10.1016/j.freeradbiomed.2014.01.009

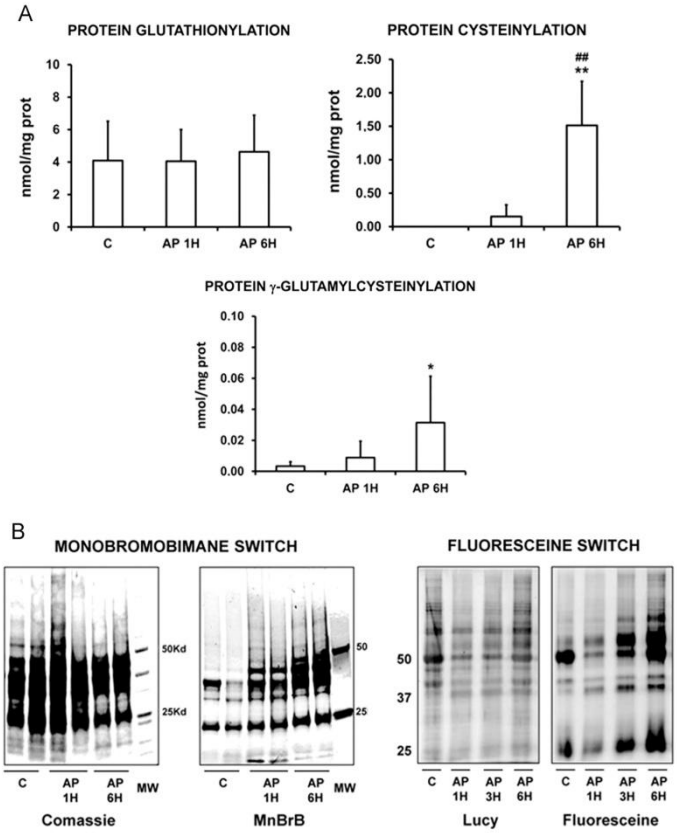


Fig. 2. Formation of protein disulfides in pancreas of rats with acute pancreatitis. (A) Protein glutathionylation, cysteinylation, and γ-glutamylcysteinylation in pancreas of rats with acute pancreatitis. GSH released from protein glutathionylation, cysteine released from protein cysteinylation, and γ-glutamylcysteine released from protein γ-glutamylcysteinylation are shown. The number of rats per group was 6–8.The statistical difference is indicated as follows: $^{*}p < 0.05$ vs C; $^{**}p < 0.01$ vs. C; $^{\#\#}p < 0.01$ vs AP1H. (B) Formation of protein disulfides in pancreas of rats with acute pancreatitis analyzed by redox monobromobimane switch (left) and redox fluorescence switch (right). The graphs show representative redox fluorescein and monobromobimane assays for three different experiments. Abbreviations used: C, control rats; AP 1 H, 1 h after induction of acute pancreatitis; AP 3 H, 3 h after induction of acute pancreatitis; AP 6 H, 6 h after induction of acute pancreatitis.

γ-glutamylcysteine/bis-γ-glutamylcystine ratio decreased upon pancreatitis. Indeed, it was 30 ± 12 in controls vs 14 ± 2 in rats at 6 h postinduction ($p < 0.05$).

We also assessed thiol modifications by using "redox fluorescence switches" in which free cysteines were first blocked with NEM and subsequently oxidized cysteines were reduced with DTT and labeled with monobromobimane (Fig. 2B) or maleimide fluorescein (Fig. 2B) [27,28]. The increase in fluorescence after pancreatitis induction (Fig. 2B) shows that reversible thiol oxidations are formed in the course of acute pancreatitis.

The next step in our work was to identify targets of disulfide stress that are oxidized in the course of acute pancreatitis. To this end we performed Western blotting of target candidates using reducing—i.e., with DTT—and nonreducing conditions, in all cases in the presence of NEM as thiol modifier. Decreased intensity of a Western blot band under nonreducing conditions relative to reducing conditions gives an indirect indication of oxidation. Our results show that ribonuclease inhibitor, serine/threonine phosphatase PP2A, tyrosine phosphatases SHP1 and SHP2, protein disulfide isomerase, thioredoxin 1, and Keap1 are oxidized in acute pancreatitis, marking them as first or earlier targets of disulfide stress (Figs. 3, 4, and 5A). In the case of APE1/Ref1 a marked decrease under both reducing and nonreducing conditions was found, probably due to degradation in the course of pancreatitis. As one of the targets of disulfide stress is Keap1, we also assessed whether targets of the Keap1/Nrf2 pathway were induced. Indeed, we found that *ho-1* and *nqo1* mRNAs were upregulated by 30- and 2.8-fold, respectively (Fig. 5B).

We also sought to identify new targets of disulfide stress by proteomic methods (see Supplementary Table 1). To this end, we performed diagonal electrophoresis using iodoacetamide as thiol modifier (Fig. 6). Three spots were found in all the samples (from

Please cite this article as: Moreno, M.L; et al. Disulfide stress: a novel type of oxidative stress in acute pancreatitis. *Free Radic. Biol. Med.* (2014), http://dx.doi.org/10.1016/j.freeradbiomed.2014.01.009

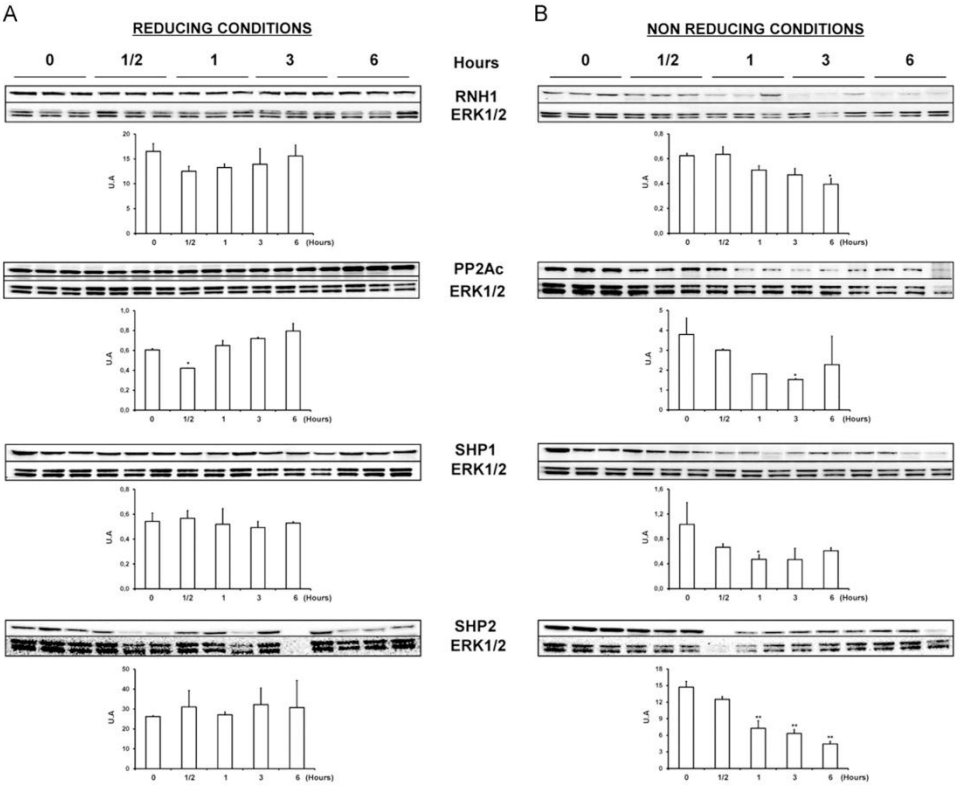


Fig. 3. Targets of disulfide stress in pancreas in acute pancreatitis identified by Western blotting under reducing or nonreducing conditions (part I). The following redox-sensitive proteins were analyzed by Western blotting under reducing or nonreducing conditions (see Materials and methods):: ribonuclease inhibitor (RNH1), Ser/Thr protein phosphatase catalytic subunit (PP2Ac), and cytosolic Tyr phosphatases SHP1 and SHP2. Western blotting of each protein is shown with its corresponding loading control, ERK1/2. Densitometries normalized by ERK1/2 are also shown below each blot. Each blot includes a set of three different pancreatic samples obtained at time 0 or after 30 min, 1 h, 3 h, and 6 h after taurocholate-induced pancreatitis. $^{*}p < 0.05$ vs C; $^{**}p < 0.01$ vs C.

both control and treated animals) above the diagonal, as hallmarks of pancreatic tissue that correspond to elastase, anionic trypsin 1, and cationic trypsin 3. They are secreted proteins with constitutive disulfide bonds [3,29]. In contrast, peroxiredoxin 4, albumin, α-amylase, and elongation factor 1α appeared under the diagonal only after pancreatitis induction, revealing that they could take part in high-molecular-mass oxidized complexes in acute inflammation.

We searched for peptides modified by redox switch labeling with biotinylated iodoacetamide that were purified before LC–MS/MS analysis with streptavidin beads. We found reversible oxidation in cysteines of mitochondrial sulfide:quinone oxidoreductase and 60 S ribosomal protein L7a (see Supplementary Fig. 3 and Supplementary Table 2). A majority of these modifications might be by low-molecular-weight thiols because the diagonal electrophoresis performed after the redox switch labeling developed with streptavidin–HRP revealed a marked increase in chemiluminescence along the diagonal (see Fig. 6B).

To confirm the formation of mixed disulfides, we searched for thiolated peptides among the proteome, and a cysteinylated peptide corresponding to a free thiol of albumin was found (see Supplementary Fig. 4). Other spectra were assigned by the software to cysteinylated peptides, but were discarded after manual inspection because the modification was not unequivocally assigned.

Serine/threonine protein phosphatase PP2Ac was indeed a target of disulfide stress, as shown by diagonal electrophoresis coupled with Western blot detection (Fig. 7). Indeed, part of the protein lay above the diagonal, which revealed that its major modification seems to be an intramolecular disulfide.

To confirm the functional relevance of disulfide stress in acute pancreatitis, we studied protein phosphatase activities. There is a clear loss of PP2A and tyrosine phosphatase activities in pancreatitis, which correlates with the marked cysteine oxidation observed (Fig. 8). The loss of PP2A and tyrosine phosphatase activities was abrogated by NAC.

Discussion

We herein propose disulfide stress as a specific type of oxidative stress in acute inflammation in mammals associated

Please cite this article as: Moreno, M.L; et al. Disulfide stress: a novel type of oxidative stress in acute pancreatitis. *Free Radic. Biol. Med.* (2014), http://dx.doi.org/10.1016/j.freeradbiomed.2014.01.009

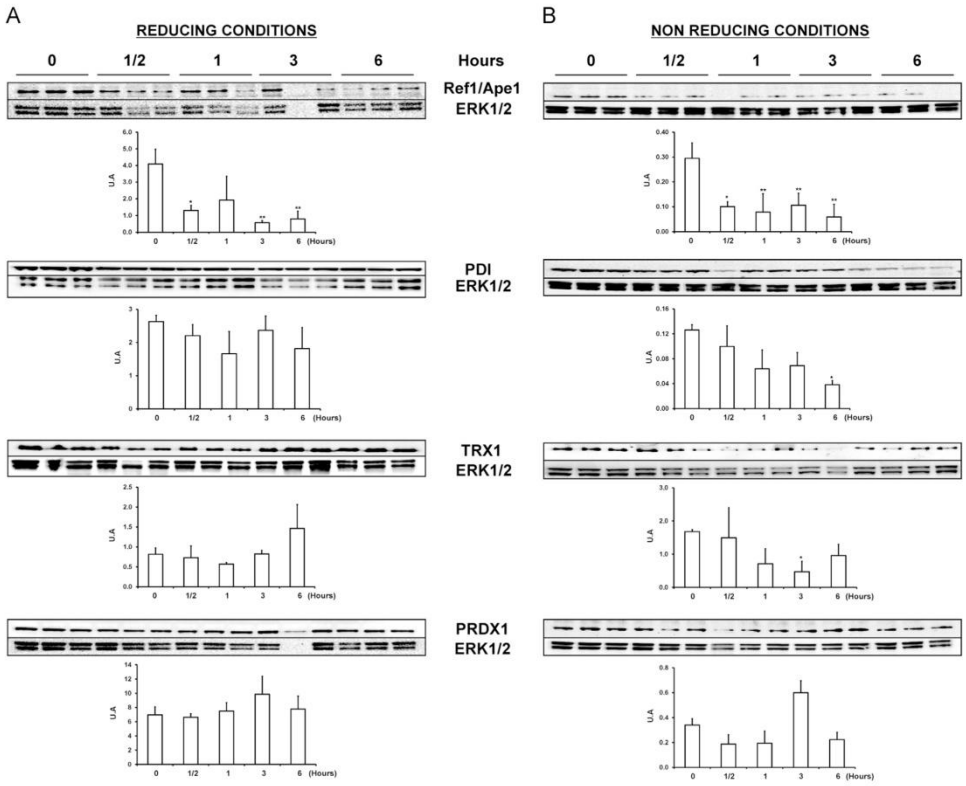


Fig. 4. Targets of disulfide stress in pancreas in acute pancreatitis identified by Western blotting under reducing or nonreducing conditions (part II). The following redox-sensitive proteins were analyzed by Western blotting under reducing or nonreducing conditions (see Materials and methods): redox effector factor 1 (Ref1/APE1), protein disulfide isomerase (PDI), thioredoxin 1 (TRX1), and peroxiredoxin 1 (PRDX1). Western blotting of each protein is shown with its corresponding loading control, ERK1/2. Densitometries normalized by ERK1/2 are also shown below each blot. Each blot includes a set of three different pancreatic samples obtained at time 0 or after 30 min, 1 h, 3 h, and 6 h after taurocholate-induced pancreatitis. $^{*}p < 0.05$ vs C; $^{**}p < 0.01$ vs C.

with oxidation of the pairs cysteine/cystine, γ-glutamylcysteine/bis-γ-glutamylcystine, and homocysteine/homocystine, as well as protein cysteinylation, but without glutathione oxidation or changes in protein glutathionylation. Toledano and co-workers [11] showed that the thiol redox state of yeast could be maintained when GSH was depleted. Our results support the hypothesis of Toledano and co-workers, who suggested that glutathione levels are not critical for thiol redox control. The term disulfide stress was already used in bacteria, referring to disulfide bonds generated by diamide [29,30]. However, diamide triggers typical oxidative stress and damage associated with glutathione oxidation, which are clearly different from the disulfide stress that we describe here associated with a pathophysiological condition such as acute inflammation.

Depletion of pancreatic glutathione may be due to activation of proteases, such as carboxypeptidase, that may cleave GSH [31]. Trypsinogen activation is accompanied by glutathione depletion in experimental acute pancreatitis [32]. Accordingly, the increase in cysteine and cystine levels that occurs in pancreatitis is likely to be ascribed to GSH breakdown by pancreatic proteases. In the case of glutathione, glutathione reductase and glutaredoxins contribute to maintaining its reduced state [32]. However, there are no specific cysteine reductases and the thioredoxin system exhibits limited activity to reduce cystine in mammalian cells [33]. This would favor oxidation of the cysteine/cystine pair and subsequent disulfide stress in proteins.

Given that the redox potentials of thioredoxin (from −270 to −300 mV) and glutathione (between −200 and −260 mV) in the cytoplasm are strongly reducing [3,29,34], large drops in the cellular redox status would be required for thiol–disulfide exchanges in proteins that may cause profound effects on protein folding and activity [29]. However, no such big drops would be required if the cysteine/cystine couple is involved. Indeed, it was reported that the intracellular redox potential of the cysteine/cystine couple is between −125 and −160 mV; i.e., it is considerably more oxidized than the GSH/GSSG couple [34]. Furthermore, the redox states of Cys/cystine and GSH/GSSG are not in equilibrium and seem to be independently regulated in cells [34]. Our

Please cite this article as: Moreno, M.L; et al. Disulfide stress: a novel type of oxidative stress in acute pancreatitis. *Free Radic. Biol. Med.* (2014), http://dx.doi.org/10.1016/j.freeradbiomed.2014.01.009

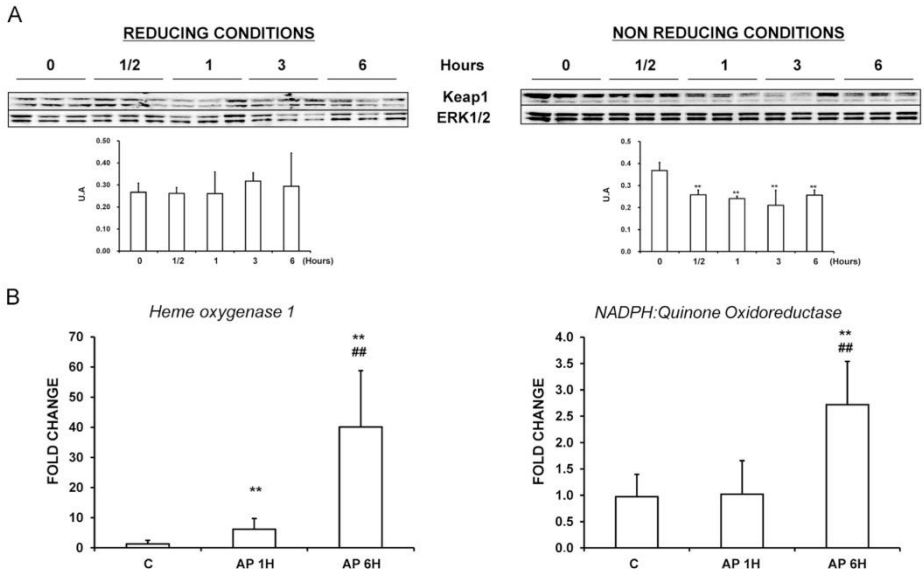


Fig. 5. Immunodetection of the Nrf2 inhibitor, Kelch-like ECH-associated protein 1 (Keap1), under reducing or nonreducing conditions and gene expression of the Nrf2 targets, heme oxygenase 1 (*ho-1*) and NADPH:quinone oxidoreductase (*nqo1*), in pancreas in acute pancreatitis. (A) The redox-sensitive Nrf2 inhibitor Keap1 was analyzed by Western blotting under reducing or nonreducing conditions (see Materials and methods). Western blotting of each condition, corresponding loading control, ERK1/2, and densitometries normalized by ERK1/2 are shown. Each blot includes a set of three different pancreatic samples obtained at time 0 or after 30 min, 1 h, 3 h, and 6 h after taurocholate-induced pancreatitis. (B) The mRNA levels of two Nrf2 targets, *ho-1* and *nqo1*, were determined by RT-PCR. AP 1 H, 1 h after induction of acute pancreatitis; AP 6 H, 6 h after induction of acute pancreatitis. The number of rats per group was 3–6 for Keap1 immunodetection and 10 for RT-PCR analysis. The statistical difference is indicated as follows: $^{**}p < 0.01$ vs C; $^{\#\#}p < 0.01$ vs AP 1 H.

findings support the hypothesis raised by Jones and co-workers [34], as GSH breakdown occurs in pancreas during acute pancreatitis without glutathione oxidation leading to an increase in cysteine levels and its oxidation to cysteine and protein cysteinylation without protein glutathionylation. Thus, the Cys/cystine pool is more prone to oxidation and accordingly Jones and colleagues suggested that oxidation of thiol moieties by cystine as well as S-cysteinylation of thiols could be new classes of redox signaling [34]. To our knowledge, the present work is the first report on disulfide stress mediated by S-cysteinylation in mammalian cells.

According to our results, two types of targets should be considered in disulfide stress: redox buffers, such as ribonuclease inhibitor or albumin, and redox-signaling thiols that would include relevant targets such as thioredoxin 1, APE1/Ref1, PTPs, and serine/threonine phosphatase PP2A. Thioredoxin is required for protein activity of many enzymes that are dependent on disulfide bond reduction, such as ribonucleotide reductase [29,35]. Thioredoxin 1 oxidation would enable disulfide stress in acute inflammation. The reducing thiol–disulfide status of the cytosol is considered mainly regulated by the thioredoxin–thioredoxin reductase pathway and the glutathione–glutaredoxin pathway [36,37]. Nevertheless, the GSH/GSSG and the thioredoxin 1 redox states seem to be regulated independently [38]. Our results support this hypothesis because they show that oxidation of thioredoxin 1 occurs without parallel glutathione oxidation in acute pancreatitis.

Our findings would provide a specific mechanism within the complexity of protein thiol–disulfide oxidoreduction in the regulation of inflammation that has been previously described by others [39,40], which was mainly ascribed to protein glutathionylation—particularly S-glutathionylation of inhibitory κB kinase-β—as well as to thioredoxin and glutaredoxin.

Ribonuclease inhibitor has a high number of redox-sensitive cysteine residues whose oxidation results in functional inactivation [41], which may contribute to the presence of ribonuclease activity in the cytosol in severe acute pancreatitis, as we reported previously [16]. Ribonuclease inhibitor plays a role in cell redox homeostasis as a redox buffer [42] and enzymes such as thioredoxin are involved in the maintenance of its redox status.

APE1, in addition to its central role in DNA repair, functions as a redox effector factor for transcription factors AP1, HIF-2α, and p53 and is involved in posttranscriptional control of the gene expression of c-myc [43–45]. Therefore, the marked APE/Ref1 oxidation and loss might contribute to the accumulation of mutations and affect the regulation of the cell cycle and cell proliferation.

PTPs are important sensors of the cellular redox state [46,47]. Importantly, inhibition of tyrosine phosphatases was sufficient to induce dissociation of adherens junctions in pancreatic acini as a prerequisite for the development of pancreatic edema in acute pancreatitis [48]. Redox changes also affect serine/threonine PP2A activity because cysteines of the active site in the catalytic subunit of PP2A can form intermolecular disulfide bonds with regulatory subunits [49] or intramolecular bonds with vicinal thiols in PP2Ac, lowering PP2A activity [50]. Moreover, the loss of PP2A and tyrosine phosphatase activities that occurs in pancreas in acute pancreatitis may be redox sensitive because it is abrogated by NAC, suggesting the involvement of disulfide stress in redox signaling through these phosphatases.

Please cite this article as: Moreno, M.L; et al. Disulfide stress: a novel type of oxidative stress in acute pancreatitis. *Free Radic. Biol. Med.* (2014), http://dx.doi.org/10.1016/j.freeradbiomed.2014.01.009

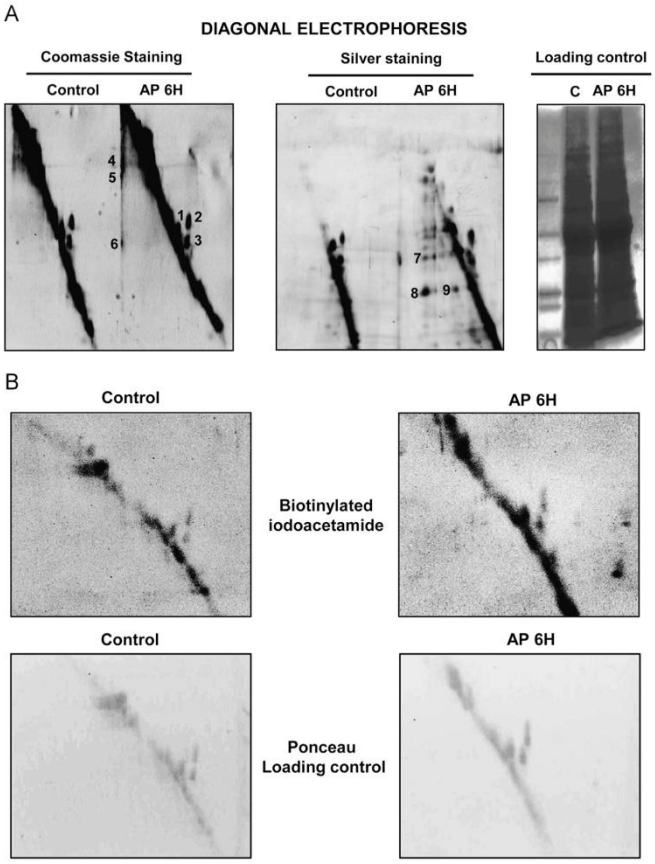


Fig. 6. Identification of proteins with disulfide modifications by diagonal electrophoresis in pancreas of rats with acute pancreatitis. (A) Identification of proteins by MALDI–TOF previously separated by diagonal electrophoresis and stained with Coomassie blue (left) or silver (middle). Right side: loading control for the diagonal. Proteins identified were 1, elastase; 2, anionic tripsin-1; 3, cationic tripsin-3; 4, α-amylase; 5, elongation factor 1α; 6, peroxiredoxin 4; and 7, 8, 9, albumin. (B) Redox-sensitive cysteines of proteins. Free thiols of proteins were blocked with iodoacetamide. Oxidized cysteines were reduced with DTT and alkylated with biotinylated iodoacetamide. Proteins with biotinylated iodoacetamide were detected by streptavidin–HRP. Membranes were stained with Ponceau as the loading control. Abbreviations used: C, control rats; AP 6 H, 6 h after induction of acute pancreatitis.

PDIs are also involved in redox control, but in contrast to the glutathione and thioredoxin systems, PDIs function in the endoplasmic reticulum to introduce disulfides as a required mechanism for protein assembly and folding in the secretory pathway [51,52]. Disulfide formation in the endoplasmic reticulum depends on recycling of peroxiredoxin 4, whose catalytic activity toward H_2O_2 depends on reduction of a disulfide within the active site to form free thiols [53]. Consequently, oxidation of PDIs and peroxiredoxin 4 might be involved in the unfolded protein response that occurs in acute pancreatitis.

The adaptive response to oxidative stress includes the activation of transcription factors, such as FoxO4 and Nrf2/Keap1 in mammals [3,5,7], through oxidation of cysteine residues to disulfide bonds [29]. The formation of these disulfides is transient and may be reversed by thioredoxin or glutathione [3]. Our data show that the Nrf2/Keap1 pathway is activated in pancreatitis by Keap1 oxidation because expression of *ho-1* and *nqo1*—two of its targets—was upregulated.

Conclusions

According to our findings, disulfide stress may be considered a specific type of oxidative stress in acute inflammation associated with certain mixed disulfides, particularly protein cysteinylation, and oxidation of low-molecular-weight thiols such as cysteine,

Please cite this article as: Moreno, M.L; et al. Disulfide stress: a novel type of oxidative stress in acute pancreatitis. *Free Radic. Biol. Med.* (2014), http://dx.doi.org/10.1016/j.freeradbiomed.2014.01.009

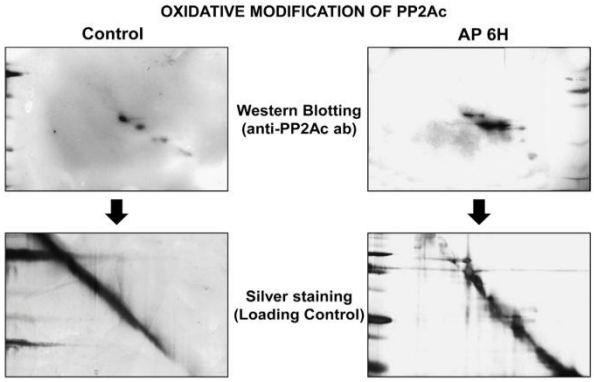


Fig. 7. PP2A oxidative modification analyzed by diagonal electrophoresis and Western blotting in pancreas from control rats and from rats at 6 h after induction of acute pancreatitis (AP 6 H). Silver staining of the gel is shown as loading control.

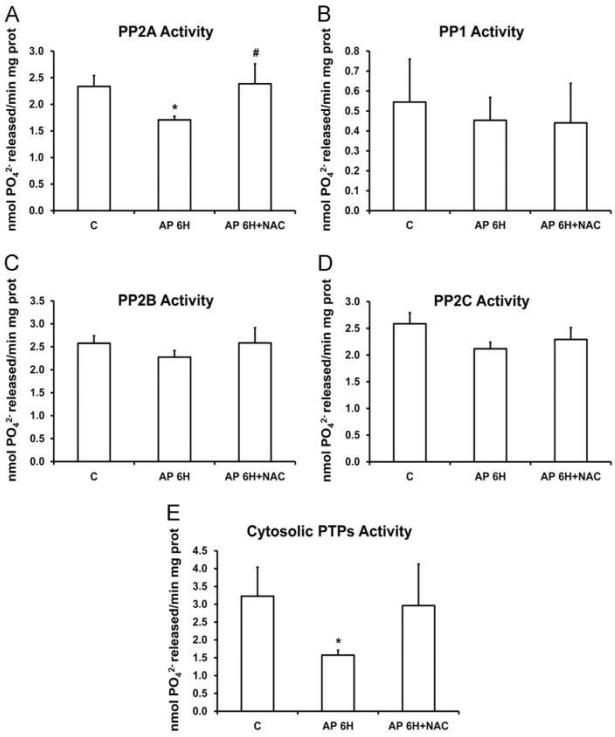


Fig. 8. Loss of Ser/Thr and Tyr protein phosphatase activities in pancreas in acute pancreatitis and its reversal by *N*-acetylcysteine. The activities of (A) PP2A, (B) PP1, (C) PP2B (calcineurin), (D) PP2C, and (E) cytosolic tyrosine phosphatases were determined by nonradioactive ELISA-based assay. Abbreviations used: C, controls; AP 6 H, 6 h after induction of acute pancreatitis; AP 6 H + NAC, rats treated with *N*-acetylcysteine and sacrificed 6 h after induction of acute pancreatitis. The number of rats per group was 4 or 5. The statistical difference is indicated as follows: $^{*}p < 0.05$ vs C; $^{\#}p < 0.05$ vs AP 6 H.

Please cite this article as: Moreno, M.L; et al. Disulfide stress: a novel type of oxidative stress in acute pancreatitis. *Free Radic. Biol. Med.* (2014), http://dx.doi.org/10.1016/j.freeradbiomed.2014.01.009

γ-glutamylcysteine, and homocysteine, but without glutathione oxidation or changes in protein glutathionylation. Two types of targets should be considered in disulfide stress: redox buffers, such as ribonuclease inhibitor or albumin, and redox-signaling thiols that include thioredoxin 1, APE1/Ref1, Keap1, tyrosine and serine/threonine phosphatases, and protein disulfide isomerases.

Our study is also the first report that shows binding of γ-glutamylcysteine to proteins. Very recently it has been demonstrated that γ-glutamylcysteine is a potent antioxidant acting as a cofactor of glutathione peroxidase 1 [20]. In the present study we show that GSH breakdown in acute pancreatitis causes an increase in cystine but not in GSSG levels, leading to protein modification by cysteinylation and γ-glutamylcysteinylation, the former being undetectable under basal physiological conditions in vivo. Therefore, we provide a novel mechanism of redox signaling involved in acute inflammation through disulfide stress independent of the glutathione redox status.

Acknowledgments

This work was supported by Grants SAF2009-09500 and SAF2012-39694 with FEDER funds to J.S., CP07/00143, PS09/00101 and PI12/00875 to A.M.R. and CSD-2007-00020 to J.S. and A.M.R, from the Spanish Ministry of Economy and Competitiveness. J.E. was a recipient of the Sara Borrell Fellowship CD11/00154 from the Instituto de Salud Carlos III. Proteomic analyses were done at the Protein Chemistry Facility of the CBMSO and at the SCIE Research Center of the University of Valencia, members of the ProteoRed-ISCIII network. We thank Mrs. Patricia Ahicart, Mrs. Elena Ramos, Mrs. Luz Valero, and Mrs. Oreto Antúnez for their helpful technical assistance and Ms. Landy Menzies for revising the manuscript.

Appendix A. Supplementary Information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.freeradbiomed.2014.01.009.

References

[1] Jones, D. P.; Go, Y. M. Mapping the cysteine proteome: analysis of redox-sensing thiols. *Curr. Opin. Chem. Biol.* **15:**103–112; 2011.
[2] Yang, Y.; Song, Y.; Loscalzo, J. Regulation of the protein disulfide proteome by mitochondria in mammalian cells. *Proc. Natl. Acad. Sci. USA* **104:**10813–10817; 2007.
[3] Wouters, M. A.; Fan, S. W.; Haworth, N. L. Disulfides as redox switches: from molecular mechanisms to functional significance. *Antioxid. Redox Signaling* **12:**53–91; 2010.
[4] Jones, D. P. Redox sensing: orthogonal control in cell cycle and apoptosis signalling. *J. Intern. Med.* **268:**32–48; 2010.
[5] Antelmann, H.; Helmann, J. D. Thiol-based redox switches and gene regulation. *Antioxid. Redox Signaling* **14:**1049–1063; 2011.
[6] Forman, H. J.; Maiorino, M.; Ursini, F. Signaling functions of reactive oxygen species. *Biochemistry* **49:**835–842; 2010.
[7] Dickinson, B. C.; Chang, C. J. Chemistry and biology of reactive oxygen species in signaling or stress responses. *Nat. Chem. Biol.* **7:**504–511; 2011.
[8] Grauschopf, U.; Winther, J. R.; Korber, P.; Zander, T.; Dallinger, P.; Bardwell, J. C. Why is DsbA such an oxidizing disulfide catalyst? *Cell* **83:**947–955; 1995.
[9] Cotgreave, I. A.; Gerdes, R. G. Recent trends in glutathione biochemistry—glutathione–protein interactions: a molecular link between oxidative stress and cell proliferation? *Biochem. Biophys. Res. Commun* **242:**1–9; 1998.
[10] Jakob, U.; Muse, W.; Eser, M.; Bardwell, J. C. Chaperone activity with a redox switch. *Cell* **96:**341–352; 1999.
[11] Kumar, C.; Igbaria, A.; D'Autreaux, B.; Planson, A. G.; Junot, C.; Godat, E.; Bachhawat, A. K.; Delaunay-Moisan, A.; Toledano, M. B. Glutathione revisited: a vital function in iron metabolism and ancillary role in thiol-redox control. *EMBO J.* **30:**2044–2056; 2011.
[12] Go, Y. M.; Park, H.; Koval, M.; Orr, M.; Reed, M.; Liang, Y.; Smith, D.; Pohl, J.; Jones, D. P. A key role for mitochondria in endothelial signaling by plasma cysteine/cystine redox potential. *Free Radic. Biol. Med.* **48:**275–283; 2010.
[13] Moriarty-Craige, S. E.; Jones, D. P. Extracellular thiols and thiol/disulfide redox in metabolism. *Annu. Rev. Nutr.* **24:**481–509; 2004.
[14] Escobar, J.; Pereda, J.; Arduini, A.; Sandoval, J.; Sabater, L.; Aparisi, L.; López-Rodas, G.; Sastre, J. Cross-talk between oxidative stress and proinflammatory cytokines in acute pancreatitis: a key role for protein phosphatases. *Curr. Pharm. Des* **15:**3027–3042; 2009.
[15] Gómez-Cambronero, L.; Camps, B.; de La Asunción, J. G.; Cerdá, M.; Pellín, A.; Pallardó, F. V.; Calvete, J.; Sweiry, J. H.; Mann, G. E.; Viña, J.; Sastre, J. Pentoxifylline ameliorates cerulein-induced pancreatitis in rats: role of glutathione and nitric oxide. *J. Pharmacol. Exp. Ther.* **293:**670–676; 2000.
[16] Pereda, J.; Escobar, J.; Sandoval, J.; Rodríguez, J. L.; Sabater, L.; Pallardó, F. V.; Torres, L.; Franco, L.; Viña, J.; López-Rodas, G.; Sastre, J. Glutamate cysteine ligase up-regulation fails in necrotizing pancreatitis. *Free Radic. Biol. Med.* **44:**1599–1609; 2008.
[17] Escobar, J.; Pereda, J.; Arduini, A. L.; Sandoval, J.; Moreno, M. L.; Pérez, S.; Sabater, L.; Aparisi, L.; Cassinello, N.; Hidalgo, J.; Joosten, L. A. B.; Vento, M.; López-Rodas, G; Sastre, J. Oxidative and nitrosative stress in acute pancreatitis: modulation by pentoxifylline and oxypurinol. *Biochem. Pharmacol.* **83:**122–130; 2012.
[18] de Dios, I.; Ramudo, L.; García-Montero, A. C.; Manso, M. A. Redox-sensitive modulation of CD45 expression in pancreatic acinar cells during acute pancreatitis. *J. Pathol.* **210:**234–239; 2006.
[19] Giustarini, D.; Dalle-Donne, I.; Colombo, R.; Milzani, A.; Rossi, R. An improved HPLC measurement for GSH and GSSG in human blood. *Free Radic. Biol. Med.* **35:**1365–1372; 2003.
[20] Quintana-Cabrera, R.; Fernández-Fernández, S.; Bobo-Jimenez, V.; Escobar, J.; Sastre, J.; Almeida, A.; Bolaños, J. P. γ-Glutamylcysteine detoxifies reactive oxygen species by acting as glutathione peroxidase-1 cofactor. *Nat. Commun* **3:**718–722; 2012.
[21] Wait, R.; Begum, S.; Brambilla, D.; Carabelli, A. M.; Conserva, F.; Rocco-Guerini, A.; Eberini, I.; Ballerio, R.; Gemeiner, M.; Miller, I.; Gianazza, E. Redox options in two-dimensional electrophoresis. *Amino Acids* **28:**239–272; 2005.
[22] Yano, H.; Wong, J. H.; Lee, Y. M.; Cho, M. J.; Buchanan, B. B. A strategy for the identification of proteins targeted by thioredoxin. *Proc. Natl. Acad. Sci. USA* **98:**4794–4799; 2001.
[23] Yano, H.; Kuroda, S.; Buchanan, B. B. Disulfide proteome in the analysis of protein function and structure. *Proteomics* **2:**1090–1096; 2002.
[24] Eaton, P. Protein thiol oxidation in health and disease: techniques for measuring disulfides and related modifications in complex protein mixtures. *Free Radic. Biol. Med* **40:**1889–1899; 2006.
[25] Rinalducci, S.; Murgiano, L.; Zolla, L. Redox proteomics: basic principles and future perspectives for the detection of protein oxidation in plants. *J. Exp. Bot.* **59:**3781–3801; 2008.
[26] Bonzon-Kulichenko, E.; Martínez-Martínez, S.; Trevisan-Herraz, M.; Navarro, P.; Redondo, J. M.; Vázquez, J. Quantitative in-depth analysis of the dynamic secretome of activated Jurkat T-cells. *J. Proteomics* **75:**561–571; 2011.
[27] Izquierdo-Álvarez, A.; Martínez-Ruiz, A. Thiol redox proteomics seen with fluorescent eyes: the detection of cysteine oxidative modifications by fluorescence derivatization and 2-DE. *J. Proteomics* **75:**329–338; 2011.
[28] Izquierdo-Álvarez, A.; Ramos, E.; Villanueva, J.; Hernansanz-Agustín, P.; Fernández-Rodríguez, R.; Tello, D.; Carrascal, M.; Martínez-Ruiz, A. Differential redox proteomics allows identification of proteins reversibly oxidized in cysteines in endothelial cells during acute response to hypoxia. *J. Proteomics* **75:**5449–5462; 2012.
[29] Aslund, F.; Beckwith, J. Bridge over troubled waters: sensing stress by disulfide bond formation. *Cell* **96:**751–753; 1999.
[30] Zhang, Y.; Zuber, P. Requirement of the zinc-binding domain of ClpX for Spx proteolysis in Bacillus subtilis and effects of disulfide stress on ClpXP activity. *J. Bacteriol* **189:**7669–7680; 2007.
[31] Meister, A. Glutathione deficiency produced by inhibition of its synthesis, and its reversal; applications in research and therapy. *Pharmacol. Ther* **51:**155–194; 1991.
[32] Mieyal, J. J.; Gallogly, M. M.; Qanungo, S.; Sabens, E. A.; Shelton, M. D. Molecular mechanisms and clinical implications of reversible protein S-glutathionylation. *Antioxid. Redox Signaling* **10:**1941–1988; 2008.
[33] Mannervik, B.; Axelsson, K.; Sundewall, A. C.; Holmgren, A. Relative contributions of thioltransferase- and thioredoxin-dependent systems in reduction of low-molecular-mass and protein disulphides. *Biochem. J.* **213:**519–523; 1983.
[34] Jones, D. P.; Go, Y. M.; Anderson, C. L.; Ziegler, T. L.; Kinkade Jr J. M.; Kirlin, W. G. Cysteine/cystine couple is a newly recognized node in the circuitry for biologic redox signaling and control. *FASEB J.* **18:**1246–1248; 2004.
[35] Holmgren, A.; Sengupta, R. The use of thiols by ribonucleotide reductase. *Free Radic. Biol. Med.* **49:**1617–1628; 2010.
[36] Prinz, W. A.; Aslund, F.; Holmgren, A.; Beckwith, J. The role of the thioredoxin and glutaredoxin pathways in reducing protein disulfide bonds in the Escherichia coli cytoplasm. *J. Biol. Chem.* **272:**15661–15667; 1997.
[37] Toledano, M. B.; Kumar, C.; Le Moan, N.; Spector, D.; Tacnet, F. The system biology of thiol redox system in Escherichia coli and yeast: differential functions in oxidative stress, iron metabolism and DNA synthesis. *FEBS Lett* **581:**3598–3607; 2007.
[38] Nkabyo, Y. S.; Ziegler, T. R.; Gu, L. H.; Watson, W. H.; Jones, D. P. Glutathione and thioredoxin redox during differentiation in human colon epithelial (Caco-2) cells. *Am. J. Physiol. Gastrointest. Liver Physiol.* **283:**1352–1359; 2002.
[39] Aesif, S. W.; Kuipers, I.; van der Velden, J.; Tully, J. E.; Guala, A. S.; Anathy, V.; Sheely, J. I.; Reynaert, N. L.; Wouters, E. F.; van der Vliet, A.; Janssen-Heininger,

Please cite this article as: Moreno, M.L; et al. Disulfide stress: a novel type of oxidative stress in acute pancreatitis. *Free Radic. Biol. Med.* (2014), http://dx.doi.org/10.1016/j.freeradbiomed.2014.01.009

Y. M. Activation of the glutaredoxin-1 gene by nuclear factor κB enhances signaling. *Free Radic. Biol. Med.* **51:**1249–1257; 2011.
[40] Coppo, L.; Ghezzi, P. Thiol regulation of pro-inflammatory cytokines and innate immunity: protein S-thiolation as a novel molecular mechanism. *Biochem. Soc. Trans* **39:**1268–1272; 2011.
[41] Ferreras, M.; Gavilanes, J. G.; López-Otín, C.; García-Segura, J. M. Thiol–disulfide exchange of ribonuclease inhibitor bound to ribonuclease A. *J. Biol. Chem.* **270:**28570–28578; 1995.
[42] Monti, D. M.; Montesano Gesualdi, N.; Matousek, J.; Esposito, F.; D'Alessio, G. The cytosolic ribonuclease inhibitor contributes to intracellular redox homeostasis. *FEBS Lett.* **581:**930–934; 2007.
[43] Bhakat, K. K.; Mantha, A. K.; Mitra, S. Transcriptional regulatory functions of mammalian AP-endonuclease (APE1/Ref-1), an essential multifunctional protein. *Antioxid. Redox Signaling* **11:**621–638; 2009.
[44] Luo, M.; He, H.; Kelley, M. R.; Georgiadis, M. M. Redox regulation of DNA repair: implications for human health and cancer therapeutic development. *Antioxid. Redox Signaling* **12:**1247–1269; 2010.
[45] Tell, G.; Wilson, D. M.; Lee, C. H. Intrusion of a DNA repair protein in the RNome world: is this the beginning of a new era? *Mol. Cell. Biol* **30:**366–371; 2010.
[46] Cho, S. H. Redox regulation of PTEN and protein tyrosine phosphatases in H_2O_2 mediated cell signaling. *FEBS Lett* **560:**7–13; 2004.
[47] Den Hertog, J.; Groen, A.; van der Wijk, T. Redox regulation of protein-tyrosine phosphatases. *Arch. Biochem. Biophys.* **434:**11–15; 2005.
[48] Schnekenburger, J.; Mayerle, J.; Krüger, B.; Buchwalow, I.; Weiss, F. U.; Albrecht, E.; Samoilova, V. E.; Domschke, W.; Lerch, M. M. Protein tyrosine phosphatase kappa and SHP-1 are involved in the regulation of cell–cell contacts at adherens junctions in the exocrine pancreas. *Gut* **54:**1445–1455; 2005.
[49] Foley, T. D.; Kintner, M. E. Brain PP2A is modified by thiol–disulfide exchange and intermolecular disulfide formation. *Biochem. Biophys. Res. Commun.* **330:**1224–1229; 2005.
[50] Foley, T. D.; Petro, L. A.; Stredny, C. M.; Coppa, T. M. Oxidative inhibition of protein phosphatase 2 A activity: role of catalytic subunit disulfides. *Neurochem. Res.* **32:**1957–1964; 2007.
[51] Frand, A. R.; Cuozzo, J. W.; Kaiser, C. A. Pathways for protein disulphide bond formation. *Trends Cell. Biol.* **10:**203–210; 2000.
[52] Park, B.; Lee, S.; Kim, E.; Cho, K.; Riddell, S. R.; Cho, S.; Ahn, K. Redox regulation facilitates optimal peptide selection by MHC class I during antigen processing. *Cell* **127:**369–382; 2006.
[53] Tavender, T. J.; Springate, J. J.; Bulleid, N. J. Recycling of peroxiredoxin IV provides a novel pathway for disulphide formation in the endoplasmic reticulum. *EMBO J.* **29:**4185–4197; 2010.
[54] Jarvis, L.A.; Toering, S.J.; Simon, M.A.; Krasnow, M.A.; Smith-Bolton, R.K. Sprouty proteins are in vivo targets of Corkscrew/SHP-2 tyrosine phosphatases. Development 133: 1133–1142; 2006.

Please cite this article as: Moreno, M.L; et al. Disulfide stress: a novel type of oxidative stress in acute pancreatitis. *Free Radic. Biol. Med.* (2014), http://dx.doi.org/10.1016/j.freeradbiomed.2014.01.009