UNIVERSITAT DE VALÈNCIA

FACULTAT DE FARMÀCIA

DEPARTAMENT DE FARMACOLOGIA

"ESTUDIO DE LA ACCIÓN PROINFLAMATORIA DE ANGIOTENSINA II Y SU POSIBLE MODULACIÓN. PAPEL DE ANGIOTENSINA II EN DISFUNCIONES ENDOTELIALES."

TESIS DOCTORAL

presentada por:

Ángeles Álvarez Ribelles

València, 2001

UNIVERSITAT DE VALÈNCIA
FACULTAT DE FARMÀCIA
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Dña. María Jesús SANZ FERRANDO, Profesora Titular de la Universitat de València y D. Juan Vicente ESPLUGUES MOTA, Catedrático de la Universitat de València.

HACEN CONSTAR:

Que el trabajo titulado "Estudio de la acción proinflamatoria de Angiotensina II y su posible modulación. Papel de Angiotensina II en disfunciones endoteliales", presentado por la Lda. **Ángeles Álvarez Ribelles** para obtener el grado de doctor, ha sido realizado en el Departamento de Farmacología de esta facultad, bajo nuestra dirección.

Concluido el trabajo experimental y bibliográfico, autorizamos la presentación de esta Tesis Doctoral, para que sea juzgada por el tribunal correspondiente.

Valencia, Diciembre de 2001

Fdo. Dra. María Jesús Sanz

Fdo. Dr. Juan Vicente Esplugues

A mi familia

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ABREVIATURAS UTILIZADAS

AA	Ácido araquidónico
AC	Adenilato ciclasa
ADN	Ácido desoxirribonucleico
AMPc	Adenosina 3': 5'-monofosfato cíclico
Ang-II	Angiotensina II
ARNm	Ácido ribonucleico mensajero
ATP	Adenosina trifosfato
CMP 48/80	Compuesto 48/80
COX	Ciclooxigenasa
$cPLA_2$	Fosfolipasa A_2 citosólica
CSFs	"Colony-stimulating factor"
DAG	Diacilglicerol
DHR-123	Dihidrorrodamina-123
ECA	Enzima convertidora de la Angiotensina I
EETs	Ácidos epoxieicosatrienoicos
EGF	"Endotelial growth factor"
EPHETs	Ácidos epoxihidroxieicosatetraenoicos
ESL-1	"E-selectin ligand-1"
ET-1	Endotelina-1
f-MLP	Formil-metionil-leucil-fenilalanina
GlyCAM-1	"Glycosylation-dependent cell adhesion molecule 1"
GMPc	Guanosina 3': 5'-monofosfato cíclico
Gp 150, 95	Glicoproteína 150, 95
H_2O_2	Peróxido de hidrógeno
HETEs	Ácidos hidroxieicosatetraenoicos
HOCl	Ácido hipocloroso
HPETEs	Ácidos hidroperoxieicosatetraenoicos (12 y 5)
HUVECs	Células endoteliales de vena de cordón umbilical humano
ICAM-1	"Intercellular adhesion molecule-1"
ICAM-2	"Intercellular adhesion molecule-2"
IECA	Inhibidores de la enzima convertidora de la Angiotensina I
IFNs	Interferones
ILs	Interleucinas (1 y 8)
IP_3	Inosina trifosfato
LDL	Lipoproteínas de baja densidad
LFA-1	"Lymphocyte-function-associated-1"
LOs	Lipooxigenasas (12 y 5)
LPS	Lipopolisacárido bacteriano
LTs	Leucotrienos (A_4, B_4, C_4, D_4 y E_4)
LXs	Lipoxinas
MadCAM-1	"Mucosal addressin cell adhesion molecule-1"
MCP-1	"Monocyte chemoattractant protein-1"
NAC	N-acetilcisteína
NO	Óxido nítrico
NOS	NO sintasa
O_2^-	Anión superóxido

O_2	Oxígeno molecular
O_3	Ozono
PAF	Factor activador de plaquetas
PDEs	Fosfodiesterasas
PECAM-1	"Platelet endothelial cell adhesion molecule-1"
PGI_2	Prostaciclina
PGs	Prostaglandinas (E_2,G_2 y H_2)
PKA	Proteincinasa A
PLA_2	Fosfolipasa A_2
PLC	Fosfolipasa C
PMNs	Polimorfonucleares
PSGL-1	"P-selectin glicoprotein ligand-1"
R-123	Rodamina-123
RLO	Radical libre oxigenado
SLe^a	Sialil Lewis ^{a}
SLe^X	Sialil Lewis ^{X}
SOD	Superóxido dismutasa
THETs	Ácidos trihidroxieicosatetraenoicos
TNF	Factor de necrosis tumoral (α y β)
TXs	Tromboxanos (A_2)
VCAM-1	"Vascular cell adhesion molecule-1"
VLA-4	"Very-late-antigen-4"
Vmean	Flujo sanguíneo medio
Vrbc	Velocidad de los glóbulos rojos

ÍNDICE

I. INTRODUCCIÓN

El proceso inflamatorio asociado a diversas patologías tales como aterosclerosis, infarto de miocardio o procesos de isquemia-reperfusión se caracteriza por una acumulación excesiva de células leucocitarias. Son principalmente las diversas sustancias liberadas por estas células como diversos agentes citotóxicos entre los que podemos encontrar radicales libres oxigenados (RLO), enzimas proteolíticos o proteínas catiónicas, cuando se encuentran en estado de activación, las principales responsables del daño tisular observado en estos procesos patológicos.

En este sentido, uno de los primeros estadíos del proceso inflamatorio lo constituye la interacción, de manera concertada, entre las moléculas de adhesión presentes en el endotelio vascular y sus correspondientes ligandos existentes en la célula leucocitaria implicada inducida por diferentes estímulos y que da lugar a la extravasación de leucocitos desde el torrente circulatorio hasta el foco inflamado. Por consiguiente, uno de los primeros niveles de actuación en el control de la respuesta inflamatoria lo constituiría la búsqueda de nuevos fármacos antiinflamatorios que actúen bloqueando las interacciones leucocito-endotelio y, por tanto, que impidan este primer paso del proceso inflamatorio.

Angiotensina II (Ang-II) es el principal mediador peptídico del sistema renina-angiotensina y, además de su potente acción vasoconstrictora y homeostática que regula la presión arterial, también puede contribuir a la activación de monocitos y de leucocitos polimorfonucleares (PMNs). Este hecho es de gran interés ya que los estados hipertensivos están asociados con la migración de monocitos a través de la pared vascular, estadio crítico en el desarrollo de la lesión aterosclerótica, la cual se ha demostrado que puede ser atenuada mediante el empleo de inhibidores de la enzima convertidora de Angiotensina I (IECA). Además, también hay evidencias de que Ang-II puede liberar factores quimiotácticos principalmente para monocitos y linfocitos y posiblemente para neutrófilos.

Por tanto, Ang-II podría ser el estímulo clave en la infiltración leucocitaria que ocurre a nivel sub-endotelial que acontece en hipertensión, aterosclerosis e infarto de miocardio.

Por tanto, el primer objetivo de esta tesis doctoral lo constituyó el establecer y caracterizar el efecto proinflamatorio de Ang-II en la microcirculación mesentérica de rata mediante el empleo de la microscopía intravital. Así, se demostró que Ang-II a dosis sub-vasoconstrictoras y que no producen alteraciones de la presión arterial sistémica, induce un significativo aumento del "rolling", adhesión y migración leucocitaria de forma dosis-dependiente. Posteriormente se procedió a determinar en procesos agudos y subagudos *in vivo* tanto los mecanismos por los cuales Ang-II induce infiltración leucocitaria como los mediadores implicados. En concreto, esclarecer si el efecto de Ang-II está mediado a través de su interacción con sus receptores específicos, identificar los factores quimiotácticos liberados, moléculas de adhesión implicadas, generación de radicales libres en estas respuestas y la posible participación de los mastocitos en estos procesos.

Este planteamiento ha dado lugar a las siguientes publicaciones:

<u>Artículo número 1:</u>

"Angiotensin II induces leukocyte-endothelial cell interactions in vivo via AT_1 and AT_2 receptor-mediated P-selectin upregulation" Piqueras L., Kubes P., Álvarez A., O'Connor E., Issekutz A.C., Esplugues J.V., Sanz M.J. Circulation (2000) **102**: 2118-2123.

Artículo número 2:

"Reactive oxygen species mediate angiotensin II-induced leukocyte-endothelial cell interactions *in vivo*" Álvarez A., Sanz M.J. Journal of Leukocyte Biology (2001) **70**: 199-206.

Artículo número 3:

"Molecular mechanisms underlying angiotensin-II induced arteriolar and venular leukocyte adhesion in vivo. A critical role for angiotensin-II in atherogenesis?" Álvarez A., Issekutz A.C., Panés J., Lobb R.R., Sanz M.J. Journal of Experimental Medicine (2002) Enviado a publicar.

Conocidos los mecanismos por los que Ang-II induce interacciones leucocito-endotelio, el segundo objetivo de esta tesis doctoral fue dirigido hacia el estudio de la modulación de la infiltración leucocitaria inducida por este péptido mediante el empleo de agentes antiinflamatorios con el fin de desarrollar nuevas estrategias terapéuticas. Así, y debido a que Ang-II induce estas interacciones a través de la generación de radicales libres se pretendió averiguar si podían ser moduladas mediante la administración de diversos agentes antioxidantes. Seguidamente y debido a que agentes que elevan los niveles intracelulares de Adenosina 3': 5'-monofosfato cíclico (AMPc) son moduladores de la acumulación leucocitaria *in vivo*, se evaluó si las interacciones leucocito-endotelio inducidas por Ang-II podían ser disminuidas o inhibidas por agentes capaces de aumentar los niveles de este nucleótido cíclico mediante el empleo tanto de fármacos que están en el mercado para el tratamiento de otras patologías como agentes en fase de experimentación. Además debido a las propiedades antiadhesivas del óxido nítrico (NO) y a que agentes que elevan los niveles de Guanosina 3': 5'-monofosfato cíclico (GMPc) también son

moduladores de la respuesta inflamatoria *in vivo* se evaluaron los efectos de varios fármacos que o bien liberan NO o bien activan directamente la guanilato ciclasa. Finalmente, y debido a que tanto las mujeres postmenopaúsicas como los hombres presentan mayor riesgo que las mujeres premenopaúsicas de desarrollo de patologías cardiovasculares, creímos de interés estudiar el efecto de la administración exógena de estrógenos.

Los resultados de estos estudios están recogidos en las siguientes publicaciones:

Artículo número 2:

"Reactive oxygen species mediate angiotensin II-induced leukocyte-endothelial cell interactions *in vivo*" Álvarez A., Sanz M.J. Journal of Leukocyte Biology (2001) **70**: 199-206.

Artículo número 4:

"Cyclic AMP elevating agents and nitric oxide modulate angiotensin II-induced leukocyte-endothelial cell interactions *in vivo*" Álvarez A., Piqueras P., Blázquez M.A., Sanz M.J. British Journal of Pharmacology (2001) **133**: 485-494.

Artículo número 5:

"Estrogens inhibit angiotensin II-induced leukocyte-endothelial cell interactions *in vivo* via rapid endothelial nitric oxide synthase and cyclooxygenase activation" Álvarez A., Issekutz A.C., Esplugues J.V., Sanz M.J. Blood (2002) Enviado a publicar.

Finalmente, en una tercera parte pretendimos caracterizar el papel que ejerce Ang-II endógena en las interacciones leucocito-endotelio inducidas por disfunciones endoteliales mediante el bloqueo de los receptores AT_1 de la misma tras la administración de un antagonista específico como losartan. Las disfunciones endoteliales estudiadas fueron la inhibición de la NO sintasa (NOS), la inhibición de la ciclooxigenasa (COX) y la falta de estrógenos en modelos agudos. La pérdida de NO endotelial está asociada con procesos de isquemia-reperfusión, shock traumático e hipertensión; paralelamente niveles bajos de prostaciclina (PGI_2) se han asociado a la enfermedad isquémica cardíaca y la lesión aterosclerótica; y finalmente la disminución de los niveles de estrógenos circulantes que ocurre en la menopausia está asociada con el desarrollo de diversas patologías cardiovasculares. Con todo ello, pretendimos investigar las interacciones leucocito-endotelio que ocurren como consecuencia de estos procesos y, en particular, esclarecer si Ang-II pudiera jugar un papel en cada una de ellas.

La caracterización del papel de Ang-II endógena sobre la infiltración leucocitaria inducida por diversas disfunciones endoteliales ha dado lugar a las publicaciones:

Artículo número 6:

"Angiotensin II is involved in nitric oxide synthase and cyclo-oxygenase inhibition-induced leukocyte-endothelial cell interactions *in vivo*" Álvarez A., Piqueras L., Bello R., Canet A., Moreno L., Kubes P., Sanz M.J. British Journal of Pharmacology (2001) **132**: 677-684.

Artículo número 5:

"Estrogens inhibit angiotensin II-induced leukocyte-endothelial cell interactions *in vivo* via rapid endothelial nitric oxide synthase and cyclooxygenase activation" Álvarez A., Issekutz A.C., Esplugues J.V., Sanz M.J. Blood (2002) Enviado a publicar.

Artículo número 3:

"Molecular mechanisms underlying angiotensin-II induced arteriolar and venular leukocyte adhesion in vivo. A critical role for angiotensin-II in atherogenesis?" Álvarez A., Issekutz A.C., Panés J., Lobb R.R., Sanz M.J. Journal of Experimental Medicine (2002) Enviado a publicar.

II. ARTÍCULOS DE INVESTIGACIÓN

II.1 ARTÍCULO 1

Angiotensin II induces leukocyte-endothelial cell interactions in vivo via AT_1 and AT_2 receptor-mediated P-selectin upregulation

Piqueras L, Kubes P, Álvarez A, O'Connor E,

Issekutz AC, Esplugues JV, Sanz MJ.

Circulation (2000) **102:** 2118-2123

Angiotensin II Induces Leukocyte–Endothelial Cell Interactions In Vivo Via AT_1 and AT_2 Receptor–Mediated P-Selectin Upregulation

Laura Piqueras, BPharm; Paul Kubes, PhD; Angeles Alvarez, BPharm; Enrique O'Connor, PhD; Andrew C. Issekutz, MD; Juan V. Esplugues, MD, PhD; Maria-Jesus Sanz, PhD

Background—Angiotensin II (Ang II) plays a critical role in the development of vascular lesions in hypertension, atherosclerosis, and several renal diseases. Because Ang II may contribute to the leukocyte recruitment associated with these pathological states, the aim of the present study was to assess the role of Ang II in leukocyte–endothelial cell interactions in vivo.

Methods and Results—Intravital microscopy of the rat mesenteric postcapillary venules was used. Sixty minutes of superfusion with 1 nmol/L Ang II induced a significant increase in leukocyte rolling flux (83.8±20.7 versus 16.4±3.1 cells/min), adhesion (11.4±1.0 versus 0.8±0.5 cells/100 μm), and emigration (4.0±0.7 versus 0.2±0.2 cells/field) without any vasoconstrictor activity. These effects were not mediated by mast cell activation. Intravenous pretreatment with AT_1 (losartan) or AT_2 (PD123,319) receptor antagonists significantly reduced Ang II–induced responses. A combination of both receptor antagonists inhibited the leukocyte rolling flux, adhesion, and extravasation elicited by Ang II at 60 minutes. Pretreatment of animals with fucoidin or an adhesion-blocking anti–rat P-selectin monoclonal antibody abolished Ang II–induced leukocyte responses. Furthermore, rat platelet P-selectin expression was not affected by Ang II stimulation.

Conclusions—Ang II induces significant leukocyte rolling, adhesion, and emigration, which may contribute not only to hypertension but also to the onset and progression of the vascular damage associated with disease states in which plasma levels of this peptide are elevated. **(*Circulation.* 2000;102:2118-2123.)**

Key Words: angiotensin ■ endothelium ■ leukocytes ■ cell adhesion molecules ■ glycoproteins

Angiotensin II (Ang II), the main effector peptide of the renin-angiotensin system, may contribute to the development of vascular lesions in hypertension, atherosclerosis, and many glomerular diseases of the kidney.[1–3] In addition to its role as a potent vasoconstrictor and regulator of blood pressure and fluid homeostasis, Ang II seems to be involved in the activation of monocytes and polymorphonuclear leukocytes (PMNs). Ang II receptors have also been demonstrated on monocytes.[4] This may be directly relevant, because hypertension is associated with migration of monocytes through the vessel wall, a critical event leading to the development of the atherosclerotic lesion that can be attenuated by ACE inhibition.[5,6] Furthermore, Ang II can promote monocyte adhesion and activation in vitro[7–9] and stimulates the expression of monocyte chemoattractant protein-1 (MCP-1) and RANTES in several animal models in vivo.[5,10,11] In addition, Ang II releases a neutrophil chemoattractant factor from cultures of arterial endothelial cells.[12]

Leukocytes migrate from the blood to sites of extravascular injury in response to locally produced stimuli that activate specific cell surface receptors. Initial capture of leukocytes is dependent on P-selectin expression.[13] Preliminary studies suggest that elevated levels of vasoconstrictors such as vasopressin or endothelin-1 (ET-1) contribute to leukocyte–endothelial cell interactions via P-selectin surface expression but do not provoke cellular migration.[14,15] Ang II is the dominant vasoconstrictor in many vascular diseases, and it might constitute a stimulus for the subendothelial infiltration of leukocytes associated with these pathological conditions. Therefore, in the present study, we investigated the capacity and profile of Ang II and the molecular mechanisms by which it elicits in vivo leukocyte–endothelial cell interactions within the rat mesenteric microcirculation.

Methods

Intravital Microscopy

The details of the experimental preparation have been described previously.[15] In short, Sprague-Dawley rats (200 to 250 g) were

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fasted for 24 hours and anesthetized with pentobarbital sodium (50 mg/kg IP), and the trachea, right carotid artery, and jugular vein were cannulated. After a midline abdominal incision, a segment of the midjejunal mesentery was exteriorized and placed over an optically clear viewing pedestal maintained at 37°C, which permitted tissue transillumination. The exposed mesentery was superfused continuously with a warmed bicarbonate-buffered saline (pH 7.4). An orthostatic microscope (Nikon Optiphot-2, SMZ1) equipped with an ×20 objective lens (Nikon SLDW) and an ×10 eyepiece allowed tissue visualization. A video camera (Sony SSC-C350P) mounted on the microscope projected the image onto a color monitor (Sony Trinitron PVM-14N2E), and the images were video recorded (Sony SVT-S3000P) for playback analysis (final magnification of the video screen was ×1300). Single unbranched mesenteric venules were selected, and the diameters (20 to 40 μm) were measured online with a video caliper (Microcirculation Research Institute, Texas A&M University). The number of rolling, adherent, and emigrated leukocytes was determined offline during playback of videotaped images. Centerline red blood cell velocity was also measured online with an optical Doppler velocimeter (Microcirculation Research Institute, Texas A&M University). Venular blood flow and wall shear rate were calculated as previously described.[15]

Experimental Protocol

Preparations were allowed to stabilize for 30 minutes before baseline (time 0) measurements of mean arterial blood pressure, red blood cell velocity, vessel diameter, shear rate, leukocyte rolling flux and velocity, and leukocyte adhesion and emigration were obtained. The superfusion buffer was then supplemented with Ang II (0.1 to 100 nmol/L), and subsequent recordings were performed for 5 minutes at 15-minute intervals over a 60-minute period. On the basis of these initial experiments, 1 nmol/L Ang II was used for the remainder of the experiments.

Involvement of mast cell activation was determined by pretreatment of a group of animals before the start of surgery with sodium cromoglycate (cromolyn, 20 mg/kg IV), a mast cell–stabilizing agent, 0.33 mg/mL of which was added to the superfusate containing Ang II, as previously described.[15]

To identify which Ang II receptor was implicated, selective antagonists of receptor subtype AT_1 (losartan, 10 mg/kg IV), subtype AT_2 (PD123,319, 10 mg/kg IV), or a combination of the 2 were administered 15 minutes before suffusion with Ang II 1 nmol/L. The doses of both antagonists were based on previous in vivo data.[16,17]

The adhesion molecules involved in these responses were determined by pretreatment of the animals 5 minutes before Ang II suffusion with fucoidin (25 mg/kg IV), a P- and L-selectin–binding carbohydrate, an adhesion-blocking monoclonal antibody (mAb) directed against rat P-selectin (RMP-1, IgG2a, 2.5 mg/kg IV), or the nonblocking anti–rat P-selectin mAb (RP-2, IgG1, 2.5 mg/kg IV).[15]

Flow Cytometry

All the analyses were performed with an EPICS XL-MCL Flow Cytometer (Coulter Electronics) as described previously.[15]

Determination of Surface Expression of CD11b/CD18 ($\alpha_M\beta_2$) Integrins and L-Selectin (CD62L) in Rat PMNs and Monocytes

Duplicated samples (100 μL) of rat citrated peripheral whole blood were incubated for 15 minutes at 25°C with vehicle, PAF (1000 nmol/L), or Ang II (10 to 10 000 nmol/L). Samples were then incubated in darkness and on ice for 20 minutes with saturating amounts (10 μL) of the corresponding FITC-conjugated mAb. Removal of red blood cells and fixation of leukocytes was performed through an automated lysing procedure with an EPICS Q-PREP system (Coulter Electronics).

Determination of Surface Expression of P-Selectin in Rat Platelets

Rat citrated peripheral whole blood with prostaglandin E_1 (PGE_1) (1 μmol/L) was diluted 1:10 in modified Tyrode's buffer, and the procedure followed was similar to that previously described.[15]

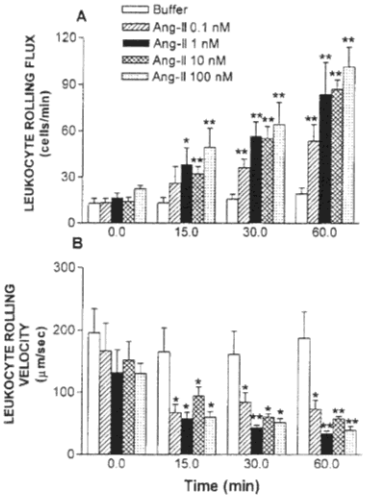

Figure 1. Dose-response and time-dependent effects of Ang II superfusion on leukocyte rolling flux (A) and rolling velocity (B) in rat mesenteric postcapillary venules. Animals were divided into 5 groups: buffer (n=6) or Ang II 0.1 nmol/L, n=4; 1 nmol/L, n=5; 10 nmol/L, n=4; and 100 nmol/L, n=5, and parameters were measured at 0, 15, 30, and 60 minutes. Results are mean±SEM. *$P<0.05$, **$P<0.01$ vs control value (0 minutes) for animals within each group.

Statistical Analysis

All data are expressed as mean±SEM. The data within groups were compared by a paired Student's *t* test. An unpaired Student's *t* test was used to compare groups. In both cases, the Student's *t* test was performed with a Bonferroni correction for multiple comparisons. A value of $P<0.05$ was considered statistically significant.

Materials

Ang II, Cromolyn, Thrombin, PGE_1, Mouse IgG_1, Goat Anti-Mouse IgG_1-FITC, and PD123,319 were purchased from Sigma Chemical Co. Losartan was kindly donated by Merck Sharp & Dohme. Antibodies RMP-1 and RP-2 were acquired as previously stated.[18] Conjugated mAb anti–rat-CD11b-FITC (OX-42) was purchased from Immunotech. Anti–rat L-selectin (HRL-3) and anti–hamster IgG FITC were supplied by LabClinics SA.

Results

The time- and concentration-dependent effects of Ang II on leukocyte rolling flux and rolling velocity are shown in Figure 1. Significant increases in leukocyte rolling and significant concomitant decreases in the leukocyte rolling velocity were observed at each stage for all concentrations with optimal changes at 1 nmol/L of Ang II (83.8±20.7 versus 16.4±3.1 cells/min and 37.3±3.5 versus 143.1±42.9 μm/s at 60 minutes, respectively). Similarly, Ang II induced a time- and concentration-dependent increase in leukocyte adhesion and emigration at 30 and 60 minutes, as shown in Figure 2 (11.4±1.0 versus 0.8±0.5 cells/100 μm and 4.0±0.7 versus 0.2±0.2 cells/field at 60 minutes, respectively, with the 1-nmol/L dose of Ang II).

Ang II 0.1 to 10 nmol/L induced no significant changes in arteriolar or venular diameter or mean arterial blood pressure (data not shown). However, 100 nmol/L Ang II, despite causing similar leukocyte responses, provoked a significant

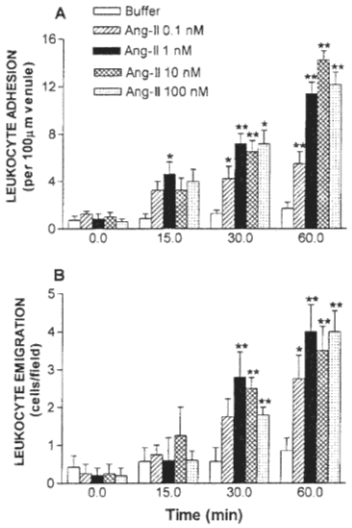

Figure 2. Dose-response and time-dependent effects of Ang II superfusion on leukocyte adhesion (A) and emigration (B) in rat mesenteric postcapillary venules. Parameters were determined at 0, 15, 30, and 60 minutes after superfusion with Ang II 0.1 nmol/L, n=4; 1 nmol/L, n=5; 10 nmol/L, n=4; or 100 nmol/L, n=5 or without Ang II (n=6). Results are mean±SEM. *$P<0.05$, **$P<0.01$ vs control value (0 minutes) for animals within each group.

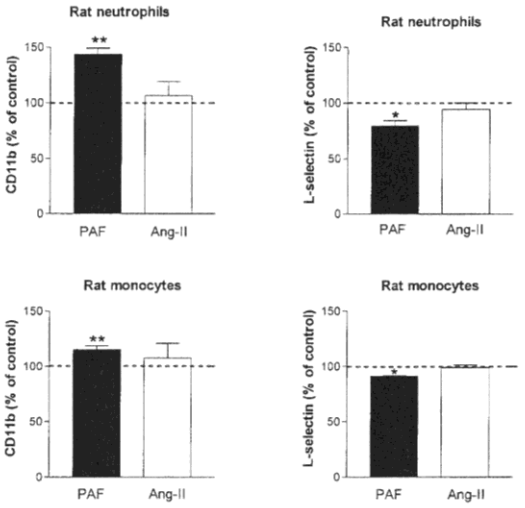

Figure 3. Effect of stimulation with PAF 1000 nmol/L or Ang II 1000 nmol/L on surface expression of CD11b/CD18 integrin and L-selectin on both rat neutrophils and monocytes. FITC fluorescence values are expressed as percentage of mean fluorescence intensities of control cells (dotted line). Data are mean±SEM of 4 experiments. *$P<0.05$, **$P<0.01$ vs absolute mean fluorescence intensity between control samples and agonist-treated samples.

vasoconstriction in both venular and arteriolar diameter, a significant decrease in shear rate, and the collapse of 40% of the vessels investigated. Therefore, a single dose of 1 nmol/L Ang II, 100-fold less than a dose that caused vasoconstriction, was selected to investigate further the mechanisms of Ang II–induced leukocyte–endothelial cell interactions.

Flow cytometry analysis showed that whereas PAF induced L-selectin shedding and increased expression of CD11b/CD18 integrins in both leukocyte subtypes, Ang II at 10 to 10 000 nmol/L had no effect on expression of either of these leukocyte surface adhesion molecules (Figure 3).

Stabilization of mast cells by cromolyn administration had no influence on Ang II–elicited effects (Figure 4). In contrast, as shown in Figure 5, both losartan and PD123,319 pretreatment significantly reduced leukocyte rolling flux, adhesion, and emigration after Ang II suffusion. However, neither antagonist completely inhibited these responses. This was obtained only after simultaneous administration of both receptor antagonists. Furthermore, fucoidin pretreatment abolished the leukocyte–endothelial cell interactions induced by Ang II (Figure 6). Although pretreatment with the P-selectin–blocking antibody RMP-1 inhibited the leukocyte rolling, adhesion, and emigration associated with Ang II superfusion, administration of a binding, nonblocking control antibody (RP-2) had little or no effect on the Ang II–induced responses (Figure 7). Finally, flow cytometry analysis on rat platelets revealed the lack of effect of Ang II on platelet P-selectin expression (Figure 8).

Discussion

Leukocyte accumulation in the vessel wall is a hallmark of early stages of atherosclerosis, acute myocardial infarction, and renal diseases of diverse causes in which Ang II seems to play a critical role.[2,10,19] In this study, we show that subvasoconstrictor and physiologically relevant doses of Ang II (0.1 to 1 nmol/L) induce a significant increase in leukocyte–endothelial cell interactions. This observation suggests that in various diseases, inappropriate leukocyte–endothelial cell interactions occur before hypertension and in response to elevated Ang II levels. Indeed, this is one of the most striking observations of this study, because it indicates that disruption of the vascular balance between vasodilators and vasoconstrictors may expose the vascular endothelium to the deleterious action of the latter. Ang II therefore could trigger the initial leukocyte recruitment that leads to the subsequent vascular damage observed in hypertension, atherosclerosis, and myocardial ischemia-reperfusion injury, constituting a prominent role for this molecule in the pathogenesis of these cardiovascular disease states.

Also significant is the fact that although Ang II elicits leukocyte adhesion and emigration, flow cytometry analysis revealed that it has no direct effect on leukocyte chemotaxis. Several explanations may account for the proadhesive effects observed. It is likely that Ang II activates the endothelium and induces leukocyte rolling, which leads to firm adhesion of leukocytes to the endothelium, resulting in their subsequent emigration. Indeed, we discovered that the initial leukocyte–endothelial cell interactions induced by Ang II are due to P-selectin expression on the endothelium. The firm adhesion could also be due to the release of endogenously generated chemotactic mediators elicited by Ang II. In this context, there is some evidence that Ang II can evoke leukocyte recruitment through increased expression and in-

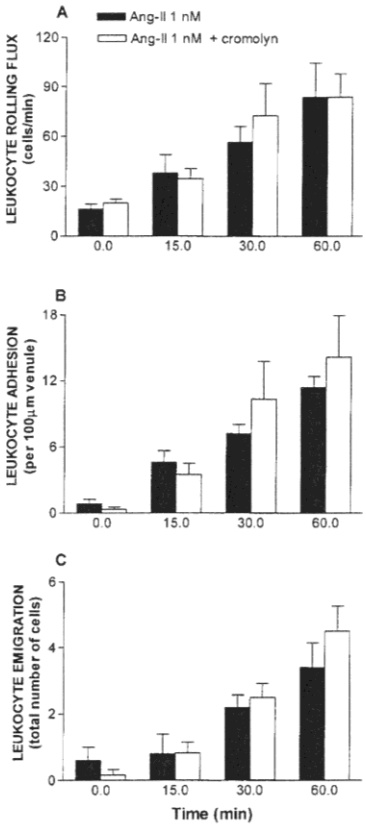

Figure 4. Effect of cromolyn treatment on Ang II–induced leukocyte rolling flux (A), adhesion (B), and emigration (C) in rat mesenteric postcapillary venules. Parameters were measured 0, 15, 30, and 60 minutes after superfusion with Ang II 1 nmol/L in animals untreated (n=5) or pretreated with cromolyn (n=6). Results are mean±SEM.

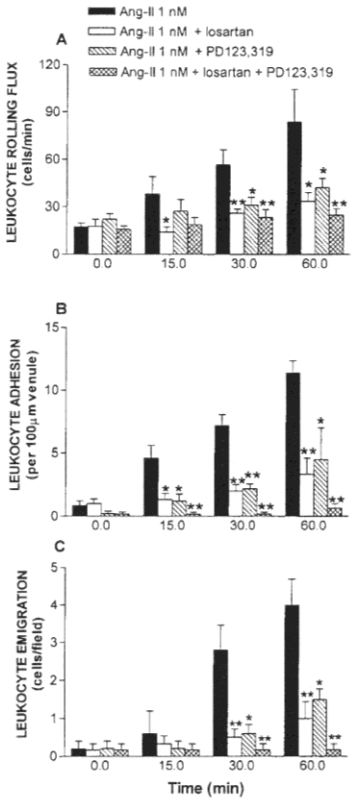

Figure 5. Effect of selective AT_1 (losartan) and AT_2 (PD123,319) receptor antagonists on Ang II–induced rolling flux (A), adhesion (B), and emigration (C) in rat mesenteric postcapillary venules. Parameters were measured 0, 15, 30, and 60 minutes after superfusion with Ang II 1 nmol/L in animals untreated (n=5) or pretreated with losartan (n=6), PD123,319 (n=5), or a combination of both antagonists (n=6). Results are mean±SEM. $^*P<0.05$, $^{**}P<0.01$ vs untreated group.

duction of chemotactic mediators such as IL-8, MCP-1, or RANTES.[5,10,12] In fact, IL-8 was recently shown to be costored with P-selectin in Weibel-Palade bodies.[20] In addition, Mangat et al[21] have showed a role for Ang II in cytosolic phospholipase A_2 activation, which is critical for the synthesis and release of potent chemotactic mediators such as PAF or leukotriene B_4. This is relevant because the release of these inflammatory mediators may constitute an amplifying mechanism for further leukocyte recruitment after Ang II stimulation.

We have also demonstrated the role of both Ang II receptor subtypes, AT_1 and AT_2, on Ang II–induced effects within the rat mesenteric microcirculation, because a combination of both receptor blockers returned all parameters to basal levels. Notably, the present findings are supported by in vitro data, albeit under static conditions, in which the involvement of both receptor subtypes in the adhesion of human monocytes to endothelial cells after incubation with Ang II has been demonstrated.[8] We have also discarded the possibility of a direct activation of mast cells on the release of mediators by Ang II and demonstrated a direct effect on the endothelium via endothelial P-selectin upregulation.

There is some evidence to suggest that Ang II induces adhesion molecule expression, but these findings are controversial. Some authors have found no role for E-selectin, vascular cell adhesion molecule-1 (VCAM-1), or intercellular cell adhesion molecule-1 (ICAM-1) in Ang II–induced monocyte adhesion to cultured endothelial cells.[8] Others have found that treatment with ACE inhibitors downregulates the expression of VCAM-1 and ICAM-1 in animal models.[6,22] Similarly, enalaprilat treatment in septic patients decreases the plasma levels of soluble adhesion molecules such as E-selectin or ICAM-1.[23] Furthermore, in an in vitro study, Gräfe et al[9] found a clear effect for Ang II in inducing E-selectin expression on coronary endothelial cells.

In this way, our data clearly demonstrate an implication of P-selectin in Ang II–mediated responses. First, pretreatment of animals with fucoidin totally inhibited the leukocyte responses elicited by Ang II. Second, administration of a blocking anti–rat P-selectin mAb (RMP-1) abolished all Ang

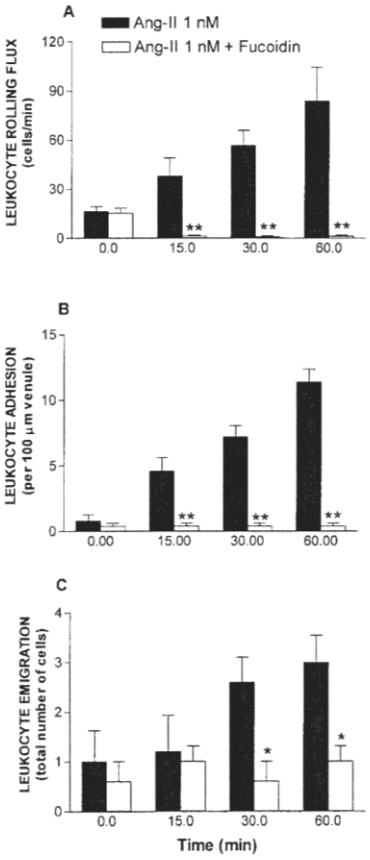

Figure 6. Effect of fucoidin pretreatment on Ang II–induced leukocyte rolling flux (A), adhesion (B), and emigration (C) in rat mesenteric postcapillary venules. Parameters were determined at 0, 15, 30, and 60 minutes after Ang II 1 nmol/L superfusion in animals untreated (n=5) or pretreated with fucoidin (n=5). Results are mean±SEM. *$P<0.05$, **$P<0.01$ vs untreated group.

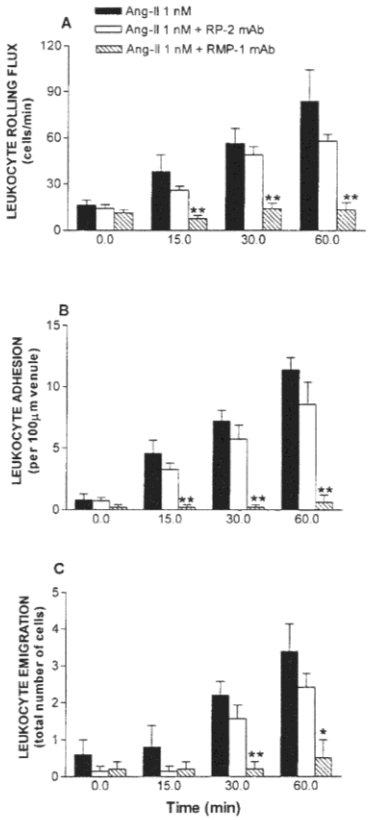

Figure 7. Effect of anti–P-selectin treatment on Ang II–induced leukocyte rolling flux (A), adhesion (B), and emigration (C) in rat mesenteric postcapillary venules. Animals were divided into 3 groups: untreated (n=5) and treated with a nonblocking (RP-2, n=5) or blocking (RMP-1, n=7) anti–P-selectin mAb. Ang II 1 nmol/L superfusion was begun 5 minutes later, and parameters were measured at 15, 30, and 60 minutes. Results are mean±SEM. *$P<0.05$, **$P<0.01$ vs untreated group.

II–induced leukocyte–endothelial cell interactions. Hence, we believe that responses induced by exogenous Ang II in our experiments are mediated through P-selectin upregulation on the endothelial cell surface. This conclusion is supported by the fact that no platelet-platelet or platelet–endothelial cell interactions were detected in this in vivo system and that flow cytometry analysis revealed no changes in rat platelet P-selectin expression after Ang II stimulation.

An explanation for all these findings is that at early stages of elevated plasma levels of Ang II, the leukocyte–endothelial cell interactions elicited by this peptide are mediated primarily through P-selectin upregulation. In chronic disorders, however, inducible adhesion molecules such as E-selectin are synthesized and may contribute to further leukocyte recruitment. Indeed, increased circulating levels of P-selectin and, to a lesser extent, E-selectin can be found in essential, renovascular, and malignant hypertension and in hypercholesterolemic patients.[24,25] Furthermore, it was recently shown that in a mouse model of atherosclerosis, there is a clear involvement of both P- and E-selectins in the development of the atherosclerotic lesion at both early and advanced stages.[26]

To summarize, we have demonstrated for the first time in vivo that Ang II elicits leukocyte–endothelial cell interactions within the rat mesenteric microcirculation at subvasoconstrictor doses. This effect is both AT_1 and AT_2 receptor–mediated and totally dependent on endothelial P-selectin expression. Thus, Ang II may play a critical role in the leukocyte attachment to and emigration through the vascular endothelium and could contribute to the vascular damage present in pathological conditions in which plasma levels of this peptide are elevated.

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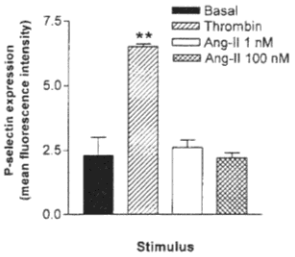

Figure 8. Effect of thrombin or Ang II stimulation on surface expression of P-selectin on rat platelets. Blood samples were incubated with vehicle, 1 U/mL thrombin, or 1 or 100 nmol/L Ang II. Anti–rat P-selectin mAb was then added, stained with FITC-conjugated mAb, and analyzed by flow cytometry. Values are expressed as mean fluorescence intensities. Data are mean±SEM of n=4 experiments. $^{**}P<0.01$ vs mean fluorescence intensity in control samples.

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II.2 ARTÍCULO 2

Reactive oxygen species mediate angiotensin II-induced leukocyte-endothelial cell interactions in vivo

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Reactive oxygen species mediate angiotensin II-induced leukocyte-endothelial cell interactions *in vivo*

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Abstract: Chronically elevated angiotensin II (Ang-II)-induced hypertension is partly mediated by superoxide production. In this study, we have investigated whether the leukocyte-endothelial cell interactions elicited by Ang-II involve reactive oxygen species (ROS) generation. Intravital microscopy within the rat mesenteric microvessels was used. Superfusion (60 min) with Ang-II (1 nM) induced significant increases in leukocyte rolling flux, adhesion, and emigration, which were inhibited by pretreatment with superoxide dismutase or catalase. Dihydrorhodamine-123 oxidation indicated that ROS are primarily produced by the vessel wall. Administration of dimethylthiourea, desferrioxamine, or *N*-acetylcisteine provoked significant reductions in Ang-II-induced leukocyte-endothelial cell interactions. In addition, a blockade of platelet-activating factor or leukotrienes also attenuated such responses significantly. The results presented indicate that *in vivo* Ang-II-induced leukocyte recruitment is dependent on the generation of intra- and extracellular ROS. Therefore, the use of anti-oxidants might constitute an alternative therapy for the control of the subendothelial leukocyte infiltration associated with hypertension and atherosclerosis. *J. Leukoc. Biol.* 70: 199–206; 2001.

Key Words: *superoxide · hydrogen peroxide · endothelium · intravital microscopy*

INTRODUCTION

The release of endothelial and leukocyte-derived reactive oxygen species (ROS) such as superoxide and hydrogen peroxide (H_2O_2) plays an active role in host-mediated destruction of foreign pathogens [1]. However, they have also been implicated as a cause of the vascular and tissue damage associated with acute inflammatory reactions [1, 2]. Indeed, there is evidence that implicates ROS in initiating and/or amplifying the inflammatory response through activation of leukocyte recruitment mechanisms. In this context, superoxide and H_2O_2 promote increases in endothelial-associated P-selectin expression, the production of platelet activating factor (PAF), and CD11/CD18-dependent leukocyte adhesion [3–5]. In addition, ROS production is involved in the pathogenesis of several cardiovascular disease states such as atherosclerosis, hypertension, and ischemia-reperfusion injury [6–8], and local leukocyte accumulation in the vessel wall constitutes a key stage in the onset and progression of such pathological conditions [8–11].

Clinical observations suggest a link between augmented renin-angiotensin system activity and the development of cardiac ischemic events [12, 13]. Angiotensin II (Ang-II), the main effector peptide in this system, has been shown to exert pro-inflammatory activity. With regard to this, angiotensin converting enzyme (ACE) inhibition has effectively been shown to reduce the number of infiltrating cells in different inflammatory conditions such as in glomerular diseases of the kidney, hypertension, and atherosclerosis [10, 14, 15]. Furthermore, we have revealed recently that Ang-II shows pro-inflammatory activity *in vivo* at sub-vasoconstrictor doses. In particular, it induces leukocyte trafficking into the rat mesenteric microvasculature through endothelial P-selectin up-regulation in the vessel wall, and this effect is mediated primarily via an Ang-II AT_1 receptor interaction [16].

Chronically elevated Ang-II-induced hypertension is mediated in part by superoxide production [7]. Moreover, in a rabbit model of early atherosclerosis, pretreatment with an AT_1 receptor antagonist normalized superoxide and endothelial function and reduced atherosclerotic lesion formation [17]. Xanthine oxidase is a prime source of endothelium-dependent superoxide production, and membrane-associated reduced nicotinamide adenine dinucleotide phosphate [NAD(P)H] oxidase is a major source of this oxygen radical in vascular smooth muscle cells (VSMCs) and myocytes [6, 18]. It has been demonstrated that vascular oxidase activity is increased by Ang-II, which can indeed augment NADH- and NADPH-driven superoxide production in cultured VSMCs and in aortic adventitial fibroblasts [6]. Furthermore, Ang-II-induced cellular hypertrophy in VSMCs is mediated by intracellular production of H_2O_2 through interaction with its subtype AT_1 receptor [6].

Therefore, in the present study, we aimed to investigate whether the leukocyte-endothelial cell interactions elicited by Ang-II are also mediated through ROS production. To test this hypothesis, we used intravital microscopy within the rat mesenteric microcirculation and examined the effect of superoxide dismutase (SOD) and catalase on Ang-II-mediated leukocyte

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responses. In addition, because anti-oxidants have the potential to limit ROS-induced cell recruitment *in vivo* and thereby regulate amplification of the inflammatory response, we have also explored the modulatory effect of different anti-oxidants and free-radical scavengers, which act at intracellular and extracellular levels on leukocyte responses induced by Ang-II. Finally, because ROS can activate phospholipase A_2 (PLA_2), we have studied the role of PAF and leukotrienes on leukocyte infiltration caused by Ang-II.

MATERIALS AND METHODS

Animal preparation

Male Sprague-Dawley rats (200–250 g) were fasted for 20–24 h prior to experiments with free access to water. The animals were anesthetized with sodium pentobarbital [Sigma Química, Madrid, Spain; 65 mg/kg, intraperitoneally (i.p.)]. A tracheotomy was performed to facilitate breathing, and the right jugular vein was cannulated for intravenous administration of drugs or additional anesthetic as required. The right carotid artery was cannulated to monitor systemic arterial blood pressure through a pressure transducer (Spectramed Stathan P-23XL) connected to a recorder (GRASS RPS7C8B, Quincy, MA).

Intravital microscopy

A mid-line, abdominal incision was made, and a segment of the mid-jejunal mesentery was exteriorized and carefully placed on an optically clear-viewing pedestal to allow transillumination of a 3-cm^2 segment of the mesenteric microvasculature. The temperature of the pedestal was maintained at 37°C. Animal temperature was monitored using a rectal electrothermometer and was maintained at the same temperature with an infrared heat lamp. The exposed intestine was superfused continuously with a bicarbonate buffer saline (BBS; pH 7.4, 2 ml/min, 37°C) and covered with a BBS-soaked gauze to prevent evaporation. Mesenteric microcirculation was observed through an orthostatic microscope (Nikon Optiphot-2, SMZ1, Badhoevedorp, The Netherlands) with a 20× objective lens (Nikon SLDW) and a 10× eyepiece as described previously [16]. A video camera (Sony SSC-C350P, Koeln, Germany) mounted on the microscope projected the image onto a color monitor (Sony Trinitron PVM-14N2E), and the images were captured on videotape (Sony SVT-S3000P) with superimposed time and date for subsequent playback analysis. The final magnification of the image on the monitor was 1300×.

Single, unbranched mesenteric venules with diameters ranging between 25 and 40 μm were studied. Venular diameter was measured on-line using a video caliper (Microcirculation Research Institute, Texas A&M University, College Station, TX). Center-line red blood cell velocity (V_{rbc}) was also measured on-line with an optical Doppler velocimeter (Microcirculation Research Institute, Texas A&M University). Venular blood flow was calculated from the product of mean V_{rbc} ($V_{mean}=V_{rbc}/1.6$) and microvascular cross-sectional area, assuming cylindrical geometry. Venular wall shear rate (γ) was calculated based on the Newtonian definition: $\gamma = 8 \times (V_{mean}/D_v)s^{-1}$, in which D_v is venular diameter [19].

The number of rolling, adherent, and emigrated leukocytes was determined off-line during playback analysis of videotaped images. Rolling leukocyte flux was determined by counting the number of rolling leukocytes passing a fixed reference point in the microvessel per min. The same reference point was used throughout the experiment, because leukocytes may roll for only a section of the vessel before rejoining the blood flow or becoming firmly adherent. Leukocyte rolling velocity (V_{wbc}) was determined by measuring the time required for a leukocyte to traverse a distance of 100 μm along the length of the venule and was expressed as μm/s. A leukocyte was considered to be adherent to venular endothelium if it remained stationary for a period equal to or exceeding 30 s. Adherent cells were expressed as the number per 100 μm length of venule. Leukocyte emigration was expressed as the number of white blood cells per microscopic field. The rate of emigration was determined from the difference between the number of any interstitial leukocytes present at the beginning of the experiment and the number of cells present in the interstitium at the end of the experiment.

Experimental protocol

After a 30-min stabilization period, baseline measurements (time 0) of mean arterial blood pressure (MABP), V_{rbc}, vessel diameter, shear rate, leukocyte rolling flux and V_{wbc}, and leukocyte adhesion and emigration were determined. The superfusion buffer was maintained or supplemented with Ang-II (Sigma Química, 1 nM) because previous studies by our laboratory have demonstrated that this dose causes the maximum and most consistent increase in leukocyte recruitment after 60-min superfusion [16]. Recordings were performed for 5 min at 15-min intervals over a 60-min period, and the aforementioned leukocyte and hemodynamic parameters were measured.

To determine the role of extracellular superoxide and H_2O_2 on Ang-II-induced leukocyte-endothelial cell interactions, animals were pretreated with SOD [Sigma Química, 8 mg/kg, intravenously (i.v.)] 5 min before the Ang-II suffusion or with catalase (Sigma Química, 5 mg/ kg, i.v.) 5 min before and 30 min after Ang-II 1-nM suffusion (to maintain adequate plasma levels) or with a combination of both enzymes. The doses of both anti-oxidants were based on those used in previous *in vivo* studies [4, 20].

To test the possible implication of intracellular ROS generation on Ang-II-induced leukocyte infiltration, one group of animals received the hydroxyl radical scavenger, dimethylthiourea (Sigma Química, 500 mg/kg, i.v.), 30 min before Ang-II superfusion. Another group of rats was pretreated with the iron-chelator, desferrioxamine mesylate (Sigma Química, 50 mg/kg, i.v.), 60 min prior to Ang-II suffusion. The doses used for the different treatments were those used by Mitchell *et al.* [20] and Suematsu *et al.* [21] in similar studies.

To examine the effect of *N*-acetylcysteine (NAC; Sigma Química), an anti-oxidant and ROS scavenger, on Ang-II-induced leukocyte responses, animals were pretreated with NAC (150 mg/kg, i.v.) 15 min before Ang-II suffusion. Schmidt *et al.* [22] have proved that this dose of NAC attenuated endotoxin-induced leukocyte adherence in rat mesenteric, postcapillary venules.

Finally, to determine the involvement of chemotactic mediators such as PAF and leukotrienes on leukocyte infiltration elicited by Ang-II, a PAF receptor antagonist (WEB2086) and a 5-lipoxygenase inhibitor (ICI 230,487) were used. Animals were pretreated with WEB2086 (10 mg/kg, i.v.), or the exposed mesentery was superfused continuously with ICI 230,487 (100 μM) 15 min before Ang-II suffusion. WEB2086 was a generous gift from Boehringer-Ingelheim (Germany), and ICI 230,487 was kindly donated by AstraZeneca (Macclesfield, U.K.). Similarly, all the doses administered were used as stated in previous *in vivo* studies [4, 23].

In vivo assessment of free-radical generation

To quantify the generation of oxidants by cells in the area under study, the oxidant-sensitive fluorochrome dihydrorhodamine (DHR)-123 (Molecular Probes, Eugene, OR; 10 μM) was superfused onto the mesentery as previously described [24].

During an initial, 30-min stabilization period, the mesenteric preparation was superfused with DHR-free BBS, and a background auto-fluorescence image was recorded. The preparation was then superfused with the working solution for 15 min and finally rinsed with DHR-123-free BBS to eliminate the precursor dye from the preparation. Fluorescence intensity (excitation wavelength, 500 nm; emission wavelength, 536 nm) was detected just before (baseline value) and following administration of BBS or Ang-II 1 nM using a charge-coupled device camera model XC-77 (Hamamatsu Photonics, Hamamatsu-City, Japan) with a C2400-68 intensifier head (Hamamatsu Photonics) and a C240-60 charge-coupled device camera control unit. Fluorescence intensity of the venule under investigation and background fluorescence were measured using an image analyzer program (analySIS 2.11, analysis DOCU). An index of free-radical generation within the venule was obtained after subtracting background fluorescence from the fluorescence intensity in the area of interest.

Statistical analysis

All data are expressed as mean ± SE. The data within groups were compared using an analysis of variance (one-way ANOVA) with a Newman-Keuls *post hoc* correction for multiple comparisons. Statistical significance was set at $^*P < 0.05$.

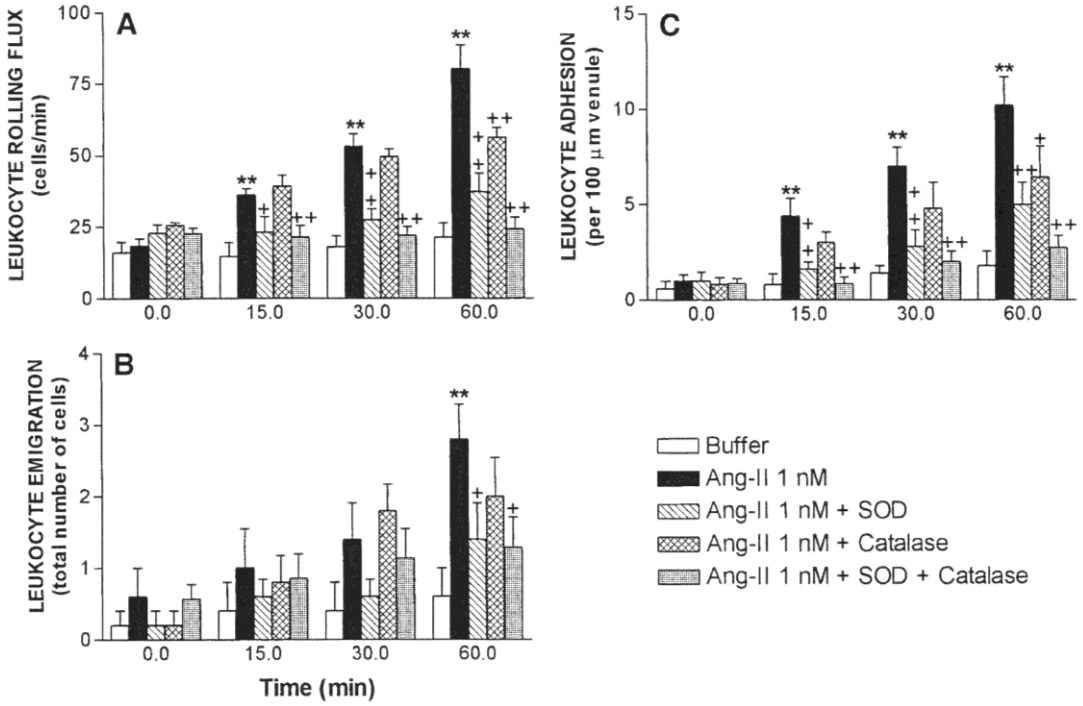

Fig. 1. Effect of the extracellular anti-oxidants superoxide dismutase and catalase on Ang-II-induced leukocyte rolling flux (A), leukocyte adhesion (B), and leukocyte emigration (C) in the rat mesenteric postcapillary venules. Parameters were measured 0, 15, 30, and 60 min after superfusion with buffer ($n=5$), with Ang-II (1 nM) in animals untreated ($n=5$) or pretreated with SOD (8 mg/kg, i.v., $n=5$), with catalase (5 mg/kg, i.v., $n=5$), or with a combination of both. Results are represented as mean ± SE. $^{**}P < 0.01$ relative to buffer group. $^{+}P < 0.05$ or $^{++}P < 0.01$ relative to the Ang-II-untreated group.

RESULTS

Ang-II promoted significant increases in leukocyte rolling flux (80.0±8.5 vs. 18.4±2.6 cells/min), adhesion (10.2±1.5 vs. 1.0±0.3 cells/100 μm), and emigration (2.8±0.5 vs. 0.6±0.4 cells/field) after 60-min superfusion compared with basal values (**Fig. 1**). Buffer superfusion did not cause significant changes in the leukocyte or the hemodynamic parameters measured during the whole experimental protocol (Fig. 1 and **Table 1**). These results are consistent with previous studies from our laboratory [16]. In addition, circulating leukocyte counts were unaltered after 60-min mesenteric superfusion with Ang-II ($82.5\pm7.8\times10^5$ cells/ml at time 0 vs. $86.5\pm9.8\times10^5$ cells/ml after 60-min superfusion). SOD pretreatment significantly reduced Ang-II induced leukocyte rolling flux, adhesion, and emigration by 79.6%, 56.5%, and 45.5%, respectively, after 60-min Ang-II suffusion (Fig. 1). When catalase was administered, leukocyte rolling flux and adhesion caused by 60-min Ang-II suffusion were attenuated significantly by 50.3% and 37.1%, respectively, but this pretreatment did not alter Ang-II-induced emigration significantly (18.1%). The co-administration of both anti-oxidants almost completely inhibited leukocyte-endothelial cell interactions elicited by Ang-II (Fig. 1). In addition, although the decrease in V_{wbc} induced by Ang-II was not reversed by pretreatment with either of these enzymes when administered separately, co-administration of the two returned V_{wbc} to basal levels (**Table 2**).

TABLE 1. Leukocyte Rolling Velocity (μm/s) and Hemodynamic Parameters before (0 min) and after (60 min) Superfusion with Buffer or Ang-II (1 nM)

	Leukocyte rolling velocity		MABP (mm Hg)		Shear rate (s^{-1})	
	0 min	60 min	0 min	60 min	0 min	60 min
Buffer	126.6 ± 10.5	114.4 ± 11.8	127.1 ± 10.2	127.1 ± 6.0	564.2 ± 48.9	527.3 ± 28.0
Ang-II	100.6 ± 9.7	41.4 ± 4.5**	102.7 ± 7.2	96.3 ± 7.4	641.2 ± 114.5	577.3 ± 91.8

** $P < 0.01$ relative to baseline value (0 min). All values are mean ± SE.

TABLE 2. Leukocyte Rolling Velocity (μm/s) in Untreated and Treated Animals before (0 min) and after (60 min) Ang-II Superfusion (1 nM)

Treatment	0 min	60 min
Untreated animals	100.6 ± 9.7	41.4 ± 4.5**
SOD	133.8 ± 12.0	83.9 ± 5.9*
Catalase	112.3 ± 4.3	58.4 ± 5.1**
SOD + catalase	122.4 ± 11.1	111.4 ± 8.2
Dimethylthiourea	106.3 ± 8.0	70.8 ± 11.7
Desferrioxamine	128.5 ± 13.7	88.0 ± 9.4
NAC	121.0 ± 8.1	119.8 ± 18.1
WEB2086	110.9 ± 10.5	105.5 ± 12.3
ICI 230,487	100.5 ± 5.0	115.1 ± 9.1

* $P < 0.05$ or ** $P < 0.01$ relative to the control group (0 min). All values are mean ± SE.

Figure 2 illustrates the changes in fluorescence intensity of the oxidant-sensitive fluorescent probe DHR-123 during superfusion of the mesentery with BBS or Ang-II 1 nM. Superfusion of the mesentery with BBS was not associated with significant DHR-123 oxidation within the venule under study (Fig. 2). In contrast, superfusion of the mesentery with Ang-II elicited a significant DHR-123 oxidation within the venule with respect to its control value and the group superfused with BBS during the first 30 min of superfusion. The increase in vascular fluorescence correlated with that of leukocyte accumulation detected in the postcapillary venule at this stage.

Figure 3 presents the effects of the intracellular anti-oxidant and hydroxyl radical scavenger, dimethylthiourea, and the iron-chelator, desferrioxamine, on Ang-II-induced leukocyte-endothelial cell interactions. Both pretreatments attenuated the Ang-II-induced leukocyte rolling flux, adhesion, and

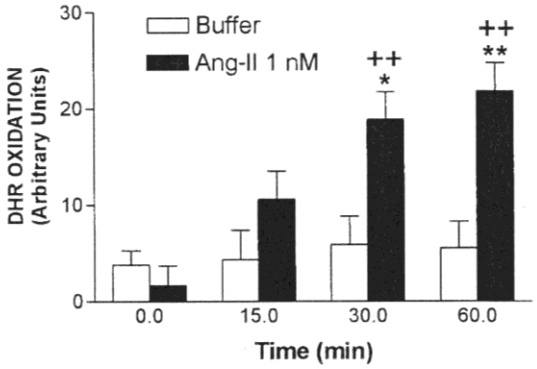

Fig. 2. Free-radical generation in response to BBS or Ang-II superfusion within the rat mesenteric postcapillary venules. Fifteen min after superfusion of DHR-123 alone, baseline DHR oxidation (0 min) was determined. Animals were divided into two groups: The BBS-superfusion was continued ($n=4$) or supplemented with Ang-II 1 nM ($n=4$), and the DHR oxidation was measured as total vessel rhodamine fluorescence intensity at 15, 30, and 60 min. Results are represented as mean ± SE. *$P < 0.05$ or **$P < 0.01$ relative to the control value (0 min) in the Ang-II-superfused group. $^{++}P < 0.01$ relative to the BBS-superfused group.

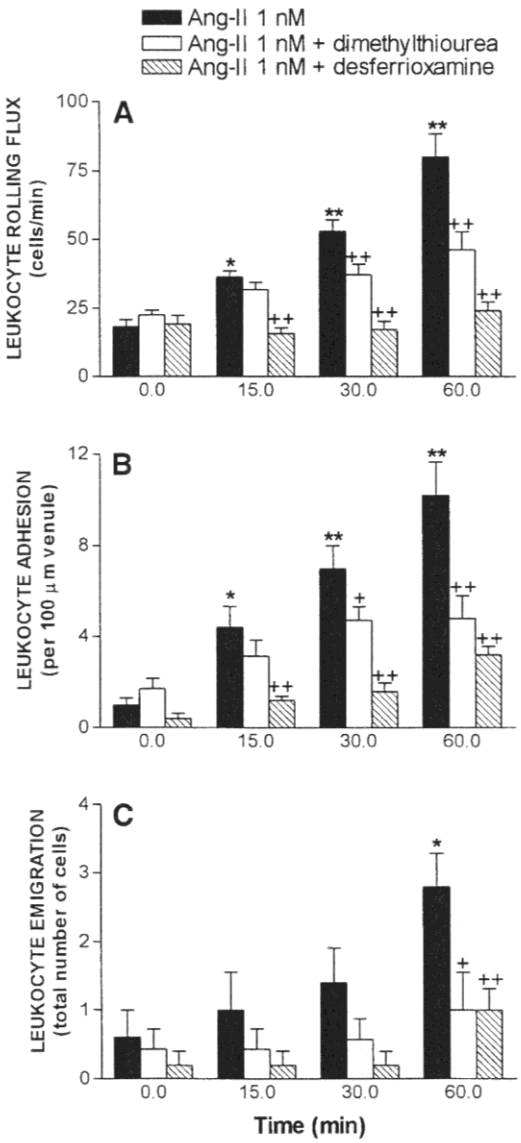

Fig. 3. Effect of the intracellular anti-oxidant, dimethylthiourea, and the iron-chelator, desferrioxamine, on Ang-II-induced leukocyte rolling flux (A), leukocyte adhesion (B), and leukocyte emigration (C) in the rat mesenteric postcapillary venules. Parameters were measured 0, 15, 30, and 60 min after Ang-II (1 nM) superfusion in animals untreated ($n=5$) or pretreated with dimethylthiourea (500 mg/kg, i.v., $n=7$) or with desferrioxamine (50 mg/kg, i.v., $n=5$). Results are represented as mean ± SE. *$P < 0.05$ or **$P < 0.01$ relative to the control value (0 min) in the untreated group. $^{+}P < 0.05$ or $^{++}P < 0.01$ relative to the untreated group.

emigration, and these parameters were inhibited by 62.7%, 69.6%, and 81.8%, respectively, in animals pretreated with dimethylthiourea and by 90.6%, 69.6%, and 63.6%, respectively, in the desferrioxamine-treated group after 60-min

Ang-II suffusion. In addition, dimethylthiourea and desferrioxamine were capable of reversing the decrease in V_{wbc} provoked by Ang-II (Table 2).

Figure 4 shows the effect of NAC on leukocyte-endothelial cell interactions caused by Ang-II. Treatment with NAC induced a significant inhibition of leukocyte rolling flux (78.9%), adhesion (91.3%), and emigration (81.8%) after 60-min Ang-II superfusion. In fact, NAC returned leukocyte adhesion and emigration to levels detected in basal conditions. In addition, treatment with NAC maintained V_{wbc} at baseline values (Table 2).

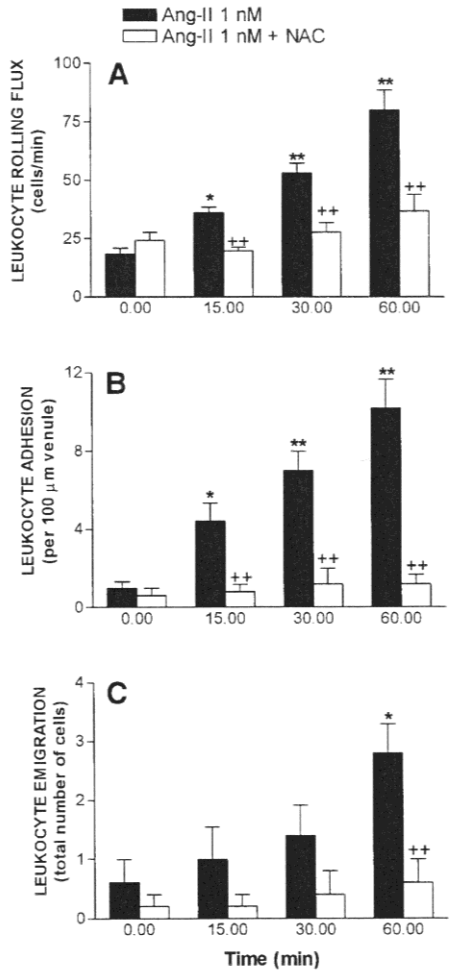

Fig. 4. Effect of the anti-oxidant and free-radical scavenger NAC on Ang-II-induced leukocyte rolling flux (A), leukocyte adhesion (B), and leukocyte emigration (C) in the rat mesenteric postcapillary venules. Parameters were measured 0, 15, 30, and 60 min after superfusion with Ang-II (1 nM) in animals untreated ($n=5$) or pretreated with NAC (150 mg/kg, i.v., $n=5$). Results are represented as mean ± SE. $^{*}P < 0.05$ or $^{**}P < 0.01$ relative to the control value (0 min) in the untreated group. $^{++}P < 0.01$ relative to the untreated group.

The effect of blocking PAF or leukotriene actions on leukocyte recruitment induced by Ang-II is shown in **Figure 5**. Treatment with the PAF receptor antagonist (WEB2086) effectively blocked leukocyte rolling flux, adhesion, and emigration elicited by Ang-II by 68.8%, 83.7%, and 43.2%, respectively, after 60-min Ang-II superfusion. Similarly, treatment of the mesentery with the 5-lipoxygenase inhibitor (ICI 230,487) significantly diminished the effects induced by 60-min Ang-II co-superfusion. This treatment inhibited leukocyte rolling flux, adhesion, and emigration by 68.5%, 95.7%, and 54.6%, respectively, at this time point. WEB2086 and ICI 230,487 also prevented the decrease in V_{wbc} caused by Ang-II superfusion (Table 2).

Finally, **Table 3** summarizes the results obtained for MABP and shear rate prior to (0 min) and 60 min after Ang-II superfusion in untreated animals and in animals subjected to different treatments. MABP or shear rate remained unaffected throughout the entire experimental period during Ang-II suffusion in untreated and treated animals.

DISCUSSION

Hypertension caused by chronically elevated Ang-II is mediated in part by superoxide generation [7]. In the present study, we have extended these findings and demonstrated that free-radical generation is also involved in Ang-II-induced leukocyte-endothelial cell interactions within the rat mesenteric postcapillary venules. Superoxide and H_2O_2 participate in these responses, because SOD and catalase can attenuate leukocyte rolling flux, adhesion, and emigration elicited by Ang-II. In addition, co-administration of both enzymes nearly abolishes the leukocyte rolling and leukocyte adhesion associated with Ang-II superfusion. In this context, it has been demonstrated clearly that the activity of the vascular oxidase is increased by Ang-II, and *in vitro* studies have shown that in VSMCs, superoxide anion and H_2O_2 production can be induced by Ang-II [6]. As previously stated, we have recently shown that Ang-II provokes leukocyte-endothelial cell interactions *in vivo* through endothelial P-selectin up-regulation [16]. In addition, different *in vivo* studies—in which the use of superoxide produced via an hypoxanthine-xanthine oxidase-generating system or H_2O_2—have been shown to induce leukocyte influx in the same microvascular bed and have proven that they occurred through increased P-selectin expression [4, 5]. Therefore, our data suggest that interaction of Ang-II with its AT_1 or AT_2 receptor subtypes results in the release of reactive oxygen metabolites, which cause the subsequent increase in P-selectin expression that in turn leads to the leukocyte recruitment observed.

To further investigate direct, free-radical formation on Ang-II stimulation in our system, we used the oxidant-sensitive fluorescent probe DHR-123, which specifically converts to a fluorescent (rhodamine) form following reaction with ROS [25] and has been successfully used in monitoring *in vivo* free-radical generation in the rat mesentery [26, 27]. In our study, the temporary responses of DHR oxidation in mesenteric tissue exposed to Ang-II suggest that there is an early rise in DHR oxidation that occurs within the first 30 min of Ang-II superfusion and is localized within the vessel. This is in agreement

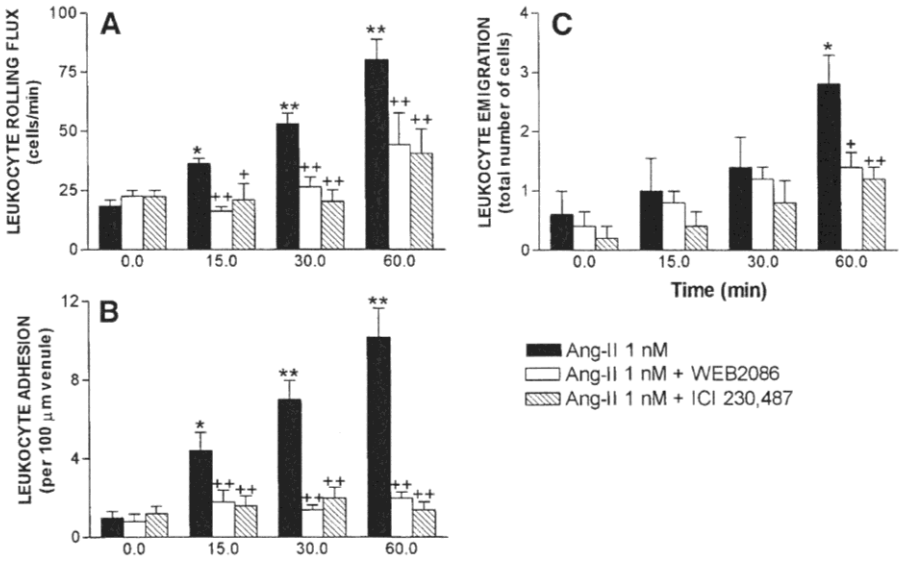

Fig. 5. Effect of the PAF receptor antagonist WEB2086 and the specific 5-lipoxygenase inhibitor ICI 230,487 on Ang-II-induced leukocyte rolling flux (A), leukocyte adhesion (B), and leukocyte emigration (C) in the rat mesenteric postcapillary venules. Parameters were measured 0, 15, 30, and 60 min after superfusion with Ang-II (1 nM) in animals untreated ($n=5$) or pretreated with WEB2086 (10 mg/kg, i.v., $n=5$) or in animals co-superfused with ICI 230,487 (100 μM, $n=5$). Results are represented as mean ± SE. $^{*}P < 0.05$ or $^{**}P < 0.01$ relative to the control value (0 min) in the untreated group. $^{+}P < 0.05$ or $^{++}P < 0.01$ relative to the untreated group.

with previous *in vitro* findings that encountered superoxide production by NADH and NADPH from cultured VSMCs after Ang-II stimulation [6]. In addition, it has been shown that lipoxygenase metabolites of arachidonic acid mediate Ang-II stimulation of the NAD(P)H oxidase in VSMCs [28], and we have demonstrated in this study that inhibition of lipoxygenase provokes a significant reduction in the leukocyte-endothelial cell interactions caused by Ang-II. Another possible source of superoxide-anion production is the endothelial cell, which can produce H_2O_2 via the mitochondrial electron transport chain, xanthine oxidase, and/or the biosynthesis of prostaglandins [21, 29]. However, because of the methodology of this study, it is difficult to see whether Ang-II acts on the endothelium, on the vascular smooth muscle, or on both, because receptors for this peptide hormone can be found in both cell types. Furthermore, the significant increase in DHR oxidation in the venule was detected at the same stage where the exacerbated leukocyte rolling and adhesion were encountered. Consequently, it seems that the vascular wall is the primary source of the Ang-II-induced oxygen free-radical generation, which leads to leukocyte recruitment through increased adhesion-molecule expression.

Superoxide can be dismutated spontaneously or by SOD to yield H_2O_2. H_2O_2 can be metabolized by catalase to form oxygen and water, or a Fenton reaction may occur in the presence of iron, resulting in the formation of hydroxyl radical. Thus, it is also relevant to this study that administration of the intracellular anti-oxidant and hydroxyl radical scavenger, di-

TABLE 3. Hemodynamic Parameters in Untreated and Treated Animals before (0 min) and after (60 min) Ang-II Superfusion (1 nM)

	MABP (mm Hg)		Shear rate (s^{-1})	
Treatment	0 min	60 min	0 min	60 min
Untreated animals	102.7 ± 7.2	96.3 ± 7.4	641.2 ± 114.5	577.3 ± 91.8
SOD	117.7 ± 7.8	109.7 ± 12.8	655.9 ± 16.6	586.0 ± 44.4
Catalase	106.7 ± 19.5	92.9 ± 11.6	662.0 ± 61.9	596.8 ± 54.6
SOD + catalase	118.1 ± 5.2	107.6 ± 5.0	677.6 ± 30.1	640.4 ± 45.0
Dimethylthiourea	96.4 ± 7.5	93.7 ± 9.3	640.3 ± 19.9	587.6 ± 53.0
Desferrioxamine	119.7 ± 5.7	110 ± 7.7	548.2 ± 56.0	594.8 ± 106.6
NAC	123.3 ± 4.2	131.1 ± 11.4	542.7 ± 83.4	556.6 ± 83.8
WEB2086	134.6 ± 5.7	141.3 ± 8.0	653.5 ± 28.1	588.9 ± 55.8
ICI 230,487	135.0 ± 10.2	136.7 ± 2.5	648.6 ± 28.7	659.6 ± 75.6

All values are mean ± SE.

methylthiourea, and inhibition of iron-catalyzed oxyradical formation by desferrioxamine pretreatment provoked significant reductions in leukocyte-endothelial cell interactions elicited by Ang-II. These results implicate intracellular secondarily derived oxygen radicals other than superoxide as important chemical mediators of the adhesive interactions observed in postcapillary venules exposed to Ang-II. Indeed, *in vitro* observations suggest that oxyradical propagation may involve ferritin-binding iron in endothelial cells, because iron-chelating reagents or free-radical scavengers can prevent the expression of adhesion molecules such as P-selectin [30]. It is conceivable that dimethylthiourea or desferrioxamine act on the endothelial cell. Consequently, the effects observed with these compounds suggest that they prevent the intracellular generation of oxygen radicals induced by Ang-II and cause the down-regulation of the increased endothelial P-selectin expression.

It is interesting that when NAC was administered, Ang-II-induced leukocyte rolling, adhesion, and emigration were nearly returned to basal levels. NAC acts as a free-radical scavenger and anti-oxidant [31]. In addition, the synthesis of cellular glutathione (GSH) can be sustained by NAC, serving as a precursor for GSH and thus replenishing the intracellular pool of cysteine [31]. Among all the compounds tested in this study, NAC exerted the most powerful inhibition of the leukocyte-endothelial cell interactions elicited by Ang-II, which is probably because of the vast array of different anti-inflammatory mechanisms that have been attributed to this molecule. In fact, its anti-adhesive properties *in vivo* have been clearly demonstrated in animal models of liver ischemia-reperfusion injury and endotoxemia [22, 32].

Finally, Ang-II-induced leukocyte-endothelial cell interactions were also ameliorated significantly by pretreatment with the PAF receptor antagonist, WEB2086, and by the 5-lipoxygenase inhibitor, ICI 230,487, in particular at the level of leukocyte adhesion and emigration. The results of these experiments support the view that PAF and leukotrienes mediate the leukocyte responses elicited by Ang-II and are consistent with the findings of Mangat *et al.* [33] that confirmed the role of Ang-II in cytosolic PLA_2 activation, which is critical to the synthesis and release of these potent chemotactic mediators. Conversely, oxidants can also peroxidize endothelial cell membranes to activate PLA_2. Indeed, superoxide and H_2O_2 induce the endothelium to synthesize PAF, and it has been proved that leukocyte-endothelial cell interactions induced by both reactive-oxygen metabolites can be inhibited by PAF receptor-antagonist pretreatment [4, 34]. Unexpectedly, in our study, pretreatment with WEB2086 was also capable of reducing Ang-II-induced leukocyte rolling flux. Although some authors could only find a role for PAF on leukocyte adhesion [35, 36], others have found that PAF superfusion causes significant increases on leukocyte rolling flux within 30 min of superfusion or that increases on the flux of rolling leukocytes induced by different mediators can be diminished significantly by PAF receptor-antagonist pretreatment [37–40]. Therefore, we suggest that in addition to the effect of PAF increasing CD11/CD18 integrin expression on leukocyte cell surface, it can also act on the endothelial cell-inducing, rapid P-selectin expression as demonstrated in previous studies [39, 40].

In conclusion, the present study indicates that *in vivo* leukocyte recruitment elicited by Ang-II is dependent on the generation of intra- and extracellular ROS. Our proposal is that upon Ang-II stimulation, superoxide and H_2O_2 are released quickly and cause endothelial P-selectin up-regulation, which leads to the observed increase in leukocyte rolling flux. In parallel, the ROS released can stimulate PLA_2, which in turn provokes the synthesis and release of PAF and leukotriene B_4 (LTB_4), explaining in part the subsequent leukocyte adhesion and emigration detected. Additionally, newly formed secondarily derived oxidants from lipoxygenase metabolism could be responsible for an amplification mechanism, which results in subsequent accumulation of adherent leukocytes and in turn contributes to the overall response to Ang-II. This is relevant, because in hypertensive states with elevated Ang-II profiles, the release of ROS by Ang-II can also participate in the oxidation of low-density lipoproteins (LDL), a key step in the development of the atherosclerotic lesion, which can also provoke leukocyte adhesion and emigration [41]. In this way, the use of anti-oxidants could constitute an alternative therapy for the control of the subendothelial leukocyte infiltration associated with the vascular damage detected in hypertension and atherosclerosis.

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II.3 ARTÍCULO 3

Molecular mechanisms underlying angiotensin-II induced arteriolar and venular leukocyte adhesion in vivo. A critical role for angiotensin II in atherogenesis?

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MOLECULAR MECHANISMS UNDERLYING ANGIOTENSIN-II INDUCED ARTERIOLAR AND VENULAR LEUKOCYTE ADHESION *IN VIVO*. A CRITICAL ROLE FOR ANGIOTENSIN-II IN ATHEROGENESIS?

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Condensed tittle: Angiotensin-II induces arteriolar and venular leukocyte recruitment

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ABSTRACT

Angiotensin II (Ang-II) may play a critical role in atherogenesis. This study was undertaken to assess the molecular mechanisms underlying Ang-II-induced leukocyte recruitment *in vivo*. Rats received a i.p injection of Ang-II (0.1-1 nM) and, 4 h later, leukocyte-endothelial cell interactions in mesenteric microcirculation were examined via intravital microscopy. We have demonstrated, for the first time *in vivo,* that 1 nM Ang-II produces a significant enhancement in arteriolar leukocyte adhesion. Using function blocking mAbs against different rat cell adhesion molecules (CAMs), this effect was primarily mediated by P- and E-selectin up-regulation and, indirectly, through increased β_2-integrin expression. Ang-II also induced a significant increase in the leukocyte-endothelial cell interactions in postcapillary venules. Venular endothelium responded differently to Ang-II, since the α_4-integrins/VCAM-1 pathway also played a clear role in this leukocyte response. Disruption of vascular balance for 4 h with L-NAME i.p. injection also provoked a significant increase in arteriolar leukocyte adhesion, which was mainly Ang-II-dependent. Therefore, Ang-II may be a key molecule in the onset and progression of the atherosclerotic lesion when its plasma levels are elevated. Interestingly, Ang-II may also be critical at the beginning of this process when endothelial dysfunction is caused by low levels of vasodilators.

INTRODUCTION

Atherosclerosis is the major cause of myocardial infarction, strokes and peripheral vascular disease, accounting for nearly half of all mortality in developed countries. Atherogenesis is a complex process characterized by the formation of a neointimal lesion that progressively occludes the arterial lumen. Different forms of insult to the vessel wall can impair the protective function of the endothelium and thus trigger this process (1, 2). Indeed, one of the earliest stages of atherogenesis is endothelial dysfunction which is thought to cause an inflammatory response consisting of lipid accumulation and increased adherence of T lymphocytes and monocyte/macrophages (3, 1, 4). These cells then migrate through the endothelium and localize subendothelially, releasing several cytokines and growth factors which contribute to the activation and proliferation of the vascular smooth muscle cells (VSMCs) and, in turn, to the formation of the atherosclerotic lesion.

In this way, vascular endothelium is the principal controller of leukocyte traffic between the blood stream, the arterial intima and the extravascular space (5). In fact, the migration of leukocytes from the blood to sites of extravascular injury in response to locally produced stimuli is mediated through a sequential cascade of leukocyte-endothelial cell adhesive interactions which involve an array of cell adhesion molecules (CAMs) present on leukocytes and on the endothelial cells. This multistage process is initiated by the tethering of leukocytes to the endothelium, followed by weak, transient adhesive interactions manifested as leukocyte rolling, leading ultimately to firm leukocyte adhesion to and subsequent transmigration through the vascular endothelium (6, 7). Arterial endothelium has been shown to express the same CAMs as those expressed in venular endothelium (8, 9, 10), thus, similar molecular mechanisms are expected in both vessel types. However, while leukocyte-endothelial cell interactions in postcapillary venules are induced by a wide range of stimuli,

arteriolar rolling and adhesion are only induced by certain stimuli such as perivascular laser injury, treatment with cytokines such as interleukin (IL)-1β or tumor necrosis factor (TNF)-α, oxidized LDL or cigarette smoke (11, 12, 13, 14, 15, 16).

Angiotensin II (Ang-II) is the main effector peptide of the renin-angiotensin system and, in addition to its role as a potent vasoconstrictor and blood pressure and fluid homeostasis regulator, it has been shown to exert pro-inflammatory activity. Ang-II receptors have been demonstrated on human monocytes and are capable of promoting monocyte adhesion and activation *in vitro* (17, 18). This may be directly relevant as hypertension is associated with migration of monocytes into the vessel wall, a critical event leading to the development of the atherosclerotic lesion which can be attenuated by angiotensin-converting enzyme (ACE) inhibition or by pretreatment with an Ang-II AT_1 receptor antagonist (19, 20, 21). Interestingly, we have recently revealed that acute Ang-II superfusion of the rat mesenteric microcirculation provokes an inflammatory response *in vivo* at sub-vasoconstrictor doses. In particular, it induces leukocyte recruitment in postcapillary venules through endothelial P-selectin up-regulation in the vessel wall and this effect is primarily mediated via an Ang-II AT_1 receptor interaction (22). Therefore, Ang-II might be a stimulus for the subendothelial infiltration of leukocytes observed in hypertension and atherosclerosis.

Hence, in the present study, we have investigated the effect of Ang-II under subacute inflammatory conditions (4h). Intravital microscopy has been used to systematically characterize the CAMs involved in leukocyte-endothelial cell interactions which occur in response to this peptide in both rat mesenteric arterioles and postcapillary venules. Finally, we have also carried out a series of experiments to further investigate whether an endothelial disruption due to absence of nitric oxide (NO) leads to the

development of the atherosclerotic lesion and to explore the possible involvement of Ang-II in this response.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (200-250 g) were purchased from Harlan Ibérica (Barcelona, Spain).

Materials

Sodium pentobarbital, Ang-II and L-NAME were purchased from Sigma Química (Madrid, Spain). The antibodies used were RMP-1, an IgG_{2a} mAb against rat P-selectin; RME-1, an IgG_1 mAb against rat E-selectin; TA-2, an IgG_1 mAb against rat α_4-integrins; 5F10, an IgG_{2a} mAb against rat VCAM-1; WT-3, an IgG_1 mAb against rat β_2-integrins and 1A29, an IgG_1 mAb against rat ICAM-1. RMP-1, RME-1, TA-2, 5F10, WT-3 and 1A29 were acquired as previously stated (23, 24, 25, 26, 27, 28). Losartan was kindly donated by Merck Sharp & Dohme (Spain).

Intravital Microscopy

Animals were prepared for intravital microscopy as previously described (29). To summarize, rats were sedated with ether and intraperitoneally injected with either 5 ml of saline or 5 ml of saline containing Ang-II (final concentration 0.1 or 1 nM). The doses selected for Ang-II were based on previous results obtained in our laboratory where it was demonstrated that 1 nM Ang-II causes the maximum and most consistent increase in

leukocyte-endothelial cell interactions *in vivo* after 60 min superfusion without provoking vasoconstriction (22). 3 h later, the rats were anesthetized with sodium pentobarbital (65 mg/kg, ip) and the trachea cannulated to maintain a patent airway throughout the experiment. A polyethylene catheter was inserted into the right jugular vein for intravenous administration of compounds and a second catheter was placed in the right carotid artery to monitor systemic mean arterial blood pressure (MABP) through a pressure transducer (Spectramed Stathan P-23XL) connected to a recorder (GRASS RPS7C8B, Quincy, MA.). A midline abdominal incision was made to expose the small intestine, a segment of the midjejunum was carefully exteriorized and the mesentery was prepared for *in vivo* microscopic observation by draping it over an optically clear viewing pedestal that permited transillumination of a 3 cm^2 segment of tissue. All exposed tissue was covered with saline-soaked gauze to minimize tissue dehydration. The temperature of the pedestal and mesentery was maintained at 37ºC with a constant temperature circulator. Rectal temperature was monitored with an electrothermometer and maintained at 37ºC using an infrared heat lamp. The exposed mesentery was continuously superfused with warmed bicarbonate buffer saline (BBS, pH 7.4) at a rate of 2 ml/min. The mesenteric microcirculation was observed through an orthostatic microscope (Nikon Optiphot-2, SMZ1, Badhoevedorp, The Netherlands) with a 20x objective lens (Nikon SLDW, Badhoevedorp, The Netherlands) and a 10x eyepiece. A video camera (Sony SSC-C350P, Alcobendas, Madrid) mounted on the microscope projected images onto a color monitor (Sony Trinitron PVM-14N2E, Alcobendas, Madrid) and these images were captured on viodeotape (Sony SVT-S3000P, Alcobendas, Madrid) with superimposed time and date for subsequent playback analysis. The final magnification of the image on the monitor was 1300x.

In each animal, leukocyte responses (leukocyte rolling flux, leukocyte rolling velocity, leukocyte adhesion and leukocyte emigration) were measured off-line during playback

analysis of videotaped images in two or three randomly selected postcapillary venules ranging between 25 and 40 μm in diameter. Leukocyte adhesion in rat mesenteric arterioles was also measured off-line during playback analysis of videotaped images in two to three randomly selected arterioles ranging between 20 and 35 μm in diameter. Rolling leukocyte flux was determined by counting the number of rolling leukocytes passing a fixed reference point in the microvessel per min. The same reference point was used throughout the experiment, as leukocytes may roll for only a section of the vessel before rejoining the blood flow or becoming firmly adherent. Leukocyte rolling velocity (V_{wbc}) was determined by measuring the time required for a leukocyte to traverse a distance of 100 μm along the venule and was expressed as μm/s. A leukocyte was considered to be adherent to venular or arteriolar endothelium if it remained stationary for a period equal to or exceeding 30 s. Adherent cells were expressed as the number per 100 μm length of venule or arteriole. Leukocyte emigration was expressed as the number of white blood cells per microscopic field. The rate of emigration was determined from the difference between the number of interstitial leukocytes present at the beginning of the experiment and the number of cells present in the intestitium at the end of the experiment.

Venular and arteriolar diameters were measured on-line using a video caliper (Microcirculation Research Institute, Texas A&M University, College Station, Texas). Centerline red blood cell velocity (V_{rbc}) was also measured on-line with an optical Doppler velocimeter (Microcirculation Research Institute, Texas A&M University, College Station, Texas). Vessel blood flow was calculated from the product of mean red blood cell velocity ($V_{mean} = V_{rbc} / 1.6$) and microvascular cross-sectional area, assuming cylindrical geometry. Vessel wall shear rate (γ) was calculated based on the Newtonian definition: $\gamma = 8 \times (V_{mean}/D)$ s^{-1}, in which D is vessel diameter (30).

Experimental protocol: The exteriorized mesentery was superfused with BBS and allowed to stabilize for 30 min in the first group of animals and was supplemented with Ang-II (final concentration 0.1 nM or 1 nM, in the superfusate respectively) in the other two groups. In all the experimental groups, 4 h after the intraperitoneal injection of the agent under investigation, measurements of venular leukocyte rolling flux, velocity, adhesion, emigration, arteriolar leukocyte adhesion, MABP, venular and arteriolar V_{rbc}, shear rate and diameter were obtained and recordings made for 5 min. On the basis of these initial experiments, 1 nM Ang-II was used for the remainder of the study as it induced arteriolar leukocyte adhesion and 0.1 nM had less profound and less consistent effects than 1 nM Ang-II in this parameter.

The adhesion molecules involved in these responses were determined by pretreatment of the animals with different mAbs directed against rat leukocyte or endothelial CAMs 15 minutes prior to Ang-II intraperitoneal injection. The doses used were 2.5 mg/kg for RMP-1 and RME-1, 4 mg/kg for TA-2, 1 mg/kg for 5F10 and WT-3 and 2 mg/kg for 1A29. All mAbs were intravenously injected via a lateral tail vein. All antibodies were assayed at doses previously described (22, 31, 32, 33, 34, 35). To determine the effect of the antibodies on circulating leukocyte numbers, blood samples were taken from the rats 4 h after Ang-II intraperitoneal injection.

In another series of experiments, rats were sedated with ether and L-NAME was intraperitoneally injected (final concentration 100 μM) in a volume of 5 ml of saline. 3.5 h later, once the rats were prepared for intravital microscopy as aforementioned, the mesentery was superfused with L-NAME 100 μM in BBS for a further 30 min. Previous studies have demonstrated that this dose of L-NAME provokes a consistent increase in leukocyte rolling and adhesion after 60 min superfusion (36). 4 h after L-NAME intraperitoneal injection, measurements were taken of the different hemodynamic and leukocyte parameters described previously. To further investigate if Ang-II is implicated in the leukocyte-endothelial cell

interactions provoked by lack of nitric oxide (NO), a second group of animals was pretreated with an AT_1 Ang-II receptor antagonist (losartan, 10 mg/kg, i.v.) 15 min prior to L-NAME intraperitoneal injection. The dose administered was that stated in previous *in vivo* studies (37).

Statistical Analysis

All values are reported as mean±SEM. Data within groups were compared using an analysis of variance (one way-ANOVA) with a Newman-Keuls *post hoc* correction for multiple comparisons. Statistical significance was set at $p < 0.05$.

RESULTS

Leukocyte-endothelial cell interactions induced by subacute Ang-II (0.1 or 1 nM) administration.

Rats intraperitoneally injected with saline showed a basal level of venular leukocyte rolling flux (29.8±8.1 cells/min), a low level of venular leukocyte adhesion (3.0±0.9 cells/100 μm) and a small number of venular leukocyte emigration (1.3±0.3 0.3±0.3 cells per field). Arteriolar leukocyte adhesion was almost absent (0.4±0.2 cells/100 μm) (Fig. 1A, 1B, 1C and 1D). When animals were treated with Ang- II for 4 h, there was a significant increase in venular leukocyte rolling flux (109.9±22.3 and 126.7±14.1 cells/min respectively), in venular leukocyte adhesion (16.1±1.9 and 18.5±1.7 cells/100 μm respectively) and in venular leukocyte emigration (11.8±3.0 and 13.8±2.5 cells per field respectively) with both of the doses assayed (0.1 and 1 nM) (Fig. 1A, 1B, 1C). Interestingly, Ang-II at 1 nM caused a significant increase in arteriolar leukocyte adhesion

(3.6±1.03 cells/100 μm) (Fig. 1D) while the dose of 0.1 nM had less profound and less consistent effects in this parameter (1.3±0.3 cells/100 μm). On the basis of these initial experiments, Ang-II 1 nM was used for the remainder of the experiments. In addition, 1 nM Ang-II induced a concomitant significant decrease in venular leukocyte rolling velocity (Table 1) but did not modify MABP, the venular shear rate or the systemic leukocyte counts (Table 2 and Table 3). Arteriolar shear rate in rats exposed to saline for 4 h (1019±71.7 s^{-1}) did not significantly differ from the value obtained in rats intraperitoneally injected with Ang-II (1 nM) for the same time period (1213.9±147.5 s^{-1}).

Effect of an anti-rat P-selectin and/or an anti-rat E-selectin mAbs on leukocyte responses in Ang-II-activated microvessels.

The increases in venular leukocyte rolling flux, venular leukocyte adhesion, venular leukocyte emigration and arteriolar leukocyte adhesion following Ang-II administration were significantly inhibited by 66.1%, 78.1%, 87.6% and 88.1% respectively when animals were pretreated with the blocking anti-rat P-selectin mAb (RMP-1) (Fig 2A, 2B, 2C and 2D). Administration of a neutralizing anti-rat E-selectin mAb (RME-1) did not modify Ang-II-induced leukocyte responses. However, a combination of these two mAbs reduced all these parameters to baseline levels (100%, 100%, 100% and 94.9% respectively) (Fig 2A, 2B, 2C and 2D and Fig. 3A, 3B and 3C). These treatments did not reduce adhesion directly, but rather prevented further recruitment by blocking the prerequisite leukocyte rolling necessary for firm adhesion. The reduction in venular leukocyte rolling velocity was also reversed by a blocking anti-rat P-selectin mAb and by a combination of these two mAbs directed against both selectins (Table 1). Hemodynamic

parameters remained the same in the three pretreated groups and systemic leukocyte counts were only affected when both mAbs were administered (Table 2 and 3).

Effect of an anti-rat α_4-integrins and an anti-rat VCAM-1 mAbs on leukocyte responses in Ang-II-activated microvessels.

Blocking mAbs directed against rat α_4-integrins (TA-2) or rat VCAM-1 (5F10) did not affect Ang-II-induced increases in venular leukocyte rolling flux, venular leukocyte emigration and arteriolar leukocyte adhesion or reductions in venular leukocyte rolling velocity (Fig. 4A, 4C, and 4D and Table 2). Although administration of a mAb directed against rat α_4-integrins significantly decreased Ang-II-induced increase in venular leukocyte adhesion by 57.7%, pretreatment of the animals with a blocking mAb directed against VCAM-1 did not affect this leukocyte response (Fig. 4B). Pretreatment of the animals with TA-2 or 5F10 had no effect on the different hemodynamic parameters determined but caused a significant elevation in the number of circulating leukocytes (Table 2 and 3).

Effect of an anti-rat β_2-integrins and an anti-rat ICAM-1 mAbs on leukocyte responses in Ang-II-activated microvessels.

As expected, β_2-integrins blockade did not cause a significant effect on the venular leukocyte rolling flux elicited by Ang-II injection (Fig.5A) but did significantly inhibit the number of adherent and emigrated leukocytes at this level by 92.7% and 89.0% respectively (Fig. 5B and 5C). Similar results were obtained after anti-ICAM-1 mAb pretreatment. Interestingly, while WT-3 administration significantly reduced the arteriolar leukocyte adhesion caused by Ang-II by 82.8% and also prevented the decrease in venular

leukocyte rolling velocity induced by this peptide hormone, 1A29 did not modify any of these parameters (Fig. 5D and Table 2). Pretreatment of the animals with WT-3 and 1A29 had no effect on either of the hemodynamic parameters determined or the number of circulating leukocytes (Table 2 and 3).

Effect of an AT_1 Ang-II receptor antagonist, losartan, on leukocyte responses in L-NAME-activated microvessels.

The effect of L-NAME intraperitoneal injection on leukocyte recruitment 4 h after its administration are shown in Figure 6. When compared with values in saline injected animals for 4 h, L-NAME administration induced a significant increase in venular leukocyte rolling flux (143.2±20.2 vs. 29.8±8.1 cells/min), venular leukocyte adhesion (22.4±2.4 vs. 3.0±0.9 cells per 100 μm), venular leukocyte emigration (21.2±3.6 vs. 1.3±0.3 cells per field) and, surprisingly, in arteriolar leukocyte adhesion (9.6±1.4 vs. 0.4±0.2 cells per 100 μm). These parameters were inhibited by 82.1%, 88.1%, 46.1% and 85.5% respectively through systemic administration of losartan. L-NAME provoked a significant decrease in leukocyte rolling velocity and venular shear rate which was maintained at basal levels in animals in which losartan was administered (Table 4). MABP, arteriolar shear rate and systemic leukocyte counts remained unchanged in the three experimental groups; saline-treated, L-NAME-treated and L-NAME–treated plus losartan pretreatment (Table 4).

DISCUSSION

Leukocyte recruitment to the arterial wall is of importance in different pathophysiological states, such as atherosclerosis and hypertension, where Ang-II seems to play a critical role. Leukocyte-endothelium interactions are generally observed in venules but

rarely in arterioles of tissues (i.e. mesentery, cremaster muscle) prepared for intravital microscopy. Of the different stimuli which cause arteriolar rolling and adhesion (11, 12, 13, 14, 15, 16), the present study shows for the first time that Ang-II at subvasoconstrictor and physiologically relevant doses (1 nM) causes arteriolar leukocyte adhesion *in vivo* in the rat mesenteric microcirculation. Interestingly, this effect is not observed under acute (1 h) stimulation with Ang-II (22) and it is necessary to expose the arteriolar endothelium to Ang-II for at least 4 h so as to obtain a significant enhancement of this parameter.

Despite this finding, rolling leukocytes were not observed at the arteriolar level, while, in contrast, increases in leukocyte rolling flux, adhesion and emigration were clearly detected in postcapillary venules after 4 h Ang-II administration. Several explanations may account for the effects observed. First, the differences in the characteristics of leukocyte-endothelium interactions between arterioles and inflamed venules is probably partly due to differences in the local hemodynamics, inasmuch as wall shear rate is higher in arteries than in venular systems. This result is supported by previous data demonstrating that wall shear rate strongly influences leukocyte rolling in various vessel types (38, 16, 12). Second, the absence of rolling leukocytes in rat mesenteric arterioles following Ang-II stimulation may be related to the diameter of the vessel under investigation. Indeed, Kunkel et al. (12) demonstrated that increases in the arteriolar leukocyte rolling flux induced by TNF-α in the mouse cremaster muscle microcirculation only occurred in arterioles ranging from 30 to 70 μm in diameter, while in the present study the arteriolar diameter ranged from 20 to 35 μm and was, therefore, below the threshold for detecting this leukocyte response. Nevertheless, the existence of leukocytes adhered to the arteriolar endothelium just 4 h after Ang-II stimulation is sufficient proof of the link between this peptide hormone and the onset and progression of the

atherosclerotic lesion, especially in pathophysiological conditions where its plasma levels can be quickly elevated and maintained for a certain period of time.

There is some evidence to suggest that Ang-II induces CAM expression, however, discrepancies between previous reports do exist. Some authors have found no role for E-selectin, VCAM-1 or ICAM-1 in Ang-II-induced monocyte adhesion to cultured endothelial cells (39). Others have found that treatment with ACE inhibitors downregulates the expression of VCAM-1 and ICAM-1 in animal models of ureteral obstruction and hypertension (19, 40). Similarly, enalaprilat treatment in septic patients decreases the plasma levels of soluble CAMs such as E-selectin and ICAM-1 (41). In addition, in an *in vitro* study, Gräfe et al. (42) found a clear role for Ang-II in inducing E-selectin expression on coronary endothelial cells without affecting VCAM-1 or ICAM-1 expression. Likewise, Pastore et al. (43), employing human umbilical vein endothelial cells (HUVECs), demonstrated increased ICAM-1 expression after Ang-II stimulation. Moreover, Tummala et al. (44) recorded increased VCAM-1 expression in the aorta of rats subjected to 6 days of Ang-II infusion. Recently, we have linked the upregulation of P-selectin to the leukocyte-endothelial cell interactions caused by acute Ang-II stimulation of rat mesenteric postcapillary venules (22).

The present study helps to clarify the discrepancies encountered between the aforementioned studies. The mechanism underlying leukocyte-endothelial cell interactions elicited by Ang-II at both arteriolar and venular levels was further investigated using function blocking mAbs directed against various rat adhesion molecules. Arteriolar leukocyte adhesion elicited by Ang-II was primarily mediated through P-selectin upregulation, since administration of a function blocking mAb against this CAM dramatically reduced, but did not abolish said response. Interestingly, while function inhibition of E-selectin had no effect on responses elicited by Ang-II, co-administration of mAbs directed against both selectins

almost abrogated the increase of the leukocytes adhered to the arteriolar endothelium. Likewise, leukocyte-endothelial cell interactions caused by Ang-II in the rat mesenteric postcapillary venules showed a similar profile to those encountered in the mesenteric arterioles, since pretreatment with a combination of mAbs against P-and E-selectin reduced these leukocyte responses to basal levels. These results indicate that *in vivo* responses do not necessarily correlate with *in vitro* data. With regard to this, it is well established that cultured endothelial cells loose the ability to express P-selectin after several passages (KD Patel, personal communication), a fact which may explain why Gräfe et al. (42) could only find a role for E-selectin in Ang-II-induced responses and not for P-selectin, as we have demonstrated in this study.

Furthermore, differences between arteriolar and venular endothelium in response to Ang-II should be taken into account. In this way, our results confirm those of Pastore et al. (43) who found that Ang-II caused increased expression of ICAM-1 in HUVECs. Indeed, in our study, Ang-II-induced leukocyte adhesion to and emigration through postcapillary venules was partly mediated through ICAM-1 increased expression. In contrast, the involvement of this CAM on the arteriolar leukocyte adhesion induced by Ang-II could not be totally confirmed as the administration of a mAb directed against ICAM-1 did not affect said response. Nevertheless, reduction in the venular and arteriolar leukocyte adhesion provoked by Ang-II was clear when β_2-integrin function was blocked, and ICAM-1 and ICAM-2 are the main endothelial ligands for these CAMs.

Although a significant decrease in Ang-II induced-increase in leukocyte adhesion in both rat mesenteric arterioles and postcapillary venules was observed after administration of a mAb directed against β_2-integrins, flow cytometry analysis revealed no changes in the expression of β_2-integrins or L-selectin on the leukocyte surface of the rat

granulocytes or monocytes exposed to Ang-II (22). These results suggest that Ang-II had no direct effect on leukocyte chemotaxis. Thus, the firm adhesion could be due to the release of endogenously generated chemotactic mediators elicited by this peptide hormone. In this context, there is some evidence that Ang-II can evoke leukocyte recruitment through increased expression and induction of chemotactic mediators such as MCP-1 or RANTES. Indeed, ACE inhibitor pretreatment and administration of Ang-II-specific receptor antagonists have been reported to inhibit chemokine production (20, 45, 46, 47). In addition, Mangat et al. (48) have also illustrated a role for Ang-II in cytosolic phospholipase A_2 ($cPLA_2$) activation, which is critical for the synthesis and release of potent chemotactic mediators such as platelet activating factor (PAF) and leukotriene B_4 (LTB_4). Furthermore, we have also demonstrated that Ang-II induced-leukocyte-endothelial cell interactions in rat mesenteric postcapillary venules can be dramatically attenuated by pretreatment with either a PAF receptor antagonist or a 5-lipoxigenase inhibitor (29).

Regarding the α_4-integrins/VCAM-1 pathway, although α_4-integrins seems to be clearly involved in the venular leukocyte adhesion elicited by Ang-II, we could not reduce the Ang-II-induced arteriolar leukocyte adhesion by inhibiting the function of both of these CAMs. Although these findings seem to sharply contrast with those of Tummala et al. (44), this is not the case. In the aforementioned study, VCAM-1 protein expression after prolonged exposure (6 days) to Ang-II was predominantly localized around the aorta adventitia and on cultured rat aortic smooth muscle cells stimulated by Ang-II was not noted in the endothelium. Therefore, although VCAM-1 can be upregulated by Ang-II, more relevant would seem to be its role in the progression of the atherosclerotic lesion, once the monocytes are subendothelialy localized, rather than in the onset of the process. In this way, our findings suggest that the arteriolar and the venular endothelium respond differently to Ang-II and that

these differences are owed to the α_4-integrins/VCAM-1 adhesion pathway, which seems to play a critical role in the leukocyte-endothelial adhesion in the mesenteric postcapillary venules but not in the arterioles.

Finally, our results are totally concordant with the "response-to-injury" theory (1, 2). According to this theory, atherosclerosis can be initiated by different forms of insult to the vessel wall which impair the protective function of the endothelial monolayer. To test this hypothesis, we disrupted the vascular balance by inhibiting NO synthase (NOS) activity with L-NAME. After 4 h intraperitoneal administration of the NOS inhibitor significant increases were detected in the leukocyte rolling flux, adhesion and emigration in the rat mesenteric postcapillary venules. However, the most striking observation was that by simply decreasing the levels of NO, leukocytes began to adhere to the rat mesenteric arterioles, thus indicating the possible onset of the atherosclerotic lesion. As expected, losartan pretreatment inhibited the leukocyte rolling flux, adhesion and emigration which occurs in rat mesenteric postcapillary venules after 4 h L-NAME administration and these results back up a previous study by our group (37). Of particular relevance is the fact that AT_1 receptor blockade dramatically attenuated the arteriolar leukocyte adhesion (85.5%) provoked by the lack of NO. Hence, we have clearly demonstrated a role for Ang-II in L-NAME-induced leukocyte–endothelial cell interactions. This is of considerable interest since it indicates that lack of NO may result in the exposure of the vascular endothelium to the deleterious actions of vasoconstrictors. These vasoconstrictors could then trigger the subsequent leukocyte recruitment that may cause the development of the vascular lesion detected in various disease states such as hypertension and atherosclerosis (19, 20).

In conclusion, we have demonstrated here for the first time *in vivo* that Ang-II induces arteriolar leukocyte adhesion within 4 h at sub-vasoconstrictor and physiologically

relevant doses. Hence, Ang-II may play a critical role in the onset and progression of the atherosclerotic lesion. This effect has been found to be primarily dependent on increased P- and E-selectin expression and, indirectly, on β_2-integrin upregulation on the leukocyte surface. Indeed, increased circulating levels of P-selectin and to a lesser extent E-selectin can be found in essential, renovascular and malignant hypertension and in hypercholesterolemic patients (49, 50). Furthermore, in several mouse models of atherosclerosis, it has been shown that there is a clear involvement of both P- and E-selectins in the development of the atherosclerotic lesion at both early and advanced stages (51, 52). On the other hand, as opposed to the arteriolar endothelium, the venular endothelium seems to respond differently to Ang-II. In this context, the α_4-integrins/VCAM-1 pathway plays a clear role in the leukocyte-endothelial cell interactions caused by this peptide hormone together with the other CAMs involved in arteriolar leukocyte adhesion. Finally, when vascular balance is disrupted by reduced levels of vasodilators, arterial leukocyte adhesion occurs and is predominantly Ang-II dependent. This mechanism may be a key to explaining the beginning of the atherosclerotic lesion and clearly implicates Ang-II in the onset of this process, despite the fact that its plasma levels are not necessarily elevated at this stage. Finally, our data also suggest that the use of Ang-II antagonists may represent a new strategy for modulating the pathophysiology of leukocyte-induced endothelial dysfunction in different circulatory disorders.

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FIGURE LEGENDS

Figure 1: Subacute Ang-II-induced leukocyte responses within rat mesenteric postcapillary venules and arterioles. Rats were treated intraperitoneally with saline (n=6), Ang-II 1 nM (n=6) or Ang-II 0.1 nM (n=4). 4 h later, the mesenteric tissue was exteriorized and superfused with buffer in the first group of rats and in the other two groups with Ang-II 1 nM and 0.1 nM respectively. Responses of venular leukocyte rolling flux (A), venular leukocyte adhesion (B), venular leukocyte emigration (C) and arteriolar leukocyte adhesion (D) were then quantified. Results are represented as mean±SEM. *p<0.05 or **p<0.01 relative to the saline group.

Figure 2: Effect of an anti P-selectin or/and an anti-E-selectin mAbs on subacute Ang-II-induced leukocyte responses within rat mesenteric postcapillary venules and arterioles. Rats were treated intraperitoneally with saline (n=6) or Ang-II 1 nM (n=6). In further groups of animals, 15 minutes before intraperitoneal administration of Ang-II 1 nM, rats were treated with RMP-1 (2.5 mg/kg, i.v., n=5) or with RME-1 (2.5 mg/kg, i.v., n=5) or with a combination of both (n=4). 4 h later, the mesenteric tissue was exteriorized and superfused with buffer in the first group of rats and with Ang-II 1 nM in the other groups. Responses of venular leukocyte rolling flux (A), venular leukocyte adhesion (B), venular leukocyte emigration (C) and arteriolar leukocyte adhesion (D) were then quantified. Results are represented as mean±SEM. *p<0.05 or **p<0.01 relative to the saline group. +p<0.05 or ++p<0.01 relative to the Ang-II 1 nM group.

Figure 3: Video photomicrographs of the effect of an anti P-selectin and an anti-E-selectin mAbs on subacute Ang-II-induced arteriolar leukocyte adhesion within rat mesenteric postcapillary arterioles. (A) Responses in an animal treated intraperitoneally with saline at 4 h; (B) responses in an animal treated intraperitoneally with Ang-II 1 nM at 4 h, clearly showing a large number of adherent leukocytes within the arteriole; and (C) responses in an animal pretreated with a combination of both RMP-1 (2.5 mg/kg, i.v.) and RME-1 (2.5 mg/kg, i.v.) before intraperitoneal administration of Ang-II 1 nM at 4 h, clearly showing a reduction in the adherent leukocytes within the arteriole (v: venule; a: arteriole). Scale bar, 50 μm.

Figure 4: Effect of an anti α_4-integrins and an anti-VCAM-1 mAbs on subacute Ang-II-induced leukocyte responses within rat mesenteric postcapillary venules and arterioles. Rats were treated intraperitoneally with saline (n=6) or Ang-II 1 nM (n=6). In further groups of animals, 15 minutes before intraperitoneal administration of Ang-II 1 nM, rats were treated with TA-2 (4 mg/kg, i.v., n=5) or with 5F10 (1 mg/kg, i.v., n=4). 4 h later, the mesenteric tissue was exteriorized and superfused with buffer in the first group of rats and with Ang-II 1 nM in the other groups. Responses of venular leukocyte rolling flux (A), venular leukocyte adhesion (B), venular leukocyte emigration (C) and arteriolar leukocyte adhesion (D) were then quantified. Results are represented as mean±SEM. *$p<0.05$ or **$p<0.01$ relative to the saline group. +$p<0.05$ or ++$p<0.01$ relative to the Ang-II 1 nM group.

Figure 5: Effect of an anti-β_2-integrins and an anti-ICAM-1 mAbs on subacute Ang-II-induced leukocyte responses within rat mesenteric postcapillary venules and arterioles. Rats were treated intraperitoneally with saline (n=6) or Ang-II 1 nM (n=6). In further groups of animals, 15 minutes before intraperitoneal administration of Ang-II 1 nM, rats were treated with WT-3 (1 mg/kg, i.v., n=4) or with 1A29 (2 mg/kg, i.v., n=5). 4 h later, the mesenteric tissue was exteriorized and superfused with buffer in the first group of rats and with Ang-II 1 nM in the other groups. Responses of venular leukocyte rolling flux (A), venular leukocyte adhesion (B), venular leukocyte emigration (C) and arteriolar leukocyte adhesion (D) were then quantified. Results are represented as mean±SEM. *p<0.05 or **p<0.01 relative to the saline group. +p<0.05 or ++p<0.01 relative to the Ang-II 1 nM group.

Figure 6: Effect of losartan treatment on subacute L-NAME-induced leukocyte responses within rat mesenteric postcapillary venules and arterioles. Rats were treated intraperitoneally with saline (n=6) or L-NAME 100 μM (n=5). In a further group of animals, 15 minutes before intraperitoneal administration of L-NAME 100 μM, rats were treated with an AT_1 Ang-II receptor antagonist losartan (10 mg/kg, i.v., n=5). 4 h later, the mesenteric tissue was exteriorized and superfused with buffer in the first group of rats and with L-NAME 100 μM in the other two groups. Responses of venular leukocyte rolling flux (A), venular leukocyte adhesion (B), venular leukocyte emigration (C) and arteriolar leukocyte adhesion (D) were then quantified. Results are represented as mean±SEM. *p<0.05 or **p<0.01 relative to the saline group. +p<0.05 or ++p<0.01 relative to the L-NAME 100 μM group.

Table 1. Leukocyte rolling velocity (μm/sec) [a].

Treatment	**4 h**
Saline	81.6±14.6
Ang II 1 nM	30.2±2.2**
Ang II 0.1 nM	50.7±5.6*
Ang II 1 nM + RMP-1	84.4±6.8++
Ang II 1 nM + RME-1	41.3±7.3**
Ang II 1 nM + RMP-1 + RME-1	105.1±9.4++
Ang II 1 nM + TA-2	52.8±6.8*
Ang II 1 nM + 5F10	42.3±4.4**
Ang II 1 nM + WT-3	70.4±7.3++
Ang II 1 nM + 1A29	38.2±4.2**

[a]Table 1 illustrates the effect of different mAb treatments on leukocyte rolling velocity reduced by intraperitoneal administration of Ang-II 1 nM. Rats were treated intraperitoneally with saline (n=6), Ang-II 1 nM (n=6) or Ang-II 0.1 nM (n=4). In further groups of animals, 15 minutes before intraperitoneal administration of Ang-II 1 nM, rats were treated with RMP-1 (n=5) or with RME-1 (n=5) or with a combination of both (n=4) or with TA-2 (n=5) or with 5F10 (n=4) or with WT-3 (n=4) or with 1A29 (n=5). 4 h later, the mesenteric tissue was exteriorized and superfused with buffer in the first group of rats, with Ang-II 0.1 nM in the third group of rats and with Ang-II 1 nM in the other groups and the responses were evaluated. *$p<0.05$ or **$p<0.01$ relative to the saline group. +$p<0.05$ or ++$p<0.01$ relative to the Ang-II 1 nM group. All values are mean±SEM.

Table 2. Hemodynamic parameters[b].

	MABP (mmHg)	Venule shear rate (s^{-1})
Treatment	**4 h**	**4 h**
Saline	108.9±16.6	774.3±181.7
Ang II 1 nM	73.6±11.1	461.4±87.0
Ang II 0.1 nM	93.3±2.5	511.9±99.1
Ang II 1 nM + RMP-1	100.3±14.8	535.7±42.7
Ang II 1 nM + RME-1	89.2±5.5	542.4±64.5
Ang II 1 nM + RMP-1 + RME-1	115.4±5.2	446.1±58.3
Ang II 1 nM + TA-2	95.3±3.3	565.1±54.0
Ang II 1 nM + 5F10	88.8±13.6	554.0±138.4
Ang II 1 nM + WT-3	102.1±10.1	473.6±79.0
Ang II 1 nM + 1A29	107.9±14.1	639.2±172.9

[b]Table 2 presents hemodynamic parameters in animals receiving mAb treatments before intraperitoneally administration of Ang-II 1 nM. Rats were treated intraperitoneally with saline (n=6), Ang-II 1 nM (n=6) or Ang-II 0.1 nM (n=4). In further groups of animals, 15 minutes before intraperitoneal administration of Ang-II 1 nM, rats were treated with RMP-1 (n=5) or with RME-1 (n=5) or with a combination of both (n=4) or with TA-2 (n=5) or with 5F10 (n=4) or with WT-3 (n=4) or with 1A29 n=5). 4 h later, the mesenteric tissue was exteriorized and superfused with buffer in the first group of rats, with Ang-II 0.1 nM in the third group of rats and with Ang-II 1 nM in the other groups and the responses were evaluated. All values are mean±SEM.

Table 3. Systemic leukocyte counts (cell/μl) [c].

Treatment	**4 h**
Saline	9500.0±1288.4
Ang II 1 nM	8366.7±558.4
Ang II 1 nM + RMP-1	9225.0±2244.4
Ang II 1 nM + RME-1	8860.0±1755.2
Ang II 1 nM + RMP-1 + RME-1	16525.0±3133.0* +
Ang II 1 nM + TA-2	18820.0±1044.7 ** ++
Ang II 1 nM + 5F10	19500.0±3153.6 ** ++
Ang II 1 nM + WT-3	10433.0±1497.0
Ang II 1 nM + 1A29	8340.0±892.5

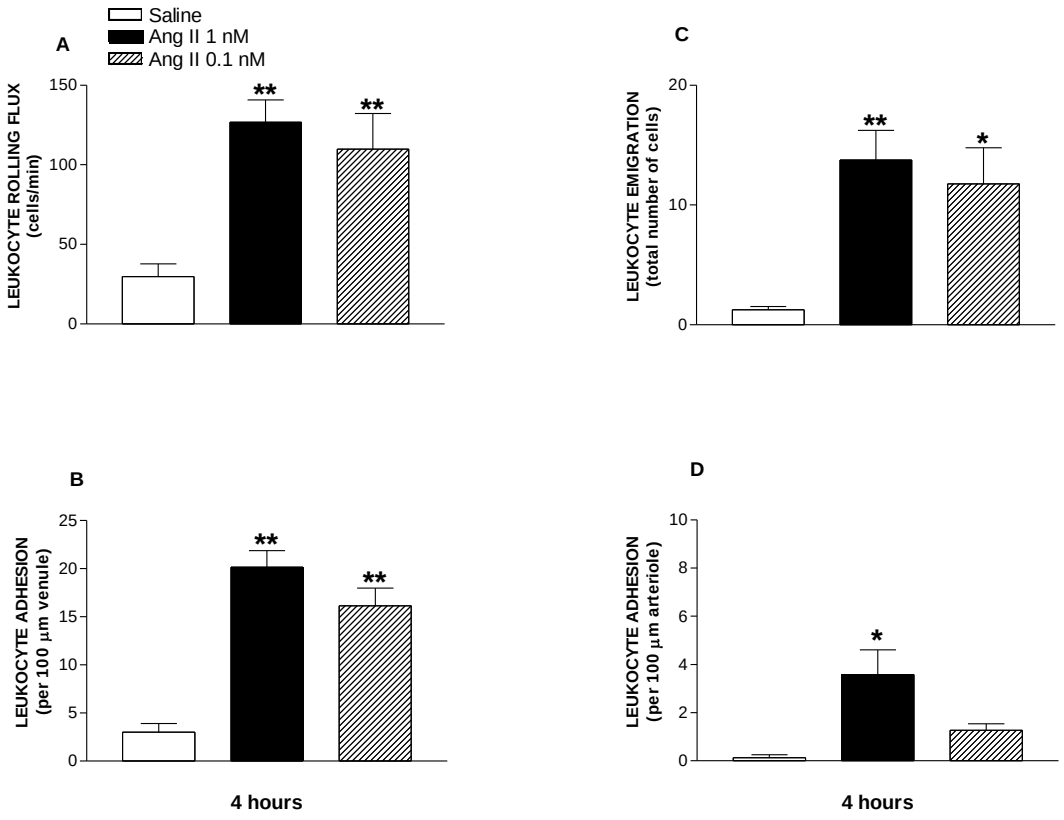
[c]Table 3 shows systemic leukocyte counts in animals receiving mAb treatments before intraperitoneal administration of Ang-II 1 nM. Rats were treated intraperitoneally with saline (n=6) or Ang-II 1 nM (n=6). In further groups of animals, 15 minutes before intraperitoneal administration of Ang-II 1 nM, rats were treated with RMP-1 (n=5) or with RME-1 (n=5) or with a combination of both (n=4) or with TA-2 (n=5) or with 5F10 (n=4) or with WT-3 (n=4) or with 1A29 (n=5). 4 h later, the mesenteric tissue was exteriorized and superfused with buffer in the first group of rats and with Ang-II 1 nM in the other groups and the responses were evaluated. *$p<0.05$ or **$p<0.01$ relative to the saline group. +$p<0.05$ or ++$p<0.01$ relative to the Ang-II 1 nM group. All values are mean±SEM.

Table 4. Leukocyte rolling velocity, hemodynamic parameters and systemic leukocyte counts [d].

	Treatment		
4 h	**Saline**	**L-NAME**	**L-NAME + losartan**
Leukocyte rolling velocity (μm/sec)	81.6±14.6	29.2±2.3**	90.5±12.2++
MABP (mm Hg)	108.9±16.6	102.7±14.8	118.3±12.0
Venule Shear rate (s^{-1})	774.3±181.7	431.4±52.4*	546.0±49.1
Arteriolar Shear rate (s^{-1})	1019.2±71.7	1423.8±419.3	1039.8±169.5
Systemic leukocyte counts (cell/μl)	9500.0±1288.4	8400.0±794.4	12640.0±4155.1

[d]Table 4 summarizes leukocyte rolling velocity, hemodynamic parameters and systemic leukocyte counts in rats treated intraperitoneally with saline (n=6) or with L-NAME 100 μM (n=5). In a further group of animals, 15 minutes before intraperitoneal administration of L-NAME 100 μM, rats were pretreated with an AT_1 Ang-II receptor antagonist, losartan (n=5). 4 h later, the mesenteric tissue was exteriorized and superfused with buffer in the first group of rats and with L-NAME 100 μM in the other two groups and the responses were evaluated. All values are mean±SEM. *$p<0.05$ or **$p<0.01$ relative to the saline group. +$p<0.05$ or ++$p<0.01$ relative to the L-NAME 100 μM group. All values are mean±SEM.

Figure 1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Ang II 0.1 nM
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150
100
50
0
**
**
C
LEUKOCYTE EMIGRATION (total number of cells)
20
10
0
**
*
B
LEUKOCYTE ADHESION (per 100 μm venule)
25
20
15
10
5
0
**
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4 hours
D
LEUKOCYTE ADHESION (per 100 μm arteriole)
10
8
6
4
2
0
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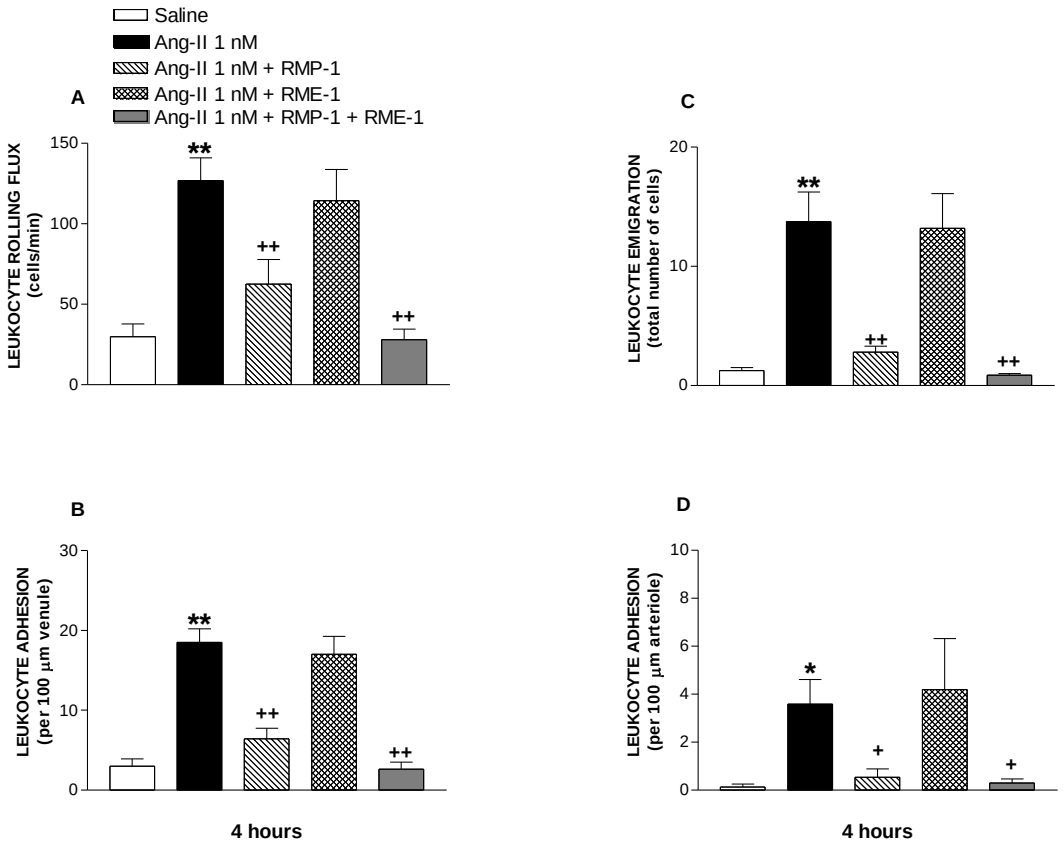
4 hours

Figure 2


Saline
Ang-II 1 nM
Ang-II 1 nM + RMP-1
Ang-II 1 nM + RME-1
Ang-II 1 nM + RMP-1 + RME-1
A
LEUKOCYTE ROLLING FLUX (cells/min)
150
100
50
0
**
++
++
C
LEUKOCYTE EMIGRATION (total number of cells)
20
10
0
**
++
++
B
LEUKOCYTE ADHESION (per 100 µm venule)
30
20
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++
++
4 hours
D
LEUKOCYTE ADHESION (per 100 µm arteriole)
10
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4 hours

Figure 3

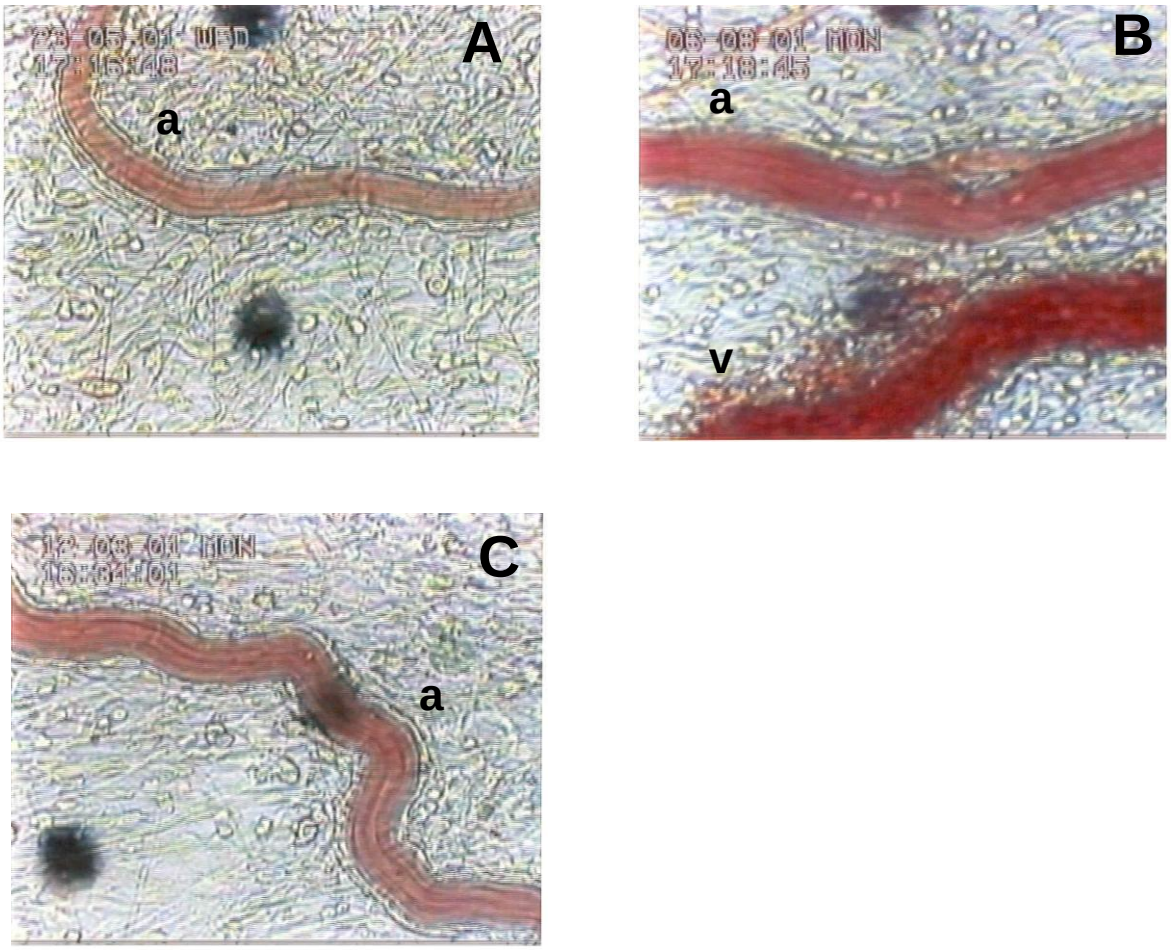
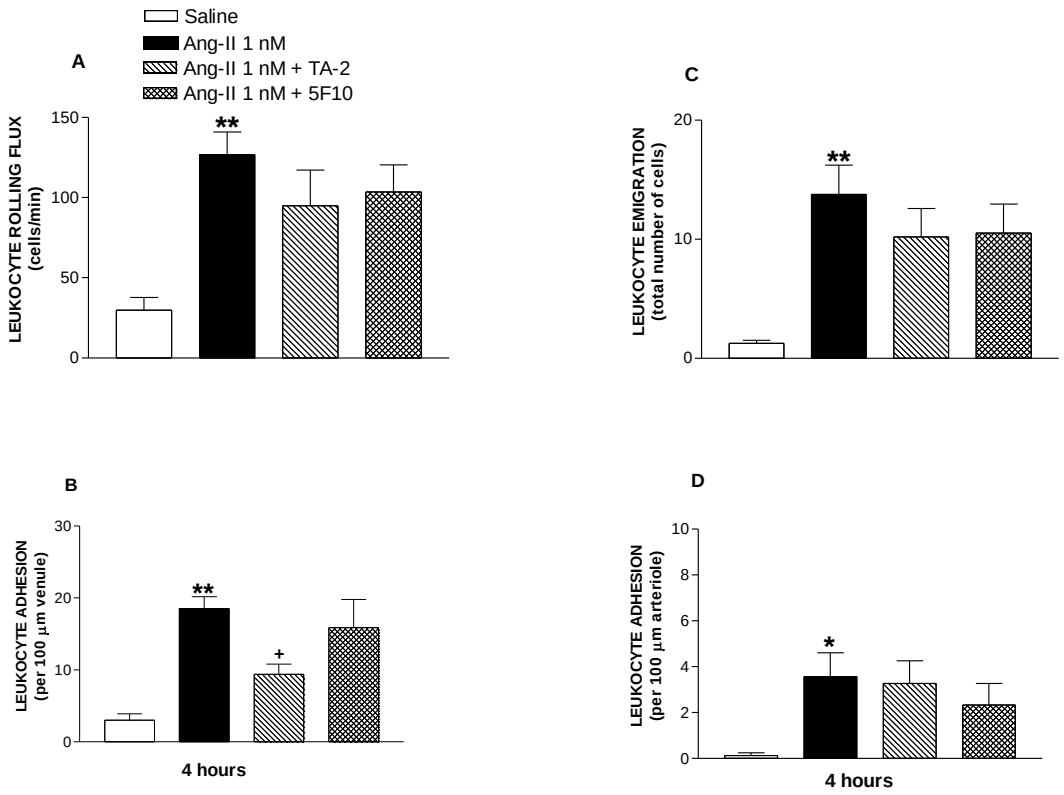


Figure 4


Saline
Ang-II 1 nM
Ang-II 1 nM + TA-2
Ang-II 1 nM + 5F10
A
LEUKOCYTE ROLLING FLUX (cells/min)
150
100
50
0
**
C
LEUKOCYTE EMIGRATION (total number of cells)
20
10
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B
LEUKOCYTE ADHESION (per 100 µm venule)
30
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LEUKOCYTE ADHESION (per 100 µm arteriole)
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4 hours

Figure 5

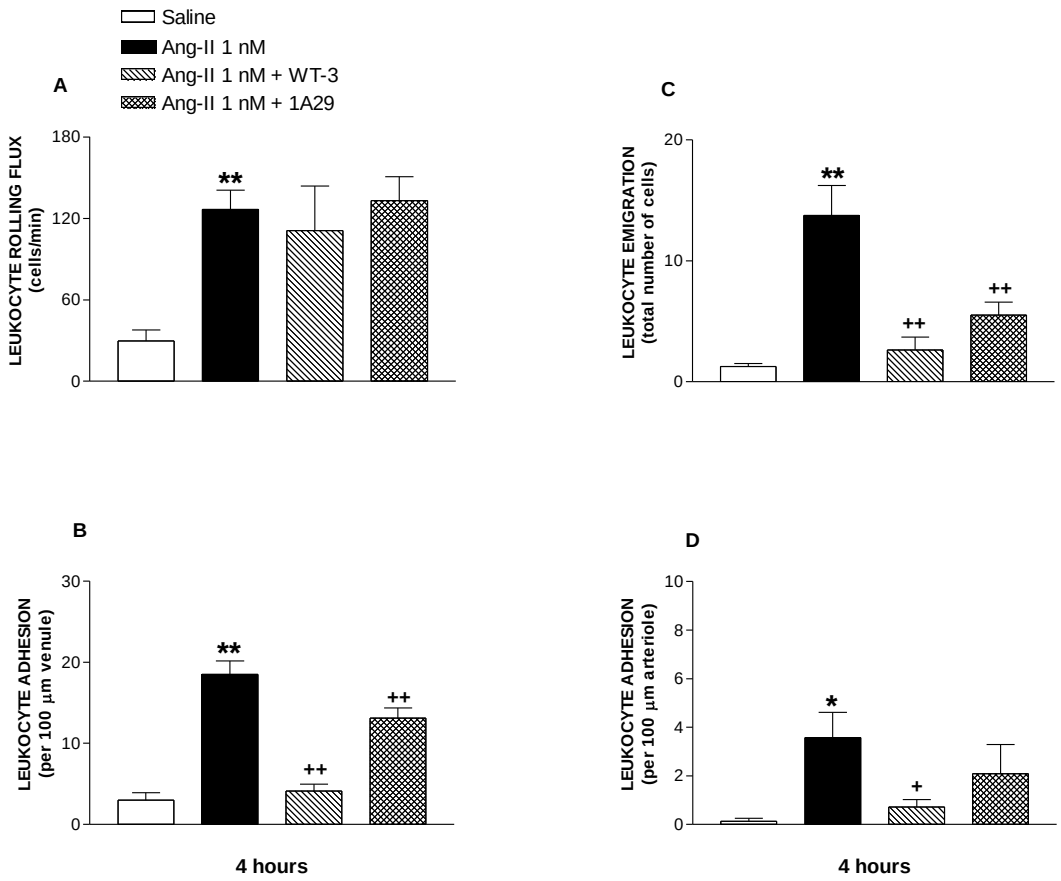
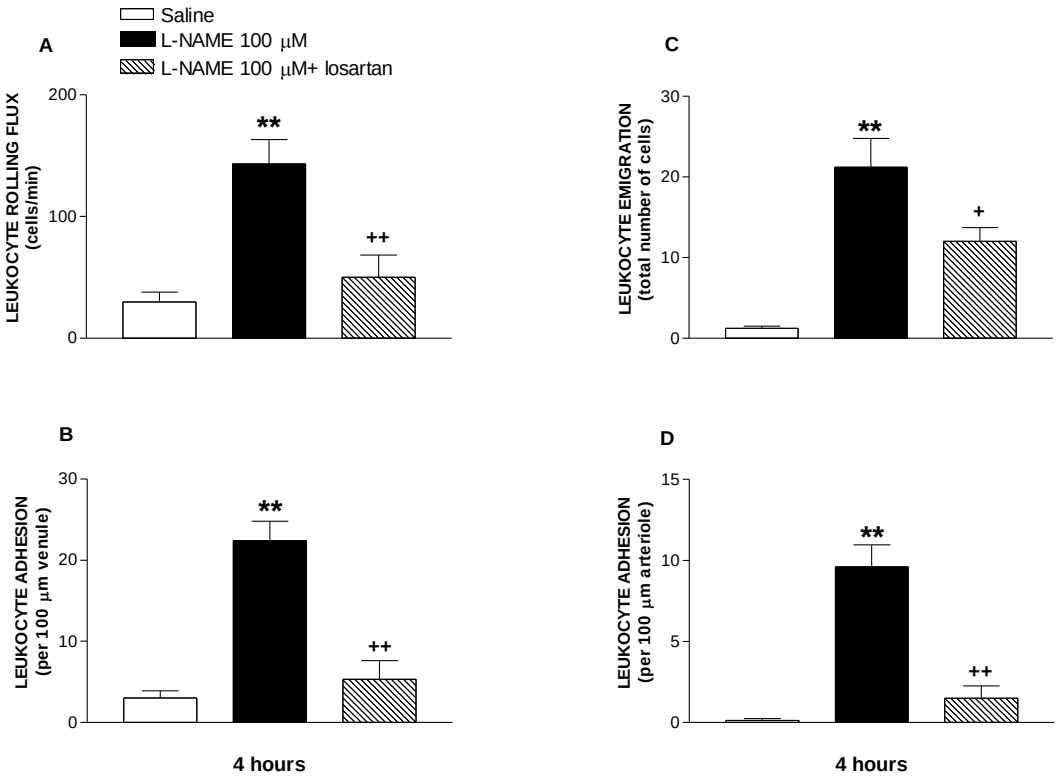

Saline
Ang-II 1 nM
Ang-II 1 nM + WT-3
Ang-II 1 nM + 1A29
A
LEUKOCYTE ROLLING FLUX (cells/min)
180
120
60
0
**
C
LEUKOCYTE EMIGRATION (total number of cells)
20
10
0
**
++
++
B
LEUKOCYTE ADHESION (per 100 µm venule)
30
20
10
0
**
++
++
4 hours
D
LEUKOCYTE ADHESION (per 100 µm arteriole)
10
8
6
4
2
0
*
+
4 hours

Figure 6


Saline
L-NAME 100 μM
L-NAME 100 μM+ losartan
A
LEUKOCYTE ROLLING FLUX (cells/min)
200
100
0
**
++
C
LEUKOCYTE EMIGRATION (total number of cells)
30
20
10
0
**
+
B
LEUKOCYTE ADHESION (per 100 μm venule)
30
20
10
0
**
++
4 hours
D
LEUKOCYTE ADHESION (per 100 μm arteriole)
15
10
5
0
**
++
4 hours

II.4 ARTÍCULO 4

Cyclic AMP elevating agents and nitric oxide modulate angiotensin II-induced leukocyte-endothelial cell interactions in vivo

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Cyclic AMP elevating agents and nitric oxide modulate angiotensin II-induced leukocyte-endothelial cell interactions *in vivo*

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1 Angiotensin (Ang-II) is a key molecule in the development of cardiac ischaemic disorders and displays proinflammatory activity *in vivo*. Since intracellular cyclic nucleotides elevating agents have proved to be effective modulators of leukocyte recruitment, we have evaluated their effect on Ang-II-induced leukocyte-endothelial cell interactions *in vivo* using intravital microscopy within the rat mesenteric microcirculation.

2 Pretreatment with iloprost significantly inhibited (1 nM) Ang-II-induced increase in leukocyte rolling flux, adhesion and emigration at 60 min by 96, 92 and 90% respectively, and returned leukocyte rolling velocity to basal levels. Pretreatment with salbutamol or co-superfusion with forskolin exerted similar effects.

3 When theophylline was administered, leukocyte rolling flux, adhesion and emigration elicited by Ang-II were significantly attenuated by 81, 89 and 71% respectively. Rolipram administration caused similar reduction of Ang-II-induced leukocyte responses.

4 Co-superfusion of Ang-II with the NO-donor, spermine-NO, or 8-Br-cyclic GMP, or pretreatment with a transdermal nytroglycerin patch, resulted in a significant reduction of the leukocyte-endothelial cell interactions elicited by Ang-II.

5 Salbutamol preadministration did not modify leukocyte-endothelial cell interactions elicited by either L-NAME or L-NAME + Ang-II, indicating that the inhibitory leukocyte effects caused by cyclic AMP-elevating agents are mediated through NO release.

6 In conclusion, we have provided evidence that cyclic AMP elevating agents and NO donors, are potent inhibitors of Ang-II-induced leukocyte-endothelial cell interactions. Thus, they could constitute a powerful therapeutical tool in the control of the leukocyte recruitment characteristic of the vascular lesions that occur in cardiovascular disease states where Ang-II plays a critical role. *British Journal of Pharmacology* (2001) **133,** 485–494

Keywords: Angiotensin II; nitric oxide; cyclic AMP; cyclic GMP; leukocyte; endothelium; intravital microscopy

Abbreviations: CAM, cell adhesion molecule; cyclic AMP, adenosine 3′:5′-cyclic monophosphate; cyclic GMP, guanosine 3′:5′-cyclic monophosphate; DMSO, dimethylsulphoxide; Dv, venular diameter; HLMVECs, human lung microvascular endothelial cells; HUVECs, human umbilical vein endothelial cells; ICAM-1, intercellular adhesion molecule-1; L-NAME , N^G-nitro-L-arginine methyl ester, PDE, phosphodiesterase; PGI_2, prostacyclin; PKA, proteinkinase A; TNFα, tumour necrosis factor-α; VCAM-1, vascular cell adhesion molecule-1; V_{mean}, mean red blood cell velocity; V_{rbc}, centreline red blood cell velocity; V_{wbc}, leukocyte rolling velocity

Introduction

Local leukocyte recruitment to the vessel wall is a hallmark of the early stages of atherosclerosis, acute myocardial infarction and several renal diseases of diverse aetiology (Badimon *et al*., 1993; Ricevuti *et al*., 1990; Klahr *et al*., 1988). Clinical observations suggest a link between augmented renin-angiotensin system activity and the development of cardiac ischemic disorders (Brunner *et al*., 1972; Cambien *et al*., 1992). In this context, we have recently revealed that angiotensin II (Ang-II) shows proinflammatory activity *in vivo* at sub-vasoconstrictor doses. In particular, it induces leukocyte trafficking into the rat mesenteric microvasculature through endothelial P-selectin up-regulation in the vessel wall (Piqueras *et al*., 2000). These findings indicate that inappropriate leukocyte-endothelial cell interactions may occur prior to hypertension and that Ang-II contributes to the onset and progression of the vascular damage associated with cardiovascular disease states such as hypertension, atherosclerosis and myocardial ischaemia-reperfusion injury.

Recent interest has focused on the intracellular signalling mechanisms involved in the regulation of cell adhesion molecule (CAM) expression as a tool for modulating inflammation. In a wide range of cells and tissues, adenosine 3′:5′-cyclic monophosphate (cyclic AMP) has proved to be an important target. Increased levels of this second messenger within the cells activates proteinkinase A (PKA) which, in turn, phosphorylates other substrates and has been shown to have anti-inflammatory effects (Giembycz & Raeburn, 1991; Teixeira *et al*., 1997).

In this way, prostacyclin (PGI_2) exerts its action through activation of adenylate cyclase and the consequent increase in

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cyclic AMP levels. Similarly, interaction with β_2-adrenoceptors is a transcriptional effect mediated through elevation of this second intracellular messenger. There is evidence that both exert anti-inflammatory effects by, among other actions, suppressing tumour necrosis factor-α (TNFα) production, leukocyte recruitment and CAM expression (Kowala *et al.*, 1993; Severn *et al.*, 1992; Teixeira & Hellewell, 1997; Derian *et al.*, 1995). On the other hand, phosphodiesterases (PDEs) are enzymes responsible for the breakdown of cyclic nucleotides within cells (Nicholson *et al.*, 1991) and inhibition of PDE enzymes has gained strong support as a possible mechanism of theophylline action (Kuehl *et al.*, 1987). Moreover, inflammatory cells contain mainly PDE type IV (PDE–IV) isoenzyme (cyclic AMP-specific) which accounts for most of the metabolism of cyclic AMP in these cells and is selectively inhibited by rollipram. Indeed, theophylline and rolipram have been shown to decrease leukocyte recruitment and function and cause downregulation of both leukocyte and endothelial CAMs expression (Spoelstra *et al.*, 1998; Braun *et al.*, 1997; Okubo *et al.*, 1997; Santamaria *et al.*, 1997; Teixeira *et al.*, 1997; Blease *et al.*, 1998). Finally, production and release of NO stimulates guanylyl cyclase, increasing guanosine 3′:5′-cyclic monophosphate (cyclic GMP) formation, therefore regulating blood pressure, blood flow, platelet aggregation and leukocyte adhesion (Moncada *et al.*, 1991; Radomski *et al.*, 1987; Kubes *et al.*, 1991).

Since agents which elevate cyclic AMP and cyclic GMP are effective modulators of leukocyte recruitment *in vivo*, the present study was undertaken to evaluate whether Ang-II-induced leukocyte-endothelial cell interactions can be reduced or inhibited by cyclic AMP or cyclic GMP elevating agents. To test this hypothesis we used intravital microscopy within the rat mesenteric microcirculation and examined the effect of various agents known to elevate cyclic nucleotides through different mechanisms of action and evaluated their effect on the leukocyte responses elicited by Ang-II. This is of relevance as new therapeutical strategies based on reduced leukocyte recruitment could be used in the control of vascular disease disorders where Ang-II plays a critical role.

Methods

Animal preparation

Male Sprague-Dawley rats (200–250 g) were deprived of food but not water for 20–24 h before each experiment. The animals were anaesthetized with pentobarbital sodium (65 mg kg^{-1}, i.p.) and a tracheotomy was performed to facilitate spontaneous breathing. A polyethylene tube was inserted into the right carotid artery and jugular vein to measure systemic arterial blood pressure (MABP) through a pressure transducer (Spectramed Stathan P-23XL) connected to a recorder (GRASS RPS7C8B, Quincy, MA, U.S.A.) and to enable intravenous administration of additional reagents (anaesthetic or drug) respectively.

Intravital microscopy

The experimental preparation used was similar to that previously described (Sanz *et al.*, 1999). The abdominal cavity was opened *via* a midline incision and a loop of the mid-jejunal mesentery was gently exteriorized and placed carefully on a heated transparent pedestal to allow transillumination of a 3 cm^2 segment of the tissue. Pedestal and animal temperature were maintained at 37°C. The exposed mesentery was superfused continuously at a rate of 1–2 ml min^{-1} with a warmed bicarbonate-buffered saline solution (pH 7.4). All exposed tissue was covered with saline-soaked gauze to prevent evaporation. An orthostatic microscope (Nikon Optiphot-2, SMZ1, Badhoevedorp, The Netherlands) with a 20 × objective lens and a 10 × eyepiece (Nikon SLDW, Badhoevedorp, The Netherlands) was used to observe the mesenteric microcirculation. A video camera (Sony SSC-C350P, Koeln, Germany) mounted on the microscope projected the image onto a color monitor (Sony Trinitron PVM-14N2E, Koeln, Germany) and the images were video recorded (Sony SVT-S3000P, Koeln, Germany) for play back analysis. The final magnification of the video screen was 1300 ×.

Single unbranched mesenteric venules with diameters of 25–40 μm were selected for study. Venular diameter (Dv) was measured on-line with a video caliper (Microcirculation Research Institute, Texas A&M University, College Station, Texas, U.S.A.). Centerline red blood cell velocity (V_{rbc}) was also measured on-line by using an optical Doppler velocimeter (Microcirculation Research Institute, Texas A&M University, College Station, Texas, U.S.A.). Venular blood flow was calculated from the product of mean red blood cell velocity ($V_{mean} = V_{rbc}/1.6$) and microvascular cross-sectional area, assuming cylindrical geometry. Venular wall shear rate (γ) was calculated based on the Newtonian definition: $\gamma = 8 \times (V_{mean}/D_v)$ s^{-1} (House & Lipowsky, 1987).

The number of rolling, adherent and emigrated leukocytes as determined off-line during playback analysis of videotaped images. Rolling leukocytes were defined as those white blood cells moving more slowly than erythrocytes in the same vessel and were determined by counting the number of rolling leukocytes min^{-1} passing a reference point in the microvessel. The same reference point was used throughout the experiment because leukocytes may roll for only a section of the vessel before rejoining the blood flow or becoming firmly adherent. Leukocyte rolling velocity (V_{wbc}) was determined from the time required for a leukocyte to transverse a 100 μm length of the venule and was expressed as μm s^{-1}. A leukocyte was considered to be adherent to venular endothelium if it remained stationary for 30 s or longer. Adherent cells were expressed as the number per 100 μm length of venule. Leukocyte emigration was expressed as the number of white blood cells per microscopic field. The rate of emigration was determined from the difference between the number of any interstitial leukocytes present at the beginning of the experiment and those present at the end of the experiment.

Experimental protocol

After a 30 min stabilization period, baseline measurements (time 0) of mean arterial blood pressure (MABP), V_{rbc}, vessel diameter, shear rate, leukocyte rolling flux and velocity and leukocyte adhesion and emigration were taken. The superfusion buffer was then supplemented with Ang-II (1 nM) and recordings were performed for 5 min at 15 min intervals over a 60 min period during which the aforementioned leukocyte and haemodynamic parameters were measured.

In the first group of experiments, animals were pretreated with the membrane receptor-dependent prostacyclin analogue iloprost (3 μg kg^{-1}, i.v.), 5 min before and 30 min after Ang-II (1 nM) suffusion, to maintain adequate plasma levels of the drug. A second group of animals was pretreated with the β_2-adrenoceptor agonist salbutamol (1 mg kg^{-1}, i.v.), 10 min before Ang-II (1 nM) superfusion. In a further group of rats, a direct adenylate cyclase activator, forskolin (1 μM) was applied topically 10 min prior to Ang-II suffusion. Forskolin was co-superfused with Ang-II to avoid the toxic effects which develop after systemic administration of this drug. The doses used for the different treatments were based on those applied in previous *in vivo* studies (Harada *et al*., 1999; Altenburg *et al*., 1994; Frisbee *et al*., 1999).

To determine the effect of PDE enzymes inhibition on leukocyte infiltration elicited by Ang-II, two PDE inhibitors were used. In the first set of experiments, animals were pretreated 10 min before Ang-II suffusion with the non-specific PDE inhibitor theophylline (5 mg kg^{-1}, i.v.). In the second group of rats, selective PDE-IV inhibitor rolipram (8 mg kg^{-1}, i.p.) was administered 30 min prior to Ang-II suffusion to achieve appropriate plasma levels of its active metabolite. Rolipram was dissolved in dimethylsulphoxide (DMSO) and diluted further in saline as previously described (Teixeira *et al*., 1994). The same percentage of DMSO was given i.p. to the rolipram control group, 30 min before Ang-II superfusion. Similarly, the doses administered were those used in previous *in vivo* studies (Teixeira *et al*., 1994; Elwood *et al*., 1995).

To investigate the ability of nitric oxide to modulate Ang-II-induced leukocyte-endothelial cell interactions, the nitric oxide donor spermine-NO was used. This compound (100 μM) was co-superfused with Ang-II, which allowed a slow release of NO and, through very local administration, complications due to alterations in haemodynamic parameters were avoided. As many actions of NO are due to the activation of soluble guanylate cyclase, a series of experiments were carried out to examine the role of cyclic GMP in leukocyte responses induced by Ang-II. The cyclic GMP analog 8-Br-cyclic GMP (100 μM) was also co-superfused with Ang-II and the effects determined. The doses administered in this experiment are the same used to inhibit leukocyte-endothelial cell interactions induced by CINC/gro in the rat mesenteric microcirculation (Johnston *et al*., 1999).

To determine the possibility of using nitroglycerin to control the leukocyte recruitment elicited by Ang-II, the effect of a transdermal nitroglycerin patch was assayed in this system. The patch was applied to the dorso-cervical area of the body which had been shaved under light ether anaesthesia the day before. Patches were cut to release a nitroglycerin dose of 160 ng min^{-1} rat^{-1} which has previously been proved to inhibit leukocyte-endothelial cell interactions induced by indomethacin (Calatayud *et al*., 1999). Control animals were treated with placebo patches of equal size. Both treatments were applied before beginning surgical procedures and were maintained in place until the end of the experiment.

Finally, to investigate whether the effects of cyclic AMP elevation on leukocyte responses are mediated through endothelial NO release, another set of experiments were carried out. In the first group of animals, after the initial 30 min stabilization period, the superfusion buffer was changed to one containing L-NAME (100 μM). Previous

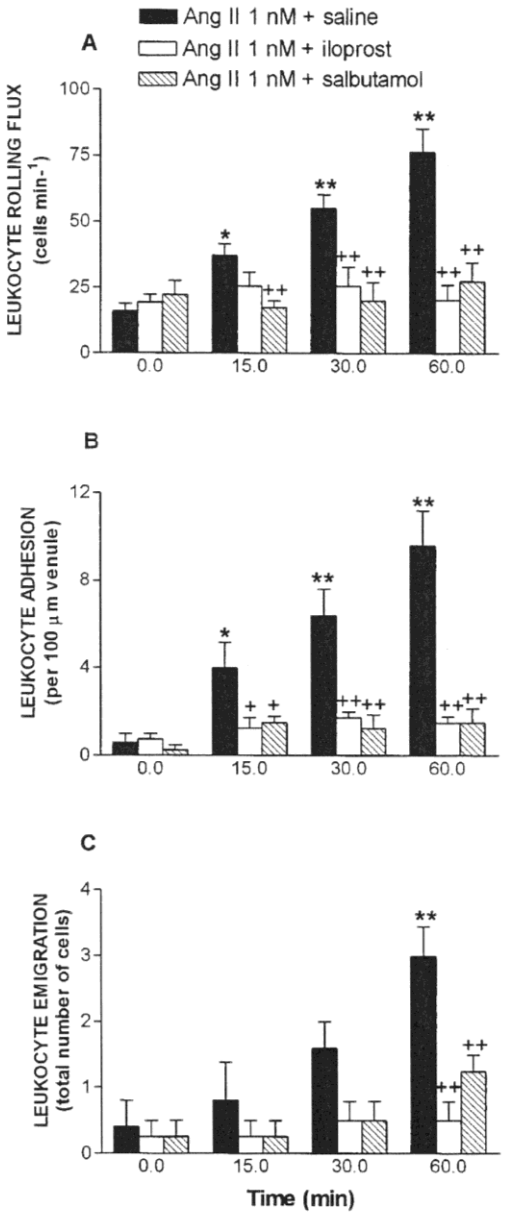

Figure 1 Effect of the prostacyclin analogue iloprost and the β_2-receptor agonist salbutamol on Ang-II-induced leukocyte rolling flux (A), leukocyte adhesion (B) and leukocyte emigration (C) in rat mesenteric postcapillary venules. Parameters were measured 0, 15, 30 and 60 min after superfusion with Ang-II (1 nM) in animals untreated ($n=5$) or pretreated with iloprost (3 μg kg^{-1}, i.v., $n=5$) or with salbutamol (1 mg kg^{-1}, i.v., $n=5$). Results are represented as mean$\pm$s.e.mean. *$P<0.05$ or **$P<0.01$ relative to the control value (0 min) in the untreated group. +$P<0.05$ or ++$P<0.01$ relative to the untreated group.

studies have demonstrated that this dose causes a consistent increase in leukocyte rolling and adhesion over a 60 min time course (Arndt *et al.*, 1993). A second group of animals was pretreated with the β_2-adrenoceptor agonist salbutamol (1 mg kg^{-1}, i.v.) 10 min before L-NAME superfusion, and a third group of animals pretreated with salbutamol at the same conditions, was then co-superfused with L-NAME and Ang-II (1 nM).

Statistical analysis

All data are expressed as mean ± s.e.mean. The data within groups were compared using an analysis of variance (one way-ANOVA) with a Newman-Keuls *post hoc* correction for multiple comparisons. A P value <0.05 was considered to be statistically significant.

Materials

Ang-II, salbutamol, forskolin, theophylline, rolipram, spermine-NO, 8-Br-cyclic GMP, L-NAME and DMSO were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.. Placebo and nitroglycerin patches (Nitro-Dur® 15) and iloprost (Ilomedin®) were generously donated by Schering-Plough Laboratories, Spain.

Results

Figure 1 illustrates the effect of Ang-II-induced leukocyte responses. Significant increases in leukocyte rolling flux (76.4 ± 8.9 vs 16.0 ± 2.9 cells min^{-1}), adhesion (9.6 ± 1.6 vs 0.6 ± 0.4 cells 100 μm^{-1}) and emigration (3.0 ± 0.4 vs 0.4 ± 0.4 cells field^{-1}) were observed at 60 min in animals subjected to 1 nM Ang-II superfusion vs buffer. Pretreatment with iloprost or salbutamol attenuated the Ang-II induced leukocyte rolling flux, adhesion and emigration and were inhibited by 96, 92 and 90% respectively when animals were pretreated with iloprost and by 83, 86 and 62% respectively, in the group pretreated with salbutamol after 60 min Ang-II suffusion. In addition, the decrease in leukocyte rolling velocity induced by Ang-II at 60 min was reversed by the administration of either of the agents under investigation (Table 1).

Figure 2 presents the effects of the direct adenylate cyclase activator forskolin on the leukocyte-endothelial cell interactions elicited by mesenteric exposure to 1 nM Ang-II. At

Table 1 Leukocyte rolling velocity in animals untreated and treated with cyclic AMP elevating agents before (0 min) and after (60 min) Ang-II superfusion (1 nM)

Treatment	*0 min*	*60 min*
Untreated animals	94.9 ± 13.2	36.0 ± 4.8**
Iloprost	105.3 ± 1.0	92.9 ± 15.1
Salbutamol	159.3 ± 35.3	128.8 ± 58.8
Forskolin	122.4 ± 8.7	105.0 ± 12.6
Theophylline	99.5 ± 3.8	114.8 ± 21.3
Untreated (rolipram vehicle)	138.1 ± 19.0	79.5 ± 15.8*
Rolipram	143.7 ± 17.4	142.6 ± 40.8

*$P<0.05$ or **$P<0.01$ relative to the control group (0 min). All values are mean ± s.e.mean.

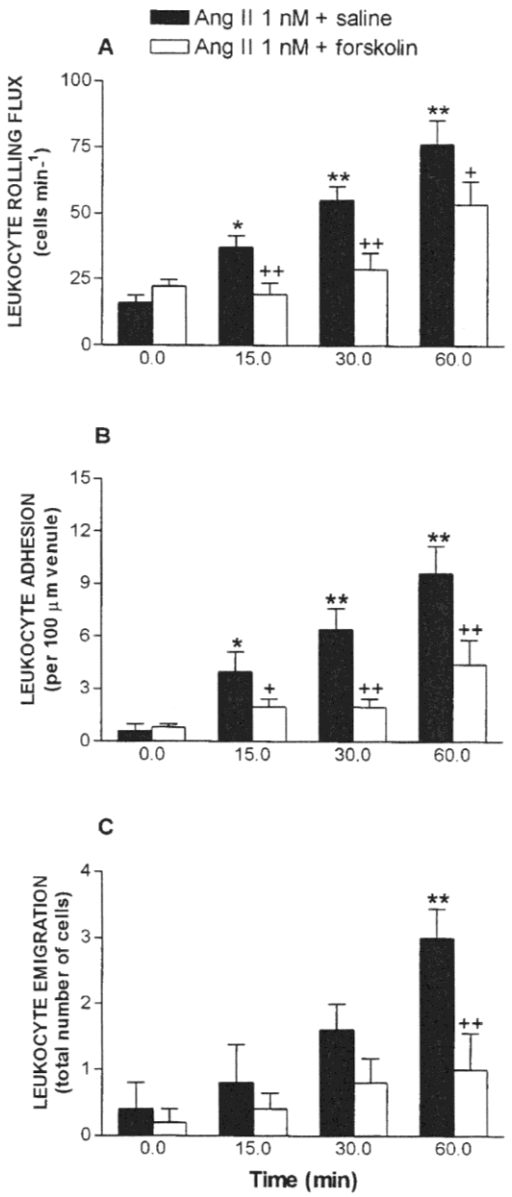

Figure 2 Effect of the adenylyl cyclase activator forskolin on Ang-II-induced leukocyte rolling flux (A), leukocyte adhesion (B) and leukocyte emigration (C) in rat mesenteric postcapillary venules. Parameters were measured 0, 15, 30 and 60 min after superfusion with Ang-II (1 nM) in animals untreated ($n=5$) or treated with forskolin (0.1 μM, $n=5$). Results are represented as mean ± s.e.mean. *$P<0.05$ or **$P<0.01$ relative to the control value (0 min) in the untreated group. +$P<0.05$ or ++$P<0.01$ relative to the untreated group.

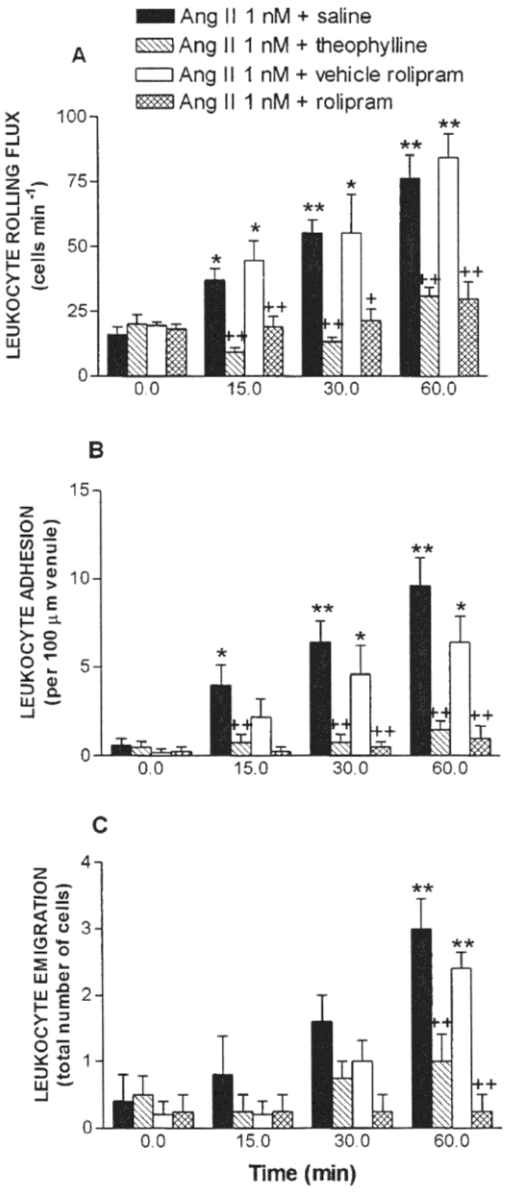

Figure 3 Effect of the PDE IV inhibitor rolipram and the non-specific PDE inhibitor theophylline on Ang-II-induced leukocyte rolling flux (A), leukocyte adhesion (B) and leukocyte emigration (C) in rat mesenteric postcapillary venules. Parameters were measured 0, 15, 30 and 60 min after superfusion with Ang-II (1 nM) in animals untreated (rolipram vehicle, $n=5$) or pretreated with rolipram (8 mg kg^{-1}, i.p., $n=5$) and in animals untreated ($n=5$) or pretreated with theophylline (5 mg kg^{-1}, i.v., $n=5$). Results are represented as mean ± s.e.mean. *$P<0.05$ or **$P<0.01$ relative to the control value (0 min) in the untreated group. +$P<0.05$ or ++$P<0.01$ relative to the untreated group.

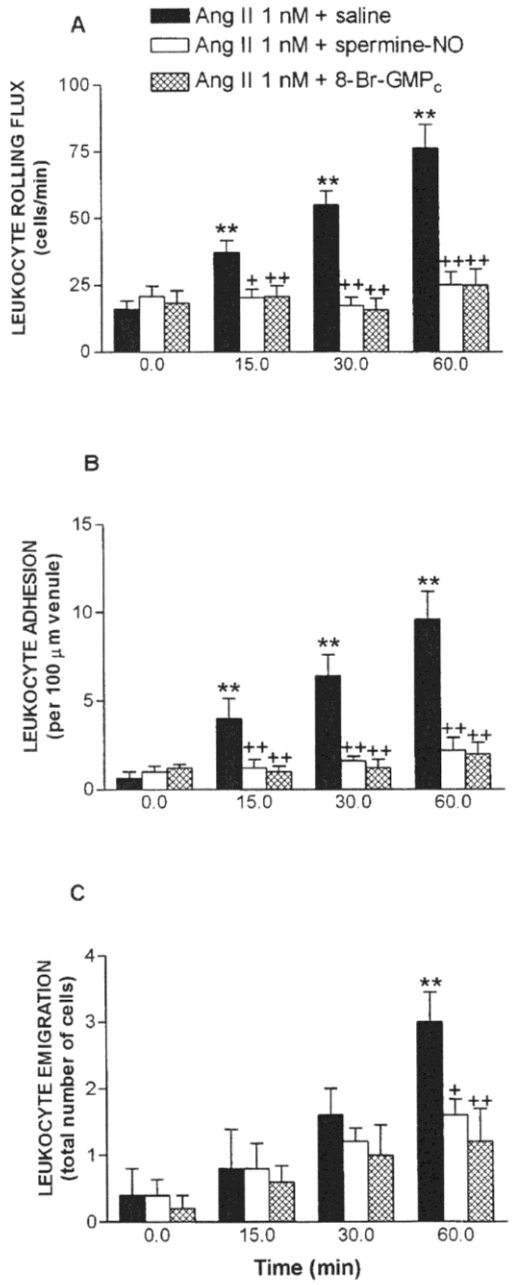

Figure 4 Effect of the NO donor spermine-NO and the cyclic GMP analog 8-Br-cyclic GMP on Ang-II-induced leukocyte rolling flux (A), leukocyte adhesion (B) and leukocyte emigration (C) in rat mesenteric postcapillary venules. Parameters were measured 0, 15, 30 and 60 min after superfusion with Ang-II (1 nM) in animals untreated ($n=5$) or co-superfused with spermine-NO (100 μM, $n=5$) or with 8-Br-cyclic GMP (100 μM, $n=5$). Results are represented as mean ± s.e.mean. *$P<0.05$ or **$P<0.01$ relative to the control value (0 min) in the untreated group. +$P<0.05$ or ++$P<0.01$ relative to the untreated group.

60 min, forskolin significantly reduced Ang-II-induced leukocyte rolling flux, adhesion and emigration by 48, 60 and 69% respectively. As previously found with iloprost and salbutamol, forskolin maintained leukocyte rolling velocity at basal levels when co-suffused with Ang-II (Table 1).

Figure 3 shows the effects of theophylline and rolipram on Ang-II induced leukocyte endothelial cell interactions. Theophylline pretreatment significantly reduced Ang-II-induced leukocyte rolling flux, adhesion and emigration by 81, 89 and 71% respectively after 60 min Ang-II superfusion. In animals pretreated with rolipram vehicle, similar values of leukocyte rolling flux (84.4±9.1 vs 19.6±1.3 cells min^{-1}), adhesion (6.4±1.5 vs 0.2±0.2 cells 100 μm^{-1}) and emigration (2.4±0.3 vs 0.2±0.2 cells $field^{-1}$) to those obtained in the Ang-II untreated group were detected. Rolipram pretreatment provoked significant reductions in leukocyte responses elicited after 60 min Ang-II suffusion, inhibiting these parameters by 80, 84 and 90% respectively. Again, decreases in leukocyte rolling velocity elicited by Ang-II were totally reversed by administration of theophylline and rolipram (Table 1).

Figure 4 summarizes the effect of the NO donor, spermine-NO and the cyclic GMP analogue, 8-Br-cyclic GMP on leukocyte recruitment caused by Ang-II. Co-superfusion of Ang-II with spermine-NO resulted in a significant reduction of the leukocyte rolling flux (83.4%), adhesion (82.2%) and emigration (57.3%) provoked by 60 min suffusion with Ang-II. Similarly, when Ang-II was co-superfused with 8-Br-cyclic GMP, these three parameters were inhibited by 83.3, 91.1 and 61.5% respectively. In addition, Table 2 shows that the diminution in leukocyte rolling velocity provoked by 60 min superfusion with Ang-II, was reversed to basal values when it was co-superfused with either spermine-NO or 8-Br-cyclic GMP.

Furthermore, as shown in Figure 5, systemic pretreatment with the transdermal nitroglycerin patch (160 ng min^{-1} rat^{-1}) evoked significant changes in leukocyte rolling flux, even in the basal value when compared with that detected in the placebo treated group (7.8±1.5 cells min^{-1} vs 19.6±4.4 cells min^{-1} respectively). In the group pretreated with the placebo patch, Ang-II superfusion induced a significant increase in leukocyte rolling flux (80.8±6.1 vs 19.6±4.4 cells min^{-1}), adhesion (12.0±1.6 vs 1.4±0.4 cells 100 μm^{-1}) and emigration (3.2±0.6 vs 0.2±0.2 cells $field^{-1}$) vs buffer after 60 min superfusion, while showing no differences with respect to the effect of Ang-II superfusion in untreated animals. Treatment with the nitroglycerin patch induced a significant inhibition in all three parameters by 94,

Table 2 Leukocyte rolling velocity in animals untreated and treated with cyclic GMP elevating agents before (0 min) and after (60 min) Ang-II superfusion (1 nM)

Treatment	*0 min*	*60 min*
Untreated animals	94.9±13.2	36.0±4.8**
Spermine-NO	102.9±7.1	105.0±13.2
8-Br-cyclic GMP	100.5±2.6	120.6±8.1
Untreated (placebo patch)	100.1±5.4	45.0±4.0**
Nitroglycerin patch	156.3±25.8	161.0±67.0

*$P<0.05$ or **$P<0.01$ relative to the control group (0 min). All values are mean±s.e.mean.

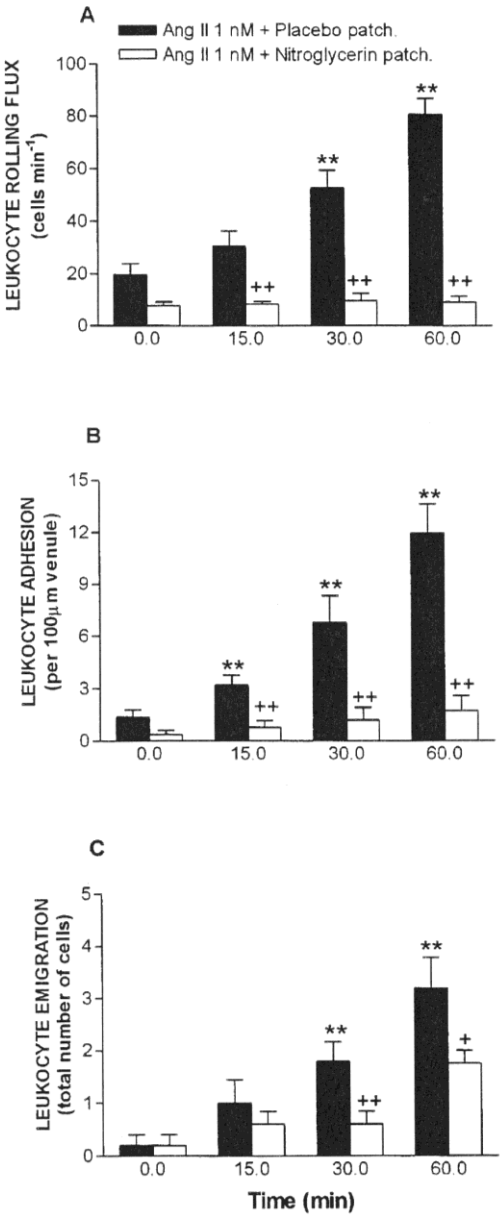

Figure 5 Effect of the nitric oxide donor nitroglycerin on Ang-II-induced leukocyte rolling flux (A), leukocyte adhesion (B) and leukocyte emigration (C) in rat mesenteric postcapillary venules. Parameters were measured 0, 15, 30 and 60 min after superfusion with Ang-II (1 nM) in animals pretreated with placebo patch ($n=5$) or with nitroglycerin patch (160 ng min^{-1} rat^{-1}, $n=5$). Results are represented as mean±s.e.mean. *$P<0.05$ or **$P<0.01$ relative to the control value (0 min) in the untreated group. +$P<0.05$ or ++$P<0.01$ relative to the untreated group.

85 and 50% respectively after 60 min Ang-II suffusion, returning leukocyte rolling flux and adhesion to levels detected in basal conditions. In addition, treatment with the nitroglycerin patch also inhibited the Ang-II induced decrease in leukocyte rolling velocity (Table 2).

Tables 3 and 4 summarize the results obtained for MABP and shear rate prior to (0 min) and 60 min following Ang-II superfusion in animals untreated and pretreated with different cyclic AMP or cyclic GMP elevating agents. MABP was unaffected by most of the agents under investigation; only salbutamol pretreatment caused a significant reduction in this hemodynamic parameter, while no changes in blood flow were detected. Similarly, the shear rate remained unchanged throughout Ang-II suffusion in most of the untreated and treated groups; only forskolin co-superfusion provoked a significant decrease in this response at the 60 min time point. Yet, despite this effect, Ang-II-induced leukocyte-endothelial cell interactions were effectively inhibited.

Finally, Figure 6 shows the effect of salbutamol on L-NAME and L-NAME + Ang-II-induced leukocyte-endothelial cell interactions. Interestingly, β_2-adrenoceptor agonist pretreatment did not modify the leukocyte recruitment caused by nitric oxide synthase (NOS) inhibition. In addition, the inhibitory effect of salbutamol on Ang-II-induced leukocyte-endothelial cell interactions was prevented when NOS was inhibited with L-NAME. As previously found, salbutamol pretreatment caused a significant decrease in MABP without affecting the decrease in shear rate provoked by L-NAME (Table 5).

Discussion

Elevated cyclic AMP levels can be attained by either activating adenylate cyclase to increase synthesis or by inhibiting metabolism *via* PDE. In the present study we have demonstrated that cyclic AMP elevating agents which activate adenylate cyclase such as iloprost, salbutamol or forskolin, can significantly reduce Ang-II-induced leukocyte rolling, adhesion and emigration *in vivo*. Similarly, when a non-selective inhibitor of PDEs such as theophylline or a selective PDE-IV inhibitor such as rolipram is administered, leukocyte responses elicited by Ang-II are also dramatically attenuated. Therefore, the ability of these compounds to exert an inhibitory effect on leukocyte-endothelial cell interactions caused by Ang-II occurs regardless of the mechanism used to elevate intracellular cyclic AMP. In addition, we have also shown that treatment of animals with the NO donor, spermine-NO, or the cyclic GMP analogue, 8-Br-GMP or systemic pretreatment with a transdermal nitroglycerin patch, almost abolished Ang-II-induced leukocyte rolling and adhesion. We have also demonstrated that the anti-inflammatory effect exerted by salbutamol disappears when NO synthesis is inhibited. Finally, none of these treatments affect the different haemodynamic parameters, with the exception of salbutamol and forskolin. The former significantly reduce MABP and the latter reduce shear rate after 60 min superfusion with Ang-II. Despite these effects, both significantly reduce leukocyte-endothelial cell interactions elicited by Ang-II.

Among the different drugs used in the present study, iloprost, salbutamol, rolipram, spermine NO, 8-Br-GMP and nitrolglycerin patch treatments exerted similarly powerful inhibitory effects on the leukocyte recruitment elicited by Ang-II. In this context, it has been shown that stimulation of prostacyclin synthesis and the use of prostacyclin agonists or NO donors can impede the leukocyte recruitment associated with cardiac ischaemic injury and the development of atherosclerotic lesions (Hohfeld *et al*., 1993; Kowala *et al*., 1993; Johnson *et al*., 1991; Massoudy *et al*., 1999). In

Table 3 Haemodynamic parameters in animals untreated and treated with cyclic AMP elevating agents before (0 min) and after (60 min) Ang-II superfusion (1 nM)

	MABP (mmHg)		*Shear rate* (s^{-1})	
Treatment	*0 min*	*60 min*	*0 min*	*60 min*
Untreated animals	114.6±11.3	104.6±10.5	600.2±27.1	540.5±117.2
Iloprost	106.7±9.2	102.8±16.4	697.2±38.8	646.6±77.8
Salbutamol	124.4±5.3	102.0±8.6*	655.8±40.9	585.3±35.9
Forskolin	123.3±1.7	130.0±5.0	641.3±53.1	544.6±48.3**
Theophylline	112.1±7.5	114.6±7.5	601.6±47.6	624.8±36.1
Untreated (rolipram vehicle)	125.0±1.7	125.0±1.7	562.7±77.4	572.6±57.9
Rolipram	94.4±5.9	85.0±8.2	572.2±66.6	638.3±102.6

*$P<0.05$ or **$P<0.01$ relative to the control group (0 min). All values are mean±s.e.mean.

Table 4 Haemodynamic parameters in animals untreated and treated with cyclic GMP elevating agents before (0 min) and after (60 min) Ang-II superfusion (1 nM)

	MABP (mmHg)		*Shear rate* (s^{-1})	
Treatment	*0 min*	*60 min*	*0 min*	*60 min*
Untreated animals	114.6±11.3	104.6±10.5	600.2±27.1	540.5±117.2
Spermine-NO	117.9±6.7	125.0±9.6	694.9±68.0	650.7±74.0
8-Br-cyclicGMP	126.0±5.1	128.7±7.4	642.0±51.4	571.6±61.6
Untreated (placebo patch)	117.7±5.4	121.0±7.7	675.6±98.8	573.4±158.7
Nitroglycerin patch	122.2±8.7	108.3±2.9	665.1±54.9	725.8±92.6

*$P<0.05$ or **$P<0.01$ relative to the control group (0 min). All values are mean±s.e.mean.

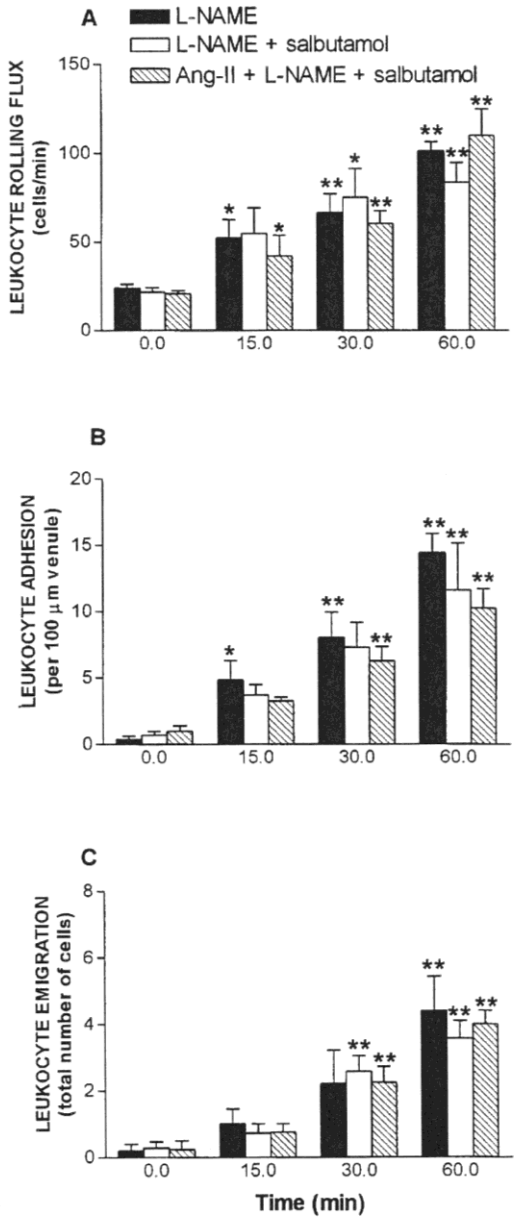

Figure 6 Effect of salbutamol pretreatment on L-NAME and L-NAME + Ang-II-induced leukocyte rolling flux (A), leukocyte adhesion (B) and leukocyte emigration (C) in the rat mesenteric postcapillary venules. Parameters were measured 0, 15, 30 and 60 min after superfusion with L-NAME (100 μM) or L-NAME + Ang-II (1 nM) in animals untreated ($n=5$) or pretreated with salbutamol (1 mg kg^{-1}, $n=5$ in both groups). Results are presented as mean ± s.e.mean. $^{*}P<0.05$ or $^{**}P<0.01$ relative to the control value (0 min) in the untreated group.

contrast, little is known about the effectiveness of β_2-adrenoceptor agonists or PDE-IV inhibition treatments in the control of inflammatory responses associated with cardiovascular diseases. With regard to this, in the present study, we have observed the effect displayed by salbutamol, which in addition to its inhibitory effect on leukocyte-endothelial cell interactions elicited by Ang-II, also reduced arterial blood pressure. Therefore, salbutamol or β_2-adrenoceptor agonist therapy may be specially useful in hypertensive states where there exists the risk of development of an atherosclerotic lesion.

The exact mechanisms by which cyclic AMP inhibits leukocyte adhesion to the vascular endothelium is not known, however, one probable mechanism is the modulation of CAM expression. In this context, most of the studies which explore regarding this possibility have used *in vitro* models and contradictory findings have been encountered based on either the origin of the endothelial cells used or the stimulus employed to provoke leukocyte activation. For example, Pober *et al.* (1993) demonstrated that combination treatment with forskolin and the non-specific PDE inhibitor isobutyl methylxanthine, suppressed the induction by cytokines of E-selectin and vascular cell adhesion molecule-1 (VCAM-1). In contrast, Morandini *et al.* (1996) showed that, while rolipram significantly suppressed the expression and release of E-selectin in TNF-α-stimulated human umbilical vein endothelial cells (HUVECs), when combined with forskolin, it had no effect on VCAM-1 expression. Similarly, Blease *et al.* (1998), using human lung microvascular endothelial cells (HLMVECs), found a significant reduction in TNFα-induced E-selectin expression with a combination of rolipram and salbutamol, whereas no effect on intercellular adhesion molecule-1 (ICAM-1) and VCAM-1 expression was detected. In addition, cyclic AMP elevating agents also prevented mediator-induced upregulation of β_2 integrins on the leukocyte surface (Derian *et al.*, 1995; Teixeira *et al.*, 1996; Berends *et al.*, 1997; Santamaria *et al.*, 1997).

Despite these findings, our results strongly suggest that effects displayed *in vitro* should not necessarily correlate with those detected in *in vivo* studies. In fact, we have shown strong inhibitory responses in Ang-II-induced leukocyte-endothelial cell interactions, using an adenylate cyclase activator, a PDE inhibitor, a guanylate cyclase activator or different NO donors, without the need of combining any of these different cyclic nucleotides elevating agents. Additionally, it seems likely that, *in vivo*, downregulation of adhesion molecules other than E-selectin or VCAM-1 are involved in the inhibitory responses observed in the present study. In this context, one possible candidate is P-selectin. Indeed, we have recently demonstrated that Ang-II-induced leukocyte-endothelial cell interactions *in vivo* occur *via* endothelial P-selectin upregulation (Piqueras *et al.*, 2000). Moreover, other studies have also shown that cyclic AMP elevating agents or NO donors can reduce P-selectin expression and release in stimulated platelets (Konstantopoulos *et al.*, 1998; Salas *et al.*, 1994). Furthermore, as witnessed in platelets, prostacyclin treatment in animals subjected to protamine administration provokes a decrease in E and P-selectin levels and a more marked preservation of left ventricular function (Katircioglu *et al.*, 1999). Similarly, treatment with NO donors has been found to attenuate homocysteine-induced P-selectin expression in rat mesenteric venules (Pruefer *et al.*, 1999).

Table 5 Haemodynamic parameters in animals untreated and treated with salbutamol (1 mg kg^{-1}) before (0 min) and after (60 min) L-NAME (100 μM) or L-NAME (100 μM) + Ang-II (1 nM) superfusion

	MABP (mmHg)		*Shear rate* (s^{-1})	
Treatment	*0 min*	*60 min*	*0 min*	*60 min*
L-NAME	115.5 ± 9.6	119.4 ± 10.6	763.2 ± 55.0	392.1 ± 58.6**
Salbutamol + L-NAME	116.4 ± 5.0	99.8 ± 7.6*	653.1 ± 77.1	408.0 ± 103.9*
Salbutamol + L-NAME + Ang-II	109.6 ± 9.7	97.4 ± 11.6*	588.2 ± 20.9	283.3 ± 55.6**

*$P<0.05$ or **$P<0.01$ relative to the control group (0 min). All values are mean ± s.e.mean.

This is of interest, since increased circulating levels of P-selectin, and to a lesser extent E-selectin, can be found in essential, renovascular and malignant hypertension and in hypercholesterolemic patients (Verhaar *et al.*, 1998; Davi *et al.*, 1998). In addition, it has recently been shown that there is a clear involvement of both P- and E-selectins in the development of the atherosclerotic lesion at both early and advanced stages in a mouse model of atherosclerosis (Dong *et al.*, 1998).

Finally, we have also demonstrated that the inhibitory leukocyte responses elicited by salbutamol are mediated through NO release. These results echo those of previous studies in which vasorelaxation induced by β_2-adrenoceptor agonist was prevented by NOS inhibition or endothelium removal, an effect found to be cyclic AMP dependent (Ferro *et al.*, 1999; Xu *et al.*, 2000). Apart from extending these findings, our results show that cyclic AMP elevating agents can reduce CAM expression through endothelial NO release.

In conclusion, in the present study we have provided evidence that cyclic AMP elevating agents and NO donors are potent *in vivo* inhibitors of Ang-II-induced leukocyte-endothelial cell interactions, regardless of the mechanism employed to increase intracellular cyclic nucleotides. The effects observed seem to be mediated through P-selectin downregulation, since Ang-II-elicited leukocyte responses are primarily mediated *via* increased endothelial expression of this adhesion molecule. As the degree of P-selectin expression determines the abundance of rolling leukocytes which may eventually adhere to the endothelium and extravasate into the tissue, these agents have the ability to impair the leukocyte infiltration associated with the vascular damage detected in different circulatory disorders. Therefore, they could constitute a powerful and alternative therapeutical tool in the control of inappropriate inflammatory responses which occur in the vasculature in cardiovascular disease states where Ang-II plays a critical role.

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II.5 ARTÍCULO 5

Estrogens inhibit angiotensin II-induced leukocyte-endothelial cell interactions in vivo via rapid endothelial nitric oxide synthase and cyclooxygenase activation

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ESTROGENS INHIBIT ANGIOTENSIN II INDUCED-LEUKOCYTE-ENDOTHELIAL CELL INTERACTIONS *IN VIVO* VIA RAPID ENDOTHELIAL NITRIC OXIDE SYNTHASE AND CYCLO-OXYGENASE ACTIVATION.

Short tittle: Estrogens inhibit Ang-II-induced leukocyte recruitment

Scientific Section Heading: Hemostasis, Thrombosis, & Vascular Biology; Immunobiology; Phagocytes.

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ABSTRACT

Angiotensin II (Ang-II) may be a key molecule in the development of atherosclerosis. Since the incidence of coronary atherosclerosis in premenopausal women is lower than that observed in men or postmenopausal women, we have investigated the effect of estrogens on Ang-II-induced leukocyte recruitment *in vivo* using intravital microscopy in the rat mesenteric microcirculation. 60 min superfusion with 1 nM Ang-II induced a significant increase in leukocyte rolling flux, adhesion and emigration. Administration of 17-β-estradiol (17-β-E, 100 nM) after 30 min Ang-II superfusion, produced a reduction of these leukocyte responses by 58.8%, 68.3% and 72.7% respectively a further 30 min later. The effect observed with 17-β-E was receptor-mediated and specific since it was inhibited by tamoxifen treatment and unaffected by 17-α-E co-superfusion. 17-β-E superfusion did not modify either L-NAME (100 μM) or indomethacin (25 μg/ml)-induced leukocyte responses. Inhibitory responses caused by 17-β-E were not altered by 7-nitroindazole co-superfusion. Finally, tamoxifen administration provoked a significant increase in leukocyte rolling flux, adhesion and emigration 90 min later, which was significantly attenuated by systemic preadministration of an anti-P-selectin mAb, an anti-CD18 mAb or an Ang-II AT_1 receptor antagonist (losartan). Hence, the anti-atherogenic effects of estrogen may be mediated by inhibition of Ang-II-induced leukocyte recruitment through endothelial NO and prostacyclin release. Furthermore, scarcity of estrogens resulted in decreased levels of vasodilators and the exposure of the endothelium to the deleterious action of Ang-II, which may explain the higher incidence of coronary atherosclerosis in men and in postmenoupausal women.

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INTRODUCTION

Atherosclerosis is the main contributor to the pathogenesis of myocardial and cerebral infarction, gangrene and loss of function in the extremities. It is the leading cause of morbidity and mortality in Western countries. This process bears several histopathologic similarities to chronic inflammation. The early atherosclerotic lesion involves an inflammatory response consisting of intimal accumulation of T lymphocytes and lipid-laden macrophages which occurs continuously throughout the entire atherogenic process (1, 2, 3). The vascular endothelium is a major controller of leukocyte traffic between the blood stream, arterial intima and extravascular space (4). In this context, the migration of leukocytes from the blood to sites of extravascular injury in response to locally produced stimuli is mediated through the interaction of different adhesive receptors present on leukocyte cell surface with their respective counterreceptors on the endothelial cell. This multistep process is initiated by the tethering of leukocytes to the endothelium, followed by leukocyte rolling, leading ultimately to firm leukocyte adhesion to and subsequent transmigration through the vascular endothelium (5, 6).

Angiotensin II (Ang-II), is the main effector peptide of the renin-angiotensin system and, in addition to its role as a potent vasoconstrictor and blood pressure and fluid homeostasis regulator, it has been shown to exert pro-inflammatory activity. Ang-II receptors have been demonstrated on human monocytes and it is capable of promoting monocyte adhesion and activation *in vitro* (7, 8). This may be relevant as hypertension is associated with migration of monocytes into the vessel wall, a critical event leading to the development of the atherosclerotic lesion which can be attenuated by angiotensin-converting enzyme (ACE) inhibition or by pretreatment with an Ang-II AT_1 receptor antagonist (9, 10, 11). Interestingly, we have recently revealed that Ang-II shows pro-inflammatory activity

in vivo at sub-vasoconstrictor doses. In particular, it induces leukocyte trafficking into the rat mesenteric microvasculature through endothelial P-selectin up-regulation in the vessel wall, an effect that is primarily mediated via an Ang-II AT_1 receptor interaction (12). Therefore, Ang-II might be a stimulus for the subendothelial infiltration of leukocytes observed in hypertension and atherosclerosis.

The incidence of coronary atherosclerosis in premenopausal women is half that observed in males of the same age (13). In contrast, postmenopausal women do not exhibit similar protection. An abundance of epidemiological data supports a role for estrogens in this atheroprotective effect, leading to recommendations for their widespread use in postmenopausal replacement therapy (14, 15). However, the mechanism whereby this protection is mediated remains obscure. In this context, avoiding the proinflammatory activity of Ang-II may constitute a plausible explanation for the effects ascribed to estrogens. Therefore, in the present study we have investigated whether 17-β-estradiol (17-β-E) can reduce and/or prevent the leukocyte-endothelial cell interactions induced by Ang-II *in vivo* and the mechanism involved in this inhibitory effect. Finally, we have also carried out a series of experiments to explore why reduced levels of these hormones, or their absence, can provoke the formation of the atherosclerotic lesion.

METHODS

Animal preparation

Male Sprague-Dawley rats 200-250 g were anesthetized with sodium pentobarbital (Sigma Química, Madrid, Spain; 65 mg/kg) injected intraperitoneally. The trachea was cannulated to mantain a patent airway throughout the experiment. A polyethylene catheter was inserted into the right jugular vein for intravenous administration of compounds and a second catheter was placed in the right carotid artery to monitor systemic mean arterial blood

pressure (MABP) through a pressure transducer (Spectramed Stathan P-23XL) connected to a recorder (GRASS RPS7C8B, Quincy, MA.).

Intravital Microscopy

A midline abdominal incision was made, a segment of the midjejunum was exteriorized and the mesentery was prepared for in vivo microscopic observation by draping it over an optically clear viewing pedestal that facilitated transillumination of a 3 cm^2 segment of tissue. All exposed tissue was covered with saline-soaked gauze to minimize tissue dehydratation. The temperature of the pedestal and mesentery was maintained at 37ºC using a constant temperature circulator. Rectal temperature was monitored using an electrothermometer and maintained at 37ºC with an infrared heat lamp. The exposed mesentery was continuously superfused with warmed bicarbonate buffer saline (BBS, pH 7.4) at a rate of 2 ml/min. Mesenteric microcirculation was observed through an orthostatic microscope (Nikon Optiphot-2, SMZ1, Badhoevedorp, The Netherlands) with a 20x objective lens (Nikon SLDW, Badhoevedorp, The Netherlands) and a 10x eyepiece as previously described (16). A video camera (Sony SSC-C350P, Alcobendas, Madrid) mounted on the microscope projected images onto a color monitor (Sony Trinitron PVM-14N2E, Alcobendas, Madrid) and these images were captured on a videotape (Sony SVT-S3000P, Alcobendas, Madrid) with superimposed time and date displayed for subsequent playback analysis. The final magnification of the image on the monitor was 1300x.

Single unbranched mesenteric venules (25-40 μm in diameter) were selected for the study. Venular diameter was measured on-line using a video caliper (Microcirculation Research Institute, Texas A&M University, College Station, Texas). Centerline red blood cell velocity (V_{rbc}) was also measured on-line with an optical Doppler velocimeter (Microcirculation Research Institute, Texas A&M University, College Station, Texas).

Venular blood flow was calculated from the product of mean red blood cell velocity (V_{mean} = V_{rbc} / 1.6) and microvascular cross-sectional area, assuming cylindrical geometry. Venular wall shear rate (γ) was calculated based on the Newtonian definition: $\gamma = 8 \times (V_{mean}/D_v)/s$, in which D_v is venular diameter (17).

Numbers of rolling, adherent and emigrated leukocytes were determined off-line during playback analysis of videotaped images. Rolling leukocyte was determined by counting the number of leukocytes rolling passing a fixed reference point in the microvessel per min. The same reference point was used throughout the experiment as leukocytes may roll for only a section of the vessel before rejoining the blood flow or becoming firmly adherent. Leukocyte rolling velocity (V_{wbc}) was determined as the time required for a leukocyte to traverse 100 μm of the venule and was expressed as μm/s. A leukocyte was considered to be adherent to venular endothelium if it remained stationary for a period equal to or exceeding 30 s. Adherent cells were expressed as the number per 100 μm length of venule. Leukocyte emigration was expressed as the number of white blood cells per microscopic field. The rate of emigration was determined from the difference between the number of any leukocytes present in the interstitium at the beginning of the experiment and the number of cells present in the intestitium at the end of the experiment.

Experimental protocol

All preparations were left to stabilize for 30 min and baseline (time 0) measurements of leukocyte rolling flux and velocity, leukocyte adhesion, leukocyte emigration, MABP, V_{rbc}, shear rate and venular diameter were obtained. The superfusion buffer was then supplemented with Ang-II 1 nM (Sigma Química), a dose that elicits the maximum and most consistent increase in leukocyte-endothelial cell interactions *in vivo* (12). Recordings were performed for 5 min at 15 min intervals over a 60 min period and the aforementioned

leukocyte and hemodynamic parameters were measured. After 30 min Ang-II-superfusion, and once its inflammatory effects are patent, 17-β-E (Sigma Química, 10-1000 nM) was added to the superfusate and recordings performed for 5 min at 15 min intervals over a further 30 min period and the different parameters measured. The doses of 17-β-E employed were based on previous studies (18). The most consistent inhibition of leukocyte-endothelial cell interactions induced by Ang-II was detected at 100 nM 17-β-E, therefore, this dose was used for the remainder of the experiments. To investigate the preventive effect of 17-β-E , in a separate group of animals 17-β-E 100 nM was co-superfused with Ang-II for 60 min at the beginning of the experiment and responses were evaluated.

In another set of experiments, animals were pretreated with the 17-β-E receptor antagonist tamoxifen (Sigma Química, 0.6 mg/kg, i.p.) 30 min before Ang-II superfusion to determine if the effects observed were receptor-mediated. The dose administered had been used in previous *in vivo* studies (19). Next, to determine the effect specificity of 17-β-E, the buffer was suplemented with 17-α-estradiol (17-α-E, Sigma Química, 100 nM) after 30 min Ang-II suffusion.

To evaluate the possible mechanism of action of 17-β-E inhibitory responses, its effect was evaluated after L-NAME (Sigma Química, 100 μM) or indomethacin (Sigma Química, 25 μg/ml) superfusion in a further group of experiments. Again, 17-β-E was added to the superfusion buffer after 30 min superfusion of the agents under investigation, once their inflammatory effects were patent, and responses were evaluated for a further 30 min period. Previous studies have demonstrated that the doses of L-NAME and indomethacin used cause consistent increase in leukocyte rolling and adhesion after 60 min superfusion and a pronounced peak in leukocyte adhesion within 30 min superfusion respectively (20, 21).

To determine the potential origin of the nitric oxide (NO) released by 17-β-E, we next evaluated the effect of neuronal derived NO on the inhibition exerted by 17-β-E on Ang-II-induced leukocyte recruitment. 7-Nitroindazole, a selective inhibitor of neuronal NO synthase (nNOS) (Sigma Química, 100 μM) was co-administered with 17-β-E after 30 min Ang-II suffusion. The dose used was found to be selective for this enzyme isoform in a previous *in vivo* study performed with endothelial NOS knock-out mice (22).

Finally, four groups of animals were pretreated with tamoxifen (0.6 mg/kg, i.p.) 30 min before buffer superfusion. The first received only tamoxifen to investigate whether an estrogen blockade produces leukocyte rolling, adhesion and emigration within the rat mesenteric microcirculation. Another group of animals was injected i.p. with saline for the same period of time as that treated with tamoxifen. A third group was pretreated with a function blocking anti-rat P-selectin mAb (RMP-1, 2.5 mg/kg, i.v.) and a fourth with a function blocking mAb against CD18 integrins (WT-3, 1 mg/kg, i.v.). In both latter cases, antibodies were administered 15 min prior to tamoxifen injection to elucidate which adhesion molecules are involved in leukocyte-endothelial cell interactions caused by estrogen receptor blockade. Antibodies against rat-P-selectin (RMP-1) and rat-CD18 (WT-3) were acquired as previously stated (23, 24) and assayed at the doses previously described (12, 25). To further investigate if Ang-II is involved in the leukocyte-endothelial cell interactions provoked by a lack of estrogens, a final group of animals was pretreated with an AT_1 Ang-II receptor antagonist (losartan, 10 mg/kg, i.v.) 10 min prior to tamoxifen administration. Losartan was kindly donated by Merck Sharp & Dohme, Spain, and the dose administered was that used in a previous *in vivo* study (12).

Statistical Analysis: All values are represented as mean±SEM. Data within groups were compared using an analysis of variance (one way-ANOVA) with a Newman-Keuls *post hoc* correction for multiple comparisions. Statistical significance was set at $p < 0.05$.

RESULTS

Ang-II 1 nM superfusion promoted a significant increase in leukocyte rolling flux (82.0±10.7 vs. 20.8±1.4 cells/min), leukocyte adhesion (10.3±1.9 vs. 0.8±0.3 cells/100 μm) and leukocyte emigration (2.8±0.6 vs. 0.3±0.3 cells/field) at 60 min compared with basal values. These results are consistent with previous studies from our laboratory (12). After 30 min superfusion, Ang-II caused significant increases in all leukocyte parameters versus values detected at time 0 (Figure 1). When 17-β-E (100 nM) was administered after 30 min Ang-II superfusion, leukocyte responses were reduced by 58.8%, 68.3% and 72.7% respectively 30 min later (Figure 1). This dose caused maximal and most consistent effect and was therefore, used for the remainder of the experiments. The decrease in leukocyte rolling velocity induced by Ang-II was reversed by co-superfusion with all of the three doses of 17-β-E employed in this study (Table 1). Concomitant superfusion of Ang-II and 17-β-E from the beginning of the experiment resulted in a complete inhibition of Ang-II-induced leukocyte-endothelial cell interactions, inhibiting leukocyte rolling flux, leukocyte adhesion and emigration by 95.1%, 86.8% and 90.0 respectively after 60 min superfusion (Figure 2). These responses were receptor-mediated since pretreatment of the animals with the estrogen receptor antagonist, tamoxifen (0.6 mg/kg) reversed the anti-inflammatory activity of 17-β-E (Figure 3 and Table 1). In addition, these effects were estrogen-specific since superfusion with the same dose of 17-α-E had no effect on either Ang-II-induced leukocyte infiltration or on the decrease in leukocyte rolling velocity provoked by Ang-II (Figure 4 and Table 1).

Table 2 summarises the results obtained for MABP and shear rate at different time points in animals where Ang-II superfusion was either prolonged or supplemented with 17-β-E at different doses, supplemented with 17-β-E in animals pretreated with tamoxifen or supplemented with 17-α-E 30 min after Ang-II superfusion. MABP and shear rates remained the same throughout the experimental period in both the untreated group and the groups subjected to varying treatments.

Since estrogens can provoke the release of NO and prostacyclin from the vascular wall, and both vasodilators have anti-adhesive properties, this possibility was further investigated. When 17-β-E was co-superfused after 30 min suffusion with a NOS or a cyclo-oxygenase (COX) inhibitor, leukocyte responses were not modified, indicating a clear role for both vasodilators in estrogen elicited anti-adhesive properties (Figure 5). Additionally, inhibitory responses induced by 17-β-E on Ang-II-induced leukocyte-endothelial cell interactions were not affected by nNOS inhibition with 7-nitroindazole. Indeed, leukocyte rolling flux (34.2±2.7cells/min), leukocyte adhesion (2.8±0.6 cells/100 μm venule) and leukocyte emigration (1.8±0.4 cells/field) after 60 min superfusion with Ang-II+17-β-E +7-nitroindazole did not differ from values detected in the Ang-II +17-β-E treated group at the same time point (34.6±6.8 cells/min, 3.4±0.4 cells/100 μm venule and 1.0±0.4 cells/field respectively). Thus, these results indicate that NO release by estrogens has an endothelial origin.

Furthermore, tamoxifen administration induced a significant increase in leukocyte rolling flux (87.0±15.1 vs. 27.7±8.1 cells/min), leukocyte adhesion (8.5±1.8 vs. 1.7±0.9 cells/100 μm) and leukocyte emigration (2.5±0.5 vs. 0.7±0.3 cells/field) 90 min after its administration compared with values detected in the saline-treated group for the same time period. Interestingly, increases in leukocyte rolling flux, adhesion and emigration

following 90 min administration of tamoxifen were significantly reduced by an anti-P-selectin mAb (RMP-1) pretreatment by 100%, 93.0% and 61.5% respectively and by 21.6%, 81.4% and 61.5% respectively after an anti-CD18 mAb (WT-3) preadministration (Figure 6). Moreover, leukocyte responses following 90 min exposure to tamoxifen were inhibited through systemic administration of losartan by 85.4%, 74.4% and 61.5% respectively (Figure 7). In addition, tamoxifen provoked a significant decrease in leukocyte rolling velocity after 90 min administration, however this parameter was maintained at basal levels when animals received the aforementioned pretreatments (Table 3).

Finally, Table 4 presents the results obtained for MABP and shear rate 90 min after saline or tamoxifen administration in animals untreated and pretreated with RMP-1, WT-3 or losartan. As shown, theses hemodynamic parameters were unaltered throughout the entire experimental period in the untreated and pretreated animals.

DISCUSSION

In the present study we have demonstrated that estrogens can not only diminish but also prevent Ang-II-induced leukocyte-endothelial cell interactions in vivo. This effect is receptor-mediated since tamoxifen pretreatment resulted in the total abolition of estrogen-induced responses and it is also specific as 17-α-E had no effect. Indeed, estrogens seem to attenuate vasoconstrictive responses to multiple agonists including Ang-II (26) and it is therefore likely that leukocyte-endothelial cell interactions induced by this peptide hormone are also be affected. The fast rate of the response observed indicate that the process does not require the classical nuclear effects of the hormone. This observation is consistent with a novel, nongenomic physiological role for estrogen receptors in endothelial cells. In this context, it has recently been shown that estrogen receptors are found in both the endothelial cell and the leukocyte surface and that they exert different

effects through interaction with their α subtype receptor (18, 27). It is also interesting to note that the protective effect exerted by estrogens is dependent on the dose employed. In fact, low doses of estrogens and close to the physiological ones inhibited leukocyte-endothelial cell interactions caused by Ang-II more than higher doses of 17-β-E such as 1 μM. Thus, these results emphasize the importance of the adequate dose of estrogens in hormone replacement therapy for postmenopausal woman, since the effects aimed may not always be those obtained.

Our results highlight that the effects of estrogens are mediated through a direct action on the endothelium and via NO and prostacyclin release. In this way, 17-β-E did not provoke any changes in the increased leukocyte-endothelial cell interactions detected after acute NOS or COX inhibition. Furthermore, we can deduce that the NO released by these hormones has an endothelial origin since the selective nNOS inhibitor, 7-nitrondazole did not modify the estrogens' diminution of Ang-II-induced responses. There is evidence that estrogens can provoke atheroprotection and this effect is thought to be primarily mediated through a paracrine phenomenon originated in the endothelium and through augmented production of prostacyclin and NO. Steinleitner *et al.* (1989) (28) demonstrated increased levels of 6-keto-prostaglandin$_{1\alpha}$, the inactive but stable metabolite of prostacyclin, in uterine arteries of premenopausal, but not postmenopausal, women. In addition, it has also been demonstrated that estrogens upregulate the expression of constitutive NOS in human umbilical vein endothelial cells (HUVECs) and human aortic endothelial cells and in the rat myocardium, and restore the regulation of wall shear stress in arterioles of male hypertensive rats by the same mechanism (29, 30, 31). However, it has recently been illustrated that estrogens can also provoke rapid NOS activation without inducing upregulation of this enzyme (18, 27) and we have revealed the strong inhibitory responses

elicited by iloprost, a prostacyclin analogue, and NO donors on Ang-II-induced leukocyte-endothelial cell interactions in vivo (16), which may account for the quick anti-inflammatory response exerted by estrogens in the present study.

Inhibition of leukocyte recruitment by estrogens has been demonstrated in several in vivo studies (32, 19, 33). Despite these findings, the adhesive mechanisms involved in these leukocyte responses are not precisely known. While some authors have found that estrogen administration can reduce intercellular adhesion molecule-1 (ICAM-1) expression (33), others have demonstrated the downregulation of vascular cell adhesion molecule-1 (VCAM-1) expression (32). Further studies have shown that inhibition of the cellular infiltration elicited by these hormones is due to decreased E-selectin expression (19) and Caulin-Glaser *et al.* (1996) (34) indicated that all three inducible adhesion molecules are downregulated by 17-β-E treatment of IL-1 stimulated HUVECs. However, the rapid responses elicited by estogens indicate that downregulation of adhesion molecules other than ICAM-1, VCAM-1 or E-selectin are involved in the inhibitory responses observed in the present study. In this context, one possible candidate is P-selectin. Indeed, we have recently demonstrated that Ang-II-induced leukocyte-endothelial cell interactions *in vivo* occur via endothelial P-selectin upregulation (12). In addition, lower plasma levels of P-selectin have been detected in premenopausal women compared to those encountered in men and the administration of a single dose of estradiol significantly decreases the plasma levels of this adhesion molecule in men (35). Furthermore, and related with the estrogen mechanism of action proposed in the present study, prostacyclin treatment in animals subjected to protamine administration provokes a decrease in E and P-selectin levels and results in a more marked preservation of left ventricular function (36). Similarly, treatment with NO donors has been found to attenuate homocysteine-induced P-selectin expression in rat mesenteric venules (37). This is of interest, since increased circulating levels of P-

selectin, and to a lesser extent E-selectin, can be found in essential, renovascular and malignant hypertension and in hypercholesterolemic patients (38, 39). In addition, a role has recently been highlighted for both P- and E-selectins in the development of the atherosclerotic lesion at both early and advanced stages in a mouse model of atherosclerosis (40).

Finally, to further investigate the consequences of estrogen deficiency occurring in men and postmenopausal women, we mimicked said condition by systemic treatment with the estrogen receptor antagonist, tamoxifen. This treatment provoked a significant increase in leukocyte-endothelial cell interactions 90 min after its administration. These effects were P-selectin and CD18-integrin dependent since administration of mAbs, which blocked the function of these adhesion molecules, dramatically reduced tamoxifen-induced leukocyte responses. These results suggest that lack of estrogens results in a deficiency of endothelial-derived vasodilators such as prostacyclin and NO. In fact, L-NAME-induced leukocyte-endothelial cell interactions are P-selectin and CD18-integrin dependent (41, 42). Similarly, COX inhibition causes significant vascular P-selectin and CD18-integrin expression within 1 hour (21, 43). Furthermore, we have recently demonstrated that COX and NOS inhibition-induced leukocyte-endothelial cell interactions are due to the deleterious action of Ang-II, since losartan can inhibit these effects (44). In addition, we have illustrated in the present study that leukocyte responses elicited by lack of estrogens are also mediated through Ang-II interaction with its AT_1 receptor subtype. Considered together, these findings suggest that low levels of estrogens, which can be encountered in men and postmenopausal women, provoke endothelial barrier dysfunction owed to lack of NO or prostacyclin. The absence of these vasodilators provokes the exposure of the endothelium to Ang-II and the upregulation of adhesion molecules such as P-selectin and

CD18-integrins, which results in the leukocyte recruitment which subsequently provokes the subendothelial leukocyte infiltration associated with the onset of the atherogenic lesion.

In conclusion, we have demonstrated in the present study that estrogen antiatherogenic activity can be explained in part by the inhibition of the inflammatory response induced by Ang-II. This effect is primarily mediated by estrogen interaction with its α receptor subtype, which is present in the endothelial cell surface, and the subsequent immediate activation of eNOS and COX. The results obtained indicate that the increased risk of cardiovascular disorders in men and postmenopausal women can be attributed to lack of estrogens which causes a disruption of vascular balance and a deficiency in endothelial vasodilators. In this way, the endothelium is exposed to the deleterious action of Ang-II, provoking the subendothelial infiltration of the adhered leukocytes, which in turn constitutes a possible mechanism responsible for the onset of the atherosclerotic lesion when a deficiency in estrogens occurs.

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FIGURE LEGENDS

Figure 1: Effect of 17-β-E on Ang-II-induced leukocyte rolling flux (A), leukocyte adhesion (B) and leukocyte emigration (C) in rat mesenteric postcapillary venules. The mesentery was superfused with Ang-II (1 nM) and, after 30 min suffusion, 17-β-E (10-1000 nM) was added to the superfusate. Animals were divided into 4 groups: the Ang-II superfusion was either continued (n=5) or supplemented with 17-β-E (10 nM, n=5; 100 nM, n=5; 1000 nM, n=5) 30 min after Ang-II superfusion and the parameters were measured 15 and 30 min later. Results are represented as mean±SEM. * $p<0.05$ or ** $p<0.01$ relative to the control value (0 min) in the untreated group. $^{+}p<0.05$ or $^{++}p<0.01$ relative to the untreated group.(Ang-II + buffer).

Figure 2: Effect of concomitant 17-β-E superfusion on Ang-II-induced leukocyte rolling flux (A), leukocyte adhesion (B) and leukocyte emigration (C) in rat mesenteric postcapillary venules. Parameters were measured 0, 15, 30 and 60 min after superfusion with Ang-II (1 nM, n=5) or with Ang-II+17-β-E (100 nM, n=5). Results are represented as mean±SEM. * $p<0.05$ or ** $p<0.01$ relative to the control value (0 min) in the untreated group. $^{+}p<0.05$ or $^{++}p<0.01$ relative to the untreated group.(Ang-II + buffer).

Figure 3: Effect of tamoxifen administration on 17-β-E inhibitory responses over Ang-II-induced leukocyte rolling flux (A), leukocyte adhesion (B) and leukocyte emigration in rat mesenteric postcapillary venules. The mesentery was superfused with Ang-II (1 nM), and after 30 min suffusion, 17-β-E (100 nM) was added to the superfusate. Animals were divided into 3 groups: the Ang-II superfusion was either continued (n=5) or supplemented with 17-β-E (100 nM) in animals untreated (n=5) or pretreated with tamoxifen (0.6 mg/kg, n=5). Parameters were measured 15 and 30 min later. Results are represented as mean±SEM. * $p<0.05$ or **$p<0.01$ relative to the control value (0 min) in the untreated group. $^{+}p<0.05$ or $^{++}p<0.01$ relative to the Ang-II + buffer group.

Figure 4: Effect of 17-α-E and 17-β-E on Ang-II-induced leukocyte rolling flux (A), leukocyte adhesion (B) and leukocyte emigration in rat mesenteric postcapillary venules. The mesentery was superfused with Ang-II (1 nM), and after 30 min suffusion, 17-β-E (100 nM) or 17-α-E (100 nM) were added to the superfusate. Animals were divided into 3 groups: the Ang-II superfusion was either continued (n=5), supplemented with 17-β-E (100 nM, n=5) or supplemented with 17-α-E (100 nM, n=5). Parameters were measured 15 and 30 min later. Results are represented as mean±SEM. * $p<0.05$ or **$p<0.01$ relative to the control value (0 min) in the untreated group. $^{+}p<0.05$ or $^{++}p<0.01$ relative to the untreated group.(Ang-II + buffer).

Figure 5: Effect of 17-β-E on L-NAME and indomethacin-induced leukocyte rolling flux (A), leukocyte adhesion (B) and leukocyte emigration (C) in rat mesenteric postcapillary venules. The mesentery was superfused with either L-NAME (100 μM) or indomethacin (25 μg/ml) and after 30 min the superfusion was either continued or 17-β-E (100 nM) was added to the superfusate. Animals were divided into 4 groups: the L-NAME superfusion was either continued (n=5), or supplemented with 17-β-E (n=4), the indomethacin superfusion was either continued (n=5) or supplemented with17-β-E (n=5). Parameters were measured 15 and 30 min later. Results are represented as mean±SEM. * $p<0.05$ or ** $p<0.01$ relative to the control value (0 min) in the untreated group.

Figure 6: Effect of an anti-P-selectin and an anti-CD18 mAb administration on tamoxifen-induced leukocyte rolling flux (A), leukocyte adhesion (B) and leukocyte emigration in rat mesenteric postcapillary venules. Animals were pretreated with tamoxifen (0.6 mg/kg, i.p.) or saline 30 min before buffer superfusion. Animals were then divided into 4 groups: saline (n=4), tamoxifen (n=5), tamoxifen + anti-P-selectin mAb (RMP-1, 2.5 mg/kg, iv., n=5) or tamoxifen + anti-CD18 mAb (WT-3, 1 mg/kg, iv., n=5). Both pretreatments were carried out 15 min prior to tamoxifen administration. Parameters were measured 30, 45, 60, 75 and 90 min after tamoxifen i.p. injection. Results are represented as mean±SEM. * $p<0.05$ or ** $p<0.01$ relative to 30 min value in the control group (saline) or + $p<0.05$ or ++ $p<0.01$ relative to its respective time point value in the tamoxifen-treated group.

Figure 7: Effect of losartan treatment on tamoxifen-induced leukocyte rolling flux (A), leukocyte adhesion (B) and leukocyte emigration in rat mesenteric postcapillary venules. Animals were pretreated with tamoxifen (0.6 mg/kg, i.p.) or saline 30 min before buffer superfusion. Animals were then divided into 3 groups: saline (n=4), tamoxifen (n=5) and tamoxifen + losartan (10 mg/kg, i.v., 10 min prior tamoxifen i.p. injection, n=5). Parameters were measured 30, 45, 60, 75 and 90 min after tamoxifen administration. Results are represented as mean±SEM. * $p<0.05$ or ** $p<0.01$ relative to 30 min value in the control group (saline) or + $p<0.05$ or ++ $p<0.01$ relative to its respective time point value in the tamoxifen-treated group.

Table 1. Leukocyte rolling velocity[a].

Treatment	0 min	30 min	60 min
Ang-II	105.3±11.0	58.2±5.6*	40.7±5.7**
Ang-II + 17-β-E 10	117.8±12.5	73.1±8.6**	102.1±10.2
Ang-II + 17-β-E 100	107.2±10.8	62.9±6.9**	94.9±12.9
Ang-II + 17-β-E 1000	122.3±11.6	63.7±5.0**	83.3±10.0
Ang-II + 17-β-E 100 + Tam	116.9±16.2	57.5±7.0*	42.6±6.8**
Ang-II + 17-α-E 100	107.7±7.5	54.8±4.1**	48.3±2.1**

[a]Table 1 illustrates leukocyte rolling velocity in animals where Ang-II superfusion was either continued (n=5) or supplemented with 17-β-E (10 nM, n=5; 100 nM, n=5; 1000 nM, n=5) or supplemented with 17-β-E (100 nM, n=5) in animals pretreated with tamoxifen (0.6 mg/kg) or supplemented with 17-α-E (100 nM, n=5) 30 min after Ang-II superfusion. Leukocyte rolling velocity was measured 30 min later.

All values are presented as mean±SEM.* $p<0.05$ or **$p<0.01$ relative to the control group (0min).

Table 2. Hemodynamic parameters[a].

	MABP (mm Hg)			Shear rate (s^{-1})		
Treatment	**0 min**	**30 min**	**60 min**	**0 min**	**30 min**	**60 min**
Ang II	99.6±8.5	99.2±7.9	92.5±8.2	705.3±122.5	701.6±103.2	656.1±60.7
Ang II + 17-β-E 10	107.5±2.8	108.8±2.0	112.9±4.9	733.7±56.4	690.0±47.1	745.6±58.0
Ang II + 17-β-E 100	95.4±4.4	85.8±2.8	85.0±5.8	756.7±70.3	621.0±83.9	662.7±69.8
Ang II + 17-β-E 1000	125.0±8.0	123.8±8.4	123.8±8.5	837.7±133.9	828.7±81.9	735.4±65.8
Ang II + 17-β-E + Tam	90.0±6.3	77.5±11.0	80.4±8.5	772.9±84.4	709.7±83.7	646.5±88.7
Ang II + 17-α-E	113.8±4.0	119.2±4.3	122.5±6.3	581.3±61.7	473.6±30.2	442.1±62.6

[a]Table 2 presents hemodynamic parameters in animals where Ang-II superfusion was either continued (n=5) or supplemented with 17-β-E (10 nM, n=5; 100 nM, n=5; 1000 nM, n=5) or supplemented with 17-β-E (100 nM, n=5) in animals pretreated with tamoxifen (0.6 mg/kg) or supplemented with 17-α-E (100 nM, n=5) 30 min after Ang-II superfusion. Hemodynamic parameters were measured 30 min later.

All values are presented as mean±SEM.

Table 3. Leukocyte rolling velocity[a].

Treatment	30 min	90 min
Saline	95.0±7.8	103.6±12.3
Tamoxifen	93.8±13.8	53.9±3.3**
Tamoxifen + RMP-1	104.5±9.0	117.6±13.5
Tamoxifen + WT-3	97.2±5.8	101.1±7.4
Tamoxifen + losartan	119.7±10.6	99.5±8.9

[a]Table 3 shows the leukocyte rolling velocity in animals pretreated with saline or tamoxifen (0.6 mg/kg, i.p.) 30 min before buffer superfusion. The animals were then divided into 5 groups: saline (n=4), tamoxifen (n=5), tamoxifen + an anti-P-selectin mAb (RMP-1, 2.5 mg /kg, iv., n=5), tamoxifen + an anti-CD18 mAb (WT-3, 1 mg /kg, iv., n=5) and tamoxifen + losartan (10 mg/kg, i.v., n=5). All treatments were carried out 10–15 min prior to tamoxifen administration. All values are presented as mean±SEM. *$p<0.05$ or **$p<0.01$ relative to the saline group at 30 min).

Table 4. Hemodynamic parameters[a].

	MABP (mm Hg)		Shear rate (s^{-1})	
Treatment	**30 min**	**90 min**	**30 min**	**90 min**
Saline	133.3±1.0	137.2±4.5	703.9±25.5	724.8±29.4
Tamoxifen	133.9±2.0	124.4±7.2	773.4±61.4	639.8±67.7
Tamoxifen + RMP-1	128.3±6.0	130.6±11.2	706.4±83.5	683.6±123.6
Tamoxifen + WT-3	116.3±5.1	121.7±9.0	644.4±60.0	615.8±56.5
Tamoxifen + losartan	106.7±5.0	93.9±21.6	729.7±109.4	543.5±28.5

[a]Table 4 summarizes hemodynamic parameters in animals pretreated with saline or tamoxifen (0.6 mg/kg, i.p.) 30 min before buffer superfusion. The animals were then divided into 5 groups: saline (n=4), tamoxifen (n=5), tamoxifen + an anti-P-selectin mAb (RMP-1, 2.5 mg /kg, iv., n=5), tamoxifen + an anti-CD18 mAb (WT-3, 1 mg /kg, iv., n=-5) and tamoxifen + losartan (10 mg/kg, i.v., n=5). All treatments were carried out 10–15 min prior to tamoxifen administration. All values are presented as mean±SEM.

Figure 1

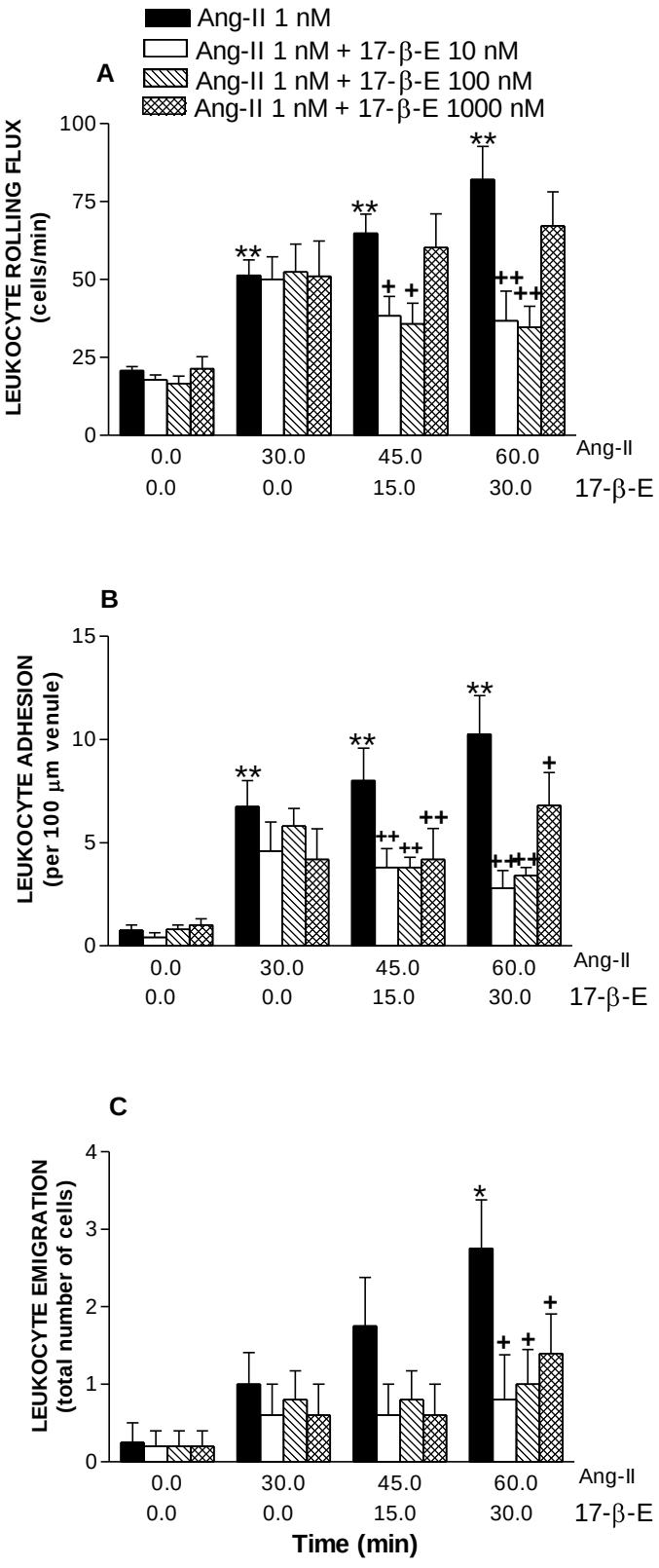

Ang-II 1 nM
Ang-II 1 nM + 17-β-E 10 nM
Ang-II 1 nM + 17-β-E 100 nM
Ang-II 1 nM + 17-β-E 1000 nM
A
LEUKOCYTE ROLLING FLUX (cells/min)
100
75
50
25
0
B
LEUKOCYTE ADHESION (per 100 μm venule)
15
10
5
0
C
LEUKOCYTE EMIGRATION (total number of cells)
4
3
2
1
0
0.0 30.0 45.0 60.0 Ang-II
0.0 0.0 15.0 30.0 17-β-E
Time (min)

Figure 2

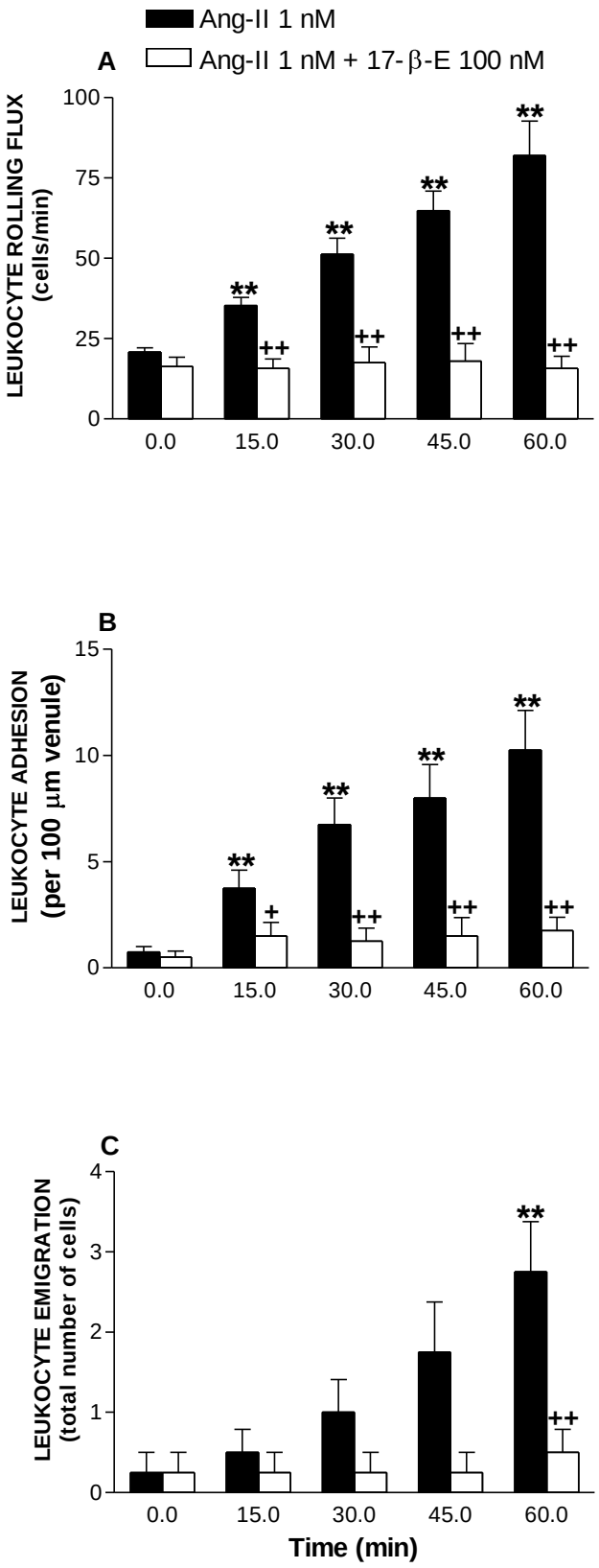

Ang-II 1 nM
Ang-II 1 nM + 17-β-E 100 nM
A
LEUKOCYTE ROLLING FLUX (cells/min)
100
75
50
25
0
0.0
15.0
30.0
45.0
60.0
**
++
B
LEUKOCYTE ADHESION (per 100 μm venule)
15
10
5
0
+
C
LEUKOCYTE EMIGRATION (total number of cells)
4
3
2
1
Time (min)

Figure 3

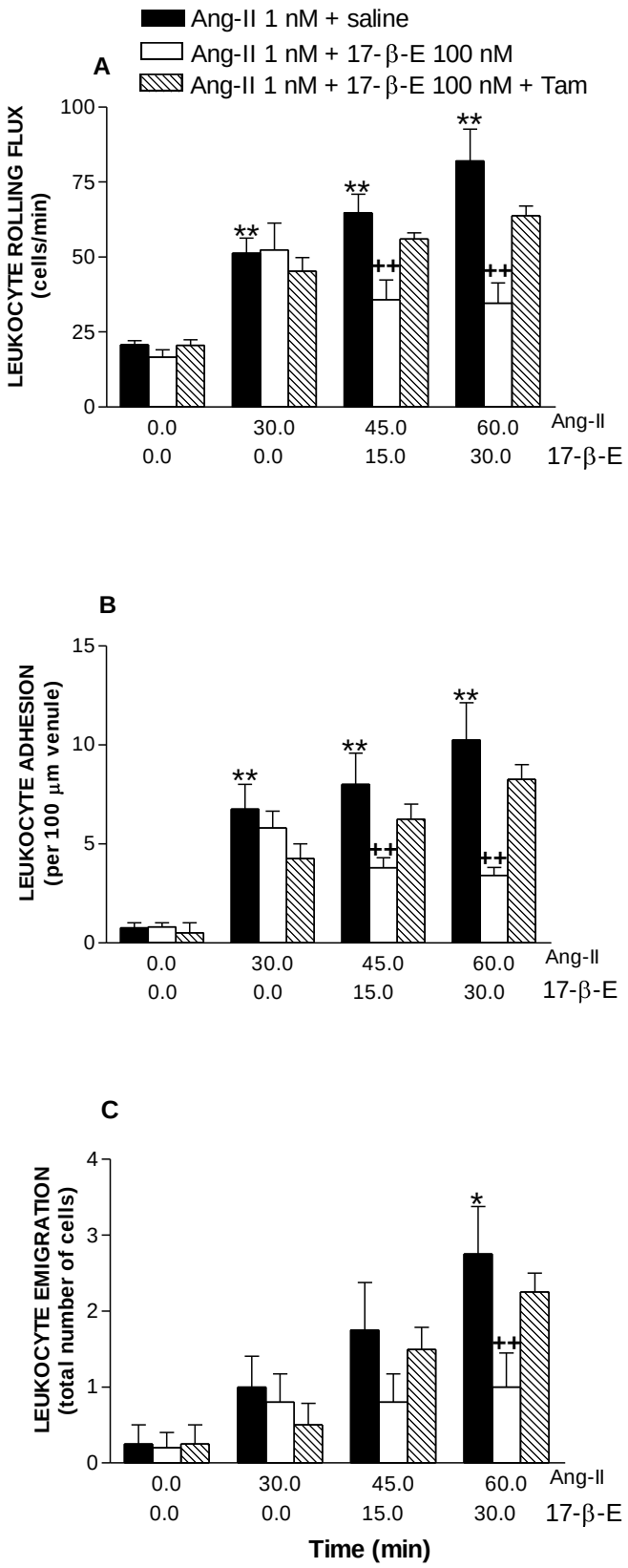

Ang-II 1 nM + saline
Ang-II 1 nM + 17-β-E 100 nM
Ang-II 1 nM + 17-β-E 100 nM + Tam
A
LEUKOCYTE ROLLING FLUX (cells/min)
100
75
50
25
0
**
**
**
**
++
++
0.0 30.0 45.0 60.0 Ang-II
0.0 0.0 15.0 30.0 17-β-E
B
LEUKOCYTE ADHESION (per 100 μm venule)
15
10
5
0
**
**
**
**
++
++
0.0 30.0 45.0 60.0 Ang-II
0.0 0.0 15.0 30.0 17-β-E
C
LEUKOCYTE EMIGRATION (total number of cells)
4
3
2
1
0
*
++
0.0 30.0 45.0 60.0 Ang-II
0.0 0.0 15.0 30.0 17-β-E
Time (min)

Figure 4

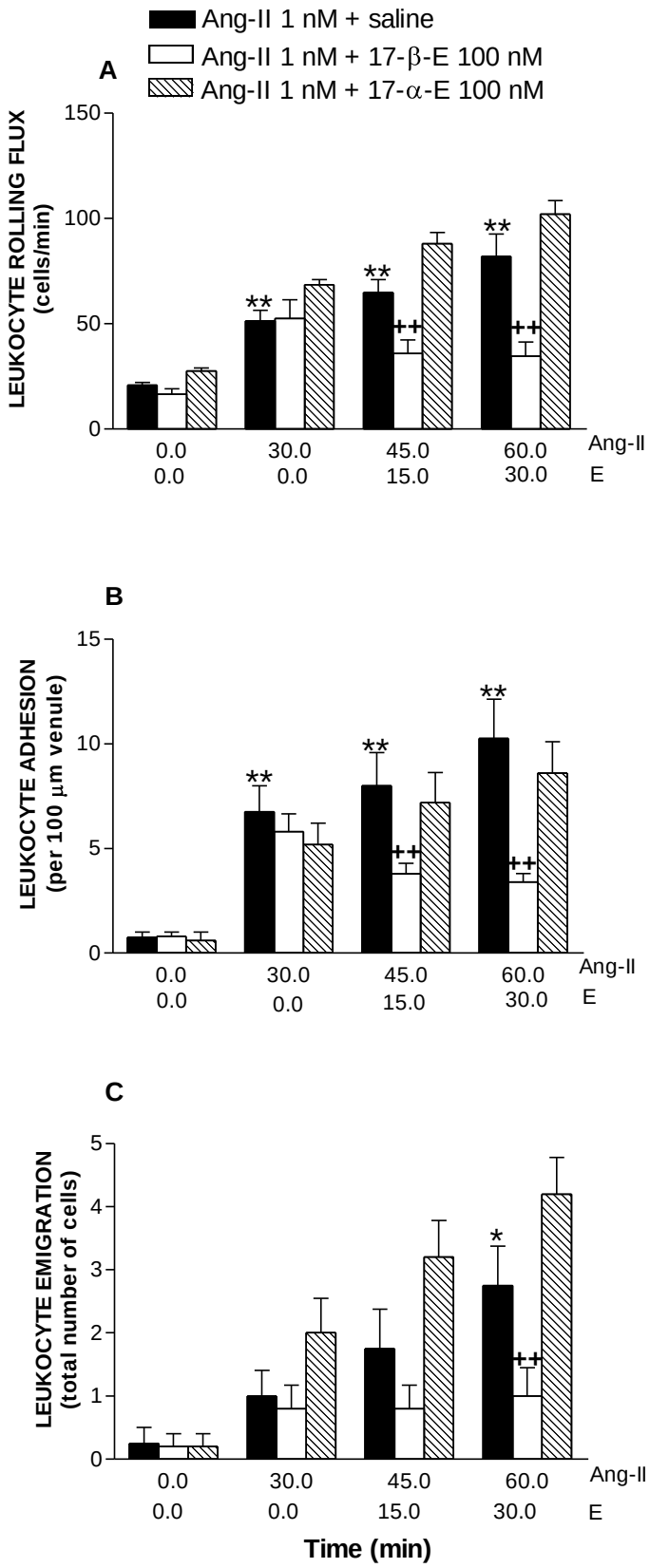

Ang-II 1 nM + saline
Ang-II 1 nM + 17-β-E 100 nM
Ang-II 1 nM + 17-α-E 100 nM
A
LEUKOCYTE ROLLING FLUX (cells/min)
150
100
50
0
**
**
**
++
++
0.0 30.0 45.0 60.0
Ang-II
0.0 0.0 15.0 30.0
E
B
LEUKOCYTE ADHESION (per 100 μm venule)
15
10
5
0
**
**
**
++
++
0.0 30.0 45.0 60.0
Ang-II
0.0 0.0 15.0 30.0
E
C
LEUKOCYTE EMIGRATION (total number of cells)
5
4
3
2
1
0
*
++
0.0 30.0 45.0 60.0
Ang-II
0.0 0.0 15.0 30.0
E
Time (min)

Figure 5

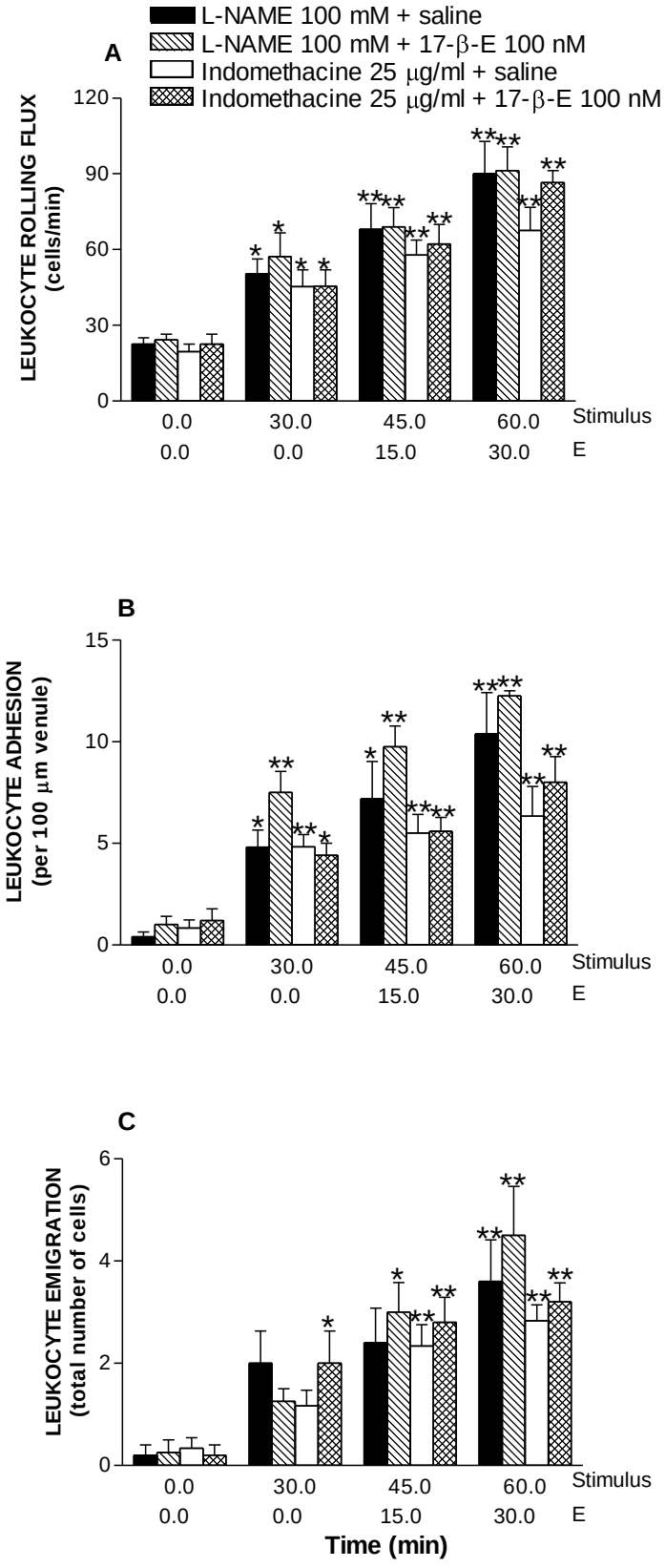

L-NAME 100 mM + saline
L-NAME 100 mM + 17-β-E 100 nM
Indomethacine 25 μg/ml + saline
Indomethacine 25 μg/ml + 17-β-E 100 nM
A
LEUKOCYTE ROLLING FLUX (cells/min)
120
90
60
30
0
B
LEUKOCYTE ADHESION (per 100 μm venule)
15
10
5
0
C
LEUKOCYTE EMIGRATION (total number of cells)
6
4
2
0
0.0 30.0 45.0 60.0 Stimulus
0.0 0.0 15.0 30.0 E
Time (min)

Figure 6

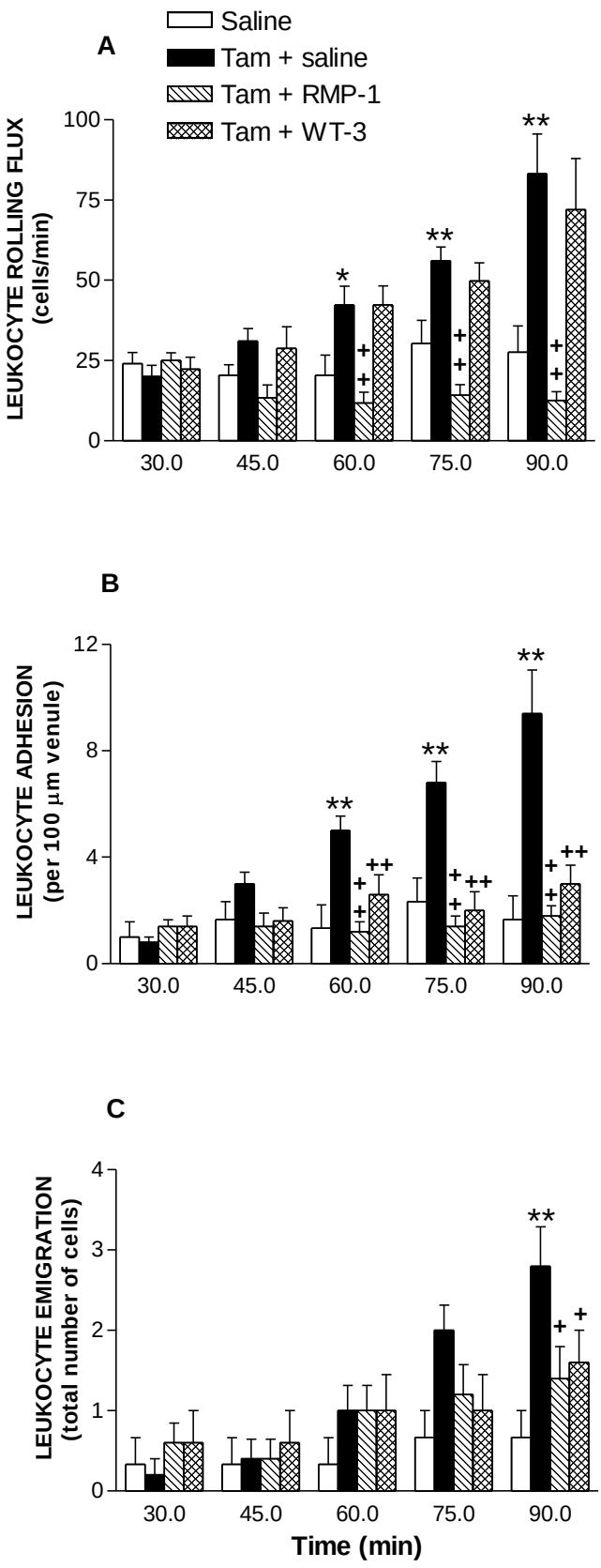
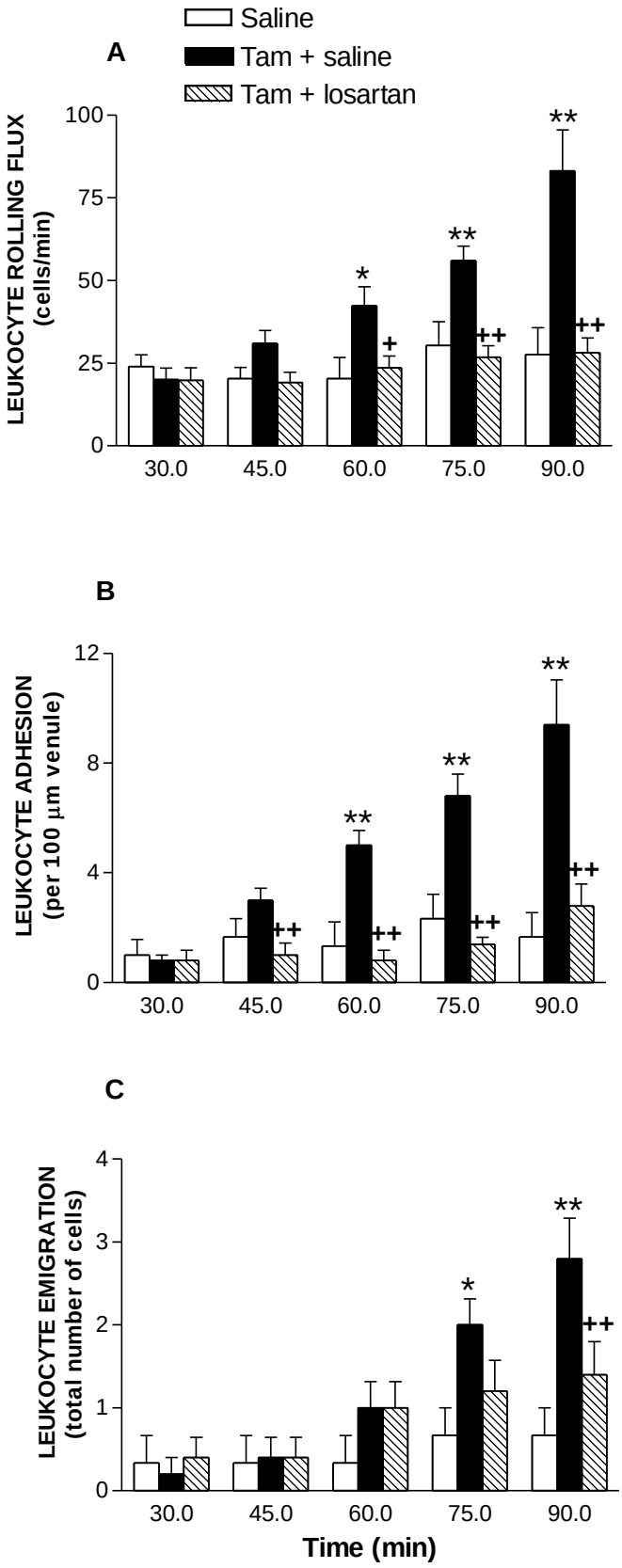

A
Saline
Tam + saline
Tam + RMP-1
Tam + WT-3
LEUKOCYTE ROLLING FLUX (cells/min)
100
75
50
25
0
30.0
45.0
60.0
75.0
90.0
B
LEUKOCYTE ADHESION (per 100 μm venule)
12
8
4
0
C
LEUKOCYTE EMIGRATION (total number of cells)
4
3
2
1
0
Time (min)

Figure 7


Saline
Tam + saline
Tam + losartan
A
LEUKOCYTE ROLLING FLUX (cells/min)
100
75
50
25
0
30.0
45.0
60.0
75.0
90.0
B
LEUKOCYTE ADHESION (per 100 μm venule)
12
8
4
0
C
LEUKOCYTE EMIGRATION (total number of cells)
4
3
2
1
0
Time (min)

II.6 ARTÍCULO 6

Angiotensin II is involved in nitric oxide synthase and cyclo-oxygenase inhibition-induced leukocyte-endothelial cell interactions in vivo

Álvarez A, Piqueras L, Bello R, Canet A, Moreno L, Kubes P, Sanz MJ.

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Angiotensin II is involved in nitric oxide synthase and cyclo-oxygenase inhibition-induced leukocyte-endothelial cell interactions *in vivo*

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1 Chronic inhibition of nitric oxide synthase (NOS) provokes a hypertensive state which has been shown to be angiotensin II (Ang-II) dependent. In addition to raising blood pressure, NOS inhibition also causes leukocyte adhesion. The present study was designed to define the role of Ang-II in hypertension and in the leukocyte-endothelial cell interactions induced by acute NOS or cyclo-oxygenase (COX) inhibition using intravital microscopy within the rat mesenteric microcirculation.

2 While pretreatment with an Ang-II AT_1 receptor antagonist (losartan) reversed the prompt increase in mean arterial blood pressure (MABP) caused by indomethacin, it had no effect on the increase evoked by systemic L-NAME administration.

3 Pretreatment with losartan inhibited the leukocyte rolling flux, adhesion and emigration which occurs after 60 min NOS inhibition by 83, 80 and 70% respectively, and returned leukocyte rolling velocity to basal levels.

4 Losartan significantly reduced the leukocyte-endothelial cell interaction elicited by COX inhibition. In contrast, leukocyte recruitment induced by acute mast cell activation was not inhibited by losartan.

5 AT_1 receptor blockade also prevented the drop in haemodynamic parameters such as mean red blood cell velocity (V_{mean}) and shear rate caused by NOS and COX inhibition.

6 In this study, we have demonstrated a clear role for Ang-II in the leukocyte-endothelial cell interactions and haemodynamic changes which arise in the absence of NO or prostacyclin (PGI_2). This is of interest since leukocyte recruitment, which culminates in the vascular lesions that occur in hypertension, atherosclerosis and myocardial ischemia-reperfusion injury, might be prevented using AT_1 Ang-II receptor antagonists.

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Keywords: Angiotensin II, nitric oxide, prostacyclin, cyclo-oxygenase, leukocyte, endothelium, intravital microscopy, mast cells.

Abbreviations: Ang-II, angiotensin-II; ACEI, angiotensin converting enzyme inhibitor; CMP 48/80, compound 48/80; Dv, venular diameter; ET-1, endothelin-1; ICAM-1, intercellular adhesion molecule-1; L-NAME, N^G-nitro-L-arginine methyl ester; MABP, mean arterial blood pressure; PGI_2, prostacyclin; TNFα, tumour necrosis factor-α; V_{mean}, mean red blood cell velocity; V_{rbc}, centreline red blood cell velocity; V_{wbc}, leukocyte rolling velocity

Introduction

Leukocyte accumulation in the vessel wall is a hallmark of the early stages of atherosclerosis, acute myocardial infarction and several renal diseases of diverse etiology (Badimon *et al.*, 1993; Ricevuti *et al.*, 1990; Klahr *et al.*, 1988) where angiotensin-II (Ang-II) seems to play a critical role (Alderman *et al.*, 1991; Badimon *et al.*, 1993; Lafayette *et al.*, 1992, Thaiss *et al.*, 1996). Ang-II is the main effector peptide of the renin-angiotensin system and we have recently revealed that it has proinflammatory activity at sub-vasoconstrictor doses *in vivo*. In particular, it induces leukocyte recruitment in the rat mesenteric microvasculature through endothelial P-selectin up-regulation in the vessel wall and this effect is primarily mediated *via* a subtype Ang-II AT_1 receptor interaction (Piqueras *et al.*, 2000).

Inhibition of endogenous NO with the NO synthase (NOS) inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME), evokes significant leukocyte-endothelial cell interactions followed by increased microvascular permeability, suggesting that constitutive NO production from microvascular endothelial cells may play a role in maintaining the functional integrity of microvascular endothelium (Kubes *et al.*, 1991; Kubes & Granger, 1992; Ardnt *et al.*, 1993). Likewise, during circulatory perturbations such as ischemia followed by reperfusion, traumatic shock or hypertension, the loss of endothelial nitric oxide (NO) is believed to underlie the development of inflammation, including enhanced leukocyte-endothelial cell interactions (Ma *et al.*, 1993; Scalia *et al.*, 1999).

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Like NO, prostacyclin (PGI_2) is an endothelium-derived vasodilator involved in maintaining the balance in systemic and regional haemodynamics by opposing endothelium-derived vasoconstrictive factors (Tolins *et al.*, 1991). In this context, patients with ischemic heart disease have decreased prostacyclin plasma levels (Neri Serneri *et al.*, 1982). In addition, it has been reported that stimulation of prostacyclin synthesis protects the reperfused myocardium from ischemic injury, thereby reducing neutrophil infiltration in the ischemic tissue (Hohfeld *et al.*, 1993). Moreover, there is evidence that prostacyclin agonists reduce early atherosclerosis in hyperlipidemic hamsters by, among other actions, suppressing monocyte adhesion to vascular endothelium, monocyte chemotaxis and tumour necrosis factor-α (TNFα) production (Kowala *et al.*, 1993). Finally, indomethacin superfusion within the rat mesenteric microvasculature elicits leukocyte-endothelial cell interactions (Wallace *et al.*, 1993).

Epidemiological evidence suggests that high blood pressure may have a direct role in enhancing atherosclerotic lesion formation (Chobanian & Alexander, 1996). Atherosclerosis is three times more common in patients with hypertension, and there is a positive, although not linear, correlation between hypertension and atherosclerosis (Doyle, 1990). There is a body of evidence which shows that inhibition of NO synthesis results in hypertension and that inhibiting Ang-II with Ang-II selective antagonists or angiotensin converting enzyme inhibitors (ACEI) can blunt, if not prevent this response (Pollock *et al.*, 1993; Michel *et al.*, 1996; Takemoto *et al.*, 1997). The primary aim of the present study was, therefore, to investigate if Ang-II is involved in the acute and systemic increase in mean arterial blood pressure (MABP) induced by L-NAME and indomethacin. To determine whether there is a relation between the acute hypertension and leukocyte recruitment witnessed after endothelial disruption by lack of vasodilators, we next investigated the possible involvement of Ang-II in the leukocyte-endothelial cell interactions caused as a result of acute NOS inhibition by L-NAME. In addition, since NO and PGI_2 are the most important mediators which preserve endothelial integrity within the vasculature, this study also aimed to determine the role of Ang-II in the leukocyte-endothelial cell interactions provoked by indomethacin-induced-COX inhibition. Furthermore, it has been shown that L-NAME activates mast cells, and that human chymase, a serin protease expressed by mast cells (Urata *et al.*, 1996), is an important alternative pathway for the local generation of Ang-II within the human heart and blood vessels. Thus, the possible participation of Ang-II in the leukocyte recruitment evoked by the acute mast cell degranulation elicited by compound 48/80 (CMP 48/80) was also investigated in the present study.

Methods

Systemic studies

Sprague-Dawley rats (200–250 g) were anaesthetized with pentobarbital sodium (50 mg kg^{-1}, i.p.). The right carotid artery and jugular vein were cannulated to measure systemic arterial blood pressure (MABP) through a pressure transducer (Spectramed Stathan P-23XL) connected to a recorder (GRASS RPS7C8B, Quincy, MA, U.S.A.) and to permit the intravenous administration of anaesthetic and drugs respectively. After a 10 min stabilization period animals received either saline or losartan (10 mg kg^{-1}) intravenously. After a further 10 min, animals received either indomethacin (20 mg kg^{-1}) or L-NAME (10 mg kg^{-1}) as a bolus intravenous injection. MABP was recorded every 20 min for 60 min and expressed as a percentage of basal values. The doses chosen for indomethacin and L-NAME in this study have previously been reported to inhibit prostaglandin synthesis and to increase systemic blood pressure respectively (Wallace *et al.*, 1993; Sigmon & Beierwaltes, 1993). The dose of 10 mg kg^{-1} i.v. of losartan has been shown to produce maximum reductions in MABP in spontaneously hypertensive rats and to inhibit the pressor response to Ang-II. Indeed, higher doses of losartan (e.g., 30 mg kg^{-1}) do not result in further reductions of MABP (Wong *et al.*, 1990).

Intravital microscopy

The experimental preparation used in this study was similar to that described previously (Sanz *et al.*, 1999). Male Sprague-Dawley rats (200–250 g) were fasted for 24 h and anaesthetized with pentobarbital sodium (50 mg kg^{-1}, i.p.). A tracheostomy was performed to maintain a patent airway throughout the experiment. A polyethylene catheter was inserted in the right carotid artery to monitor mean arterial blood pressure (MABP) through a pressure transducer (Spectramed Stathan P-23XL) connected to a recorder (GRASS RPS7C8B, Quincy, MA, U.S.A.), and a second catheter was placed in the contralateral jugular vein to permit the intravenous administration of additional reagents (anaesthetic or drug). A midline abdominal incision was made and the rats were then placed in a supine position on an adjustable Plexiglass microscope stage. A segment of the midjejunum was exteriorized and draped over an optically clear viewing pedestal which allowed transillumination of a 2 cm^2 segment of the tissue. The temperature of the pedestal was maintained at 37°C and the exposed tissue was covered with saline-soaked gauze to minimize tissue dehydration. The exposed mesentery was suffused continuously at a rate of 1 ml min^{-1} with a warmed bicarbonate-buffered salt solution (pH 7.4).

The mesenteric preparation was then observed using an intravital orthostatic microscope (Nikon Optiphot-2, SMZ1, Badhoevedorp, The Netherlands) equipped with a 20× objective lens (Nikon SLDW, Badhoevedorp, The Netherlands) and a 10× eyepiece. A video camera (Sony SSC-C350P, Cologne, Germany) mounted on the microscope projected the image onto a colour monitor (Sony Trinitron PVM-14N2E, Cologne, Germany) and the images were video recorded (Sony SVT-S3000P, Cologne, Germany) for playback analysis. The final magnification of the video screen was 1300×. Animal temperature, monitored using a rectal electrothermometer, was maintained at 37°C with an infrared heat lamp.

Single unbranched mesenteric venules (20–40 μm in diameter) were selected for study and their diameters were measured on-line using a video caliper (Microcirculation Research Institute, Texas A&M University, College Station, Texas, U.S.A.). Centreline red blood cell velocity (V_{rbc}) was also measured on-line using an optical Doppler velocimeter (Microcirculation Research Institute, Texas A&M Univer-

sity). Venular blood flow was calculated from the product of mean red blood cell velocity ($V_{mean} = V_{rbc}\ 1.6^{-1}$) and cross sectional area, assuming cylindrical geometry. Venular wall shear rate (γ) was calculated based on the Newtonian definition: $\gamma = 8 \times (V_{mean}\ D_v^{-1})\ s^{-1}$, in which D_v is venular diameter (House & Lipowsky, 1987).

The number of rolling, adherent and emigrated leukocytes was determined off-line during playback of videotaped images. Rolling leukocytes were defined as white blood cells moving at a slower velocity than erythrocytes. Leukocyte rolling velocity (V_{wbc}) was determined as the time required for a leukocyte to move along 100 μm length of the microvessel and is expressed as $\mu m\ s^{-1}$. Rolling leukocyte flux was defined as those cells crossing a defined reference point in the vessel. The same reference point was used throughout the experiment as leukocytes may roll for only a section of the vessel before rejoining the blood flow or becoming firmly adherent. A leukocyte was defined as adherent to venular endothelium if it was stationary for at least 30 s. Leukocyte adhesion was expressed as the number per 100 μm length of venule. Leukocyte emigration was expressed as the number of white blood cells per microscopic field surrounding the venule.

Experimental protocol

After stabilization of the mesentery for 30 min, a baseline recording was taken to establish basal values (time 0) of mean arterial blood pressure (MABP), V_{rbc}, D_v, shear rate and leukocyte rolling flux, velocity, adhesion and emigration. The superfusion buffer was then supplemented with L-NAME (100 μM), since previous studies have demonstrated that this dose causes the maximum and most consistent increase in leukocyte rolling and adhesion after 60 min superfusion (Arndt *et al*., 1993). Recordings were performed for 5 min at 15 min intervals over a 60 min period and the aforementioned leukocyte and haemodynamic parameters were measured at each stage. To determine if Ang-II is involved in L-NAME-mediated events, a second group of animals was pretreated 10 min before starting L-NAME superfusion with a selective antagonist of subtype AT_1 Ang-II receptor, losartan (10 mg kg^{-1}, i.v.).

To further investigate if Ang-II is implicated in the leukocyte-endothelial cell interactions provoked by a lack of prostaglandins, we performed another set of experiments in which 25 $\mu g\ ml^{-1}$ dose of indomethacin was suffused onto the exposed mesentery. The dose chosen for this study was based on previously reported data where a pronounced peak of leukocyte adhesion was observed within 30 min of indomethacin superfusion and blood levels of PGE_2 were reduced by more than 90% (Wallace *et al*., 1993). Similarly, a second group of animals was administered with a bolus injection of losartan (10 mg kg^{-1}, i.v.) 10 min prior to indomethacin superfusion. Video recordings were again made at the same time points as those previously described for L-NAME.

Finally, as Ang-II can be locally generated *via* mast cell chymase, its possible role in the leukocyte recruitment evoked by acute mast cell degranulation was also investigated in the present study. The mesentery was suffused for 60 min with CMP 48/80 at 1 $\mu g\ ml^{-1}$, a dose previously found to achieve maximal responses (Gaboury *et al*., 1995). Responses were determined throughout 1 h at the same time points as previously described for the other agents under investigation. Similarly, losartan was administered at the same dose and time point as in the aforementioned protocols.

Statistical analysis

All data are expressed as mean ± s.e.mean. The data within groups were compared using an analysis of variance (1 way-ANOVA) with a Bonferroni *post hoc* correction for multiple comparisons. A P value <0.05 was considered to be statistically significant.

Materials

L-NAME, indomethacin and CMP 48/80 were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Losartan was kindly donated by Merck Sharp & Dohme, Spain.

Results

Figure 1 shows that a bolus injection of L-NAME (10 mg kg^{-1}) induced a significant increase in MABP which lasted the remainder of the 60 min experimental period (Figure 1a). Pretreatment with losartan did not prevent L-NAME-induced MABP elevation. Interestingly, when animals were administered with a bolus dose of indomethacin (20 mg kg^{-1}), a significant increase in MABP was observed during the 30 min after NSAID injection, while a return to basal levels was witnessed after this time period. Losartan pretreatment prevented the increase detected in MABP by COX inhibition and normalized it to control values (Figure 1b).

Figure 2 illustrates the time course of changes in leukocyte rolling flux, adhesion and emigration induced by superfusion of the rat mesentery with L-NAME. Significant increases in leukocyte rolling (85.4 ± 16.0 vs 19.7 ± 2.1 cells min^{-1}), adhesion (10.4 ± 2.8 vs 0.3 ± 0.2 cells 100 μm^{-1}) and emigration (4.0 ± 1.7 vs 0.0 ± 0.0 cells $field^{-1}$) were observed at 60 min with a 100 μM dose of L-NAME vs buffer. Concomitant significant decreases in leukocyte rolling velocity were also detected (46.5 ± 5.0 vs 97.3 ± 5.2 $\mu m\ s^{-1}$ at 60 min). Administration of losartan inhibited L-NAME-induced leukocyte rolling flux and adhesion by 83 and 80% respectively at 60 min (Figure 2). In addition, losartan significantly decreased L-NAME-induced leukocyte extravasation by 70% at the same time point and returned leukocyte rolling velocity to basal levels (113.4 ± 15 vs 97.3 ± 5.2 $\mu m\ s^{-1}$ at 60 min).

Table 1 summarizes the results obtained for different haemodynamic parameters prior to (0 min) and 60 min following L-NAME superfusion. As expected, local L-NAME induced no significant changes in MABP, however, a significant decrease in V_{rbc}, V_{mean} and shear rate in single mesenteric venules was observed 60 min after L-NAME superfusion. The venular diameter did not change. Losartan pretreatment significantly attenuated the effect of L-NAME superfusion on V_{rbc}, V_{mean} and shear rate.

Figure 3 shows the effect of leukocyte responses elicited by indomethacin superfusion. Indomethacin induced a significant increase in leukocyte rolling flux, adhesion and

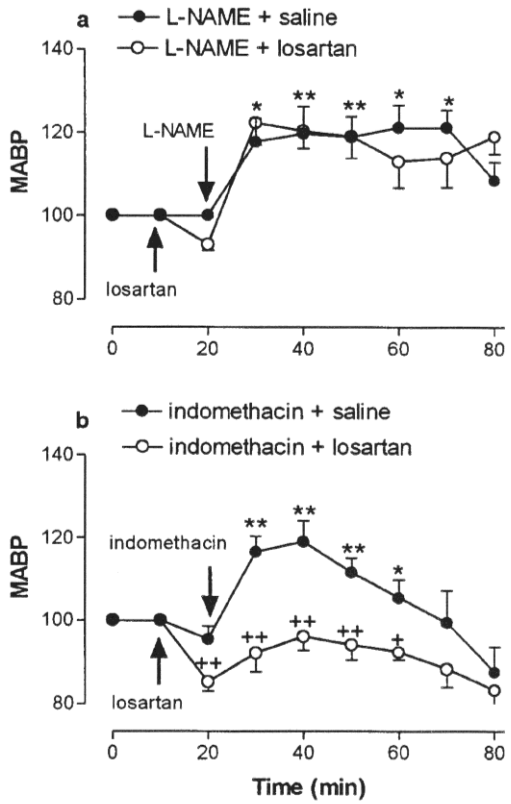

Figure 1 Effects of losartan (10 mg kg^{-1}, i.v.) on mean arterial blood pressure (MABP) in anaesthetized rats treated with L-NAME (10 mg kg^{-1}, i.v.) (a) or with indomethacin (20 mg kg^{-1}, i.v.) (b). Results are expressed as percentage of basal values. Each point and bar represent the mean±s.e.mean of $n=4-6$ animals per group. *$P<0.05$ or **$P<0.01$ relative to the control value (0 min) in the untreated group. $^{+}P<0.05$ or $^{++}P<0.01$ relative to the untreated group.

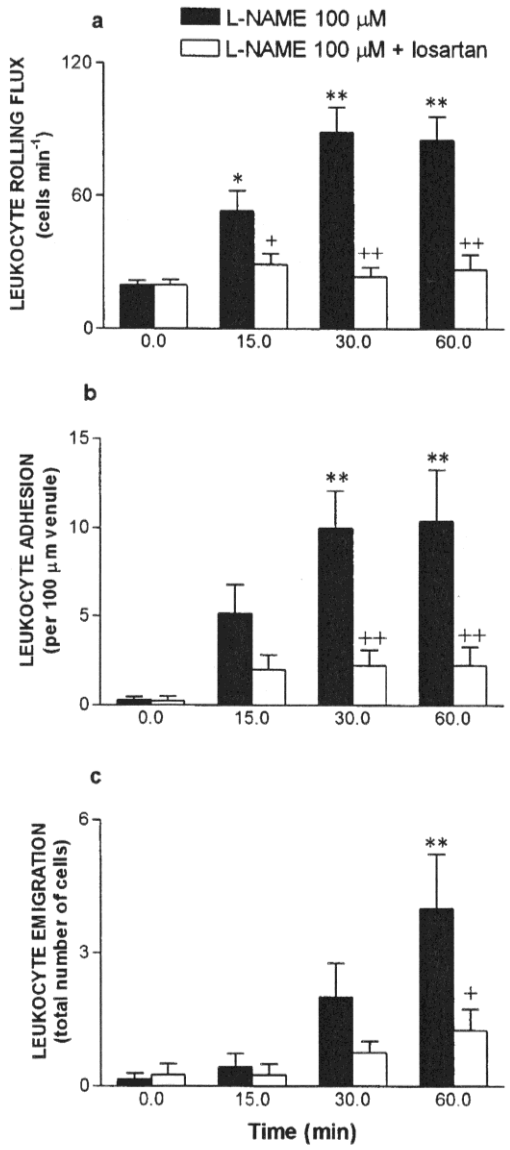

Figure 2 Effect of losartan pretreatment on L-NAME-induced leukocyte rolling flux (a), leukocyte adhesion (b) and leukocyte emigration (c) in the rat mesenteric postcapillary venules. The mesentery was superfused with bicarbonate-buffered saline. Baseline parameters (0 min) were determined after a 30 min stabilization period. The superfusion buffer was then supplemented with L-NAME (100 μM). Parameters were measured 15, 30 and 60 min after superfusion with L-NAME in animals untreated ($n=5$) or pretreated with losartan (10 mg kg^{-1}, $n=5$). Results are presented as mean± s.e.mean. *$P<0.05$ or **$P<0.01$ relative to the control value (0 min) in the untreated group. $^{+}P<0.05$ or $^{++}P<0.01$ relative to the untreated group.

emigration after 60 min superfusion vs buffer (122.4±29.1 vs 17.0±5.4 cells min^{-1}, 8.6±1.9 vs 0.2±0.2 cells 100 μm^{-1} and 1.4±0.2 vs 0.0±0.0 cells field^{-1}) which was accompanied by a significant decrease in leukocyte rolling velocity (76.6±12.5 vs 141.3±15.3 μm s^{-1}). Pretreatment with losartan significantly reduced indomethacin-induced increase in leukocyte rolling flux and adhesion by 98 and 88% respectively at this time point (Figure 3) and again returned leukocyte rolling velocity to basal levels (130.6±18.7 vs 144.7±8.2 μm s^{-1}). Although the decrease in indometacin-induced leukocyte emigration in the losartan pretreated group was not statistically significant (Figure 3), it should be noted that the leukocyte emigration evoked by indomethacin was also very subtle.

Local indomethacin suffusion induced no significant changes in D_v, V_{rbc} or MABP, however, it caused a significant diminution of V_{mean} and shear rate after 60 min superfusion. Pretreatment with losartan inhibited the decrease observed in both haemodynamic parameters elicited by indomethacin (Table 2).

As shown in Figure 4, CMP 48/80 elicited a significant increase in leukocyte rolling flux (104.8±8.7 vs 26.8±1.0

Table 1 Haemodynamic parameters in untreated and losartan (10 mg kg^{-1}) treated animals before (0 min) and after (60 min) L-NAME superfusion (100 μM)

	Untreated animals		*Losartan-treated animals*	
	0 min	*60 min*	*0 min*	*60 min*
D_V (μm)	25.4±0.8	26.4±0.7	25.0±1.0	25.8±2.5
V_{rbc} (mm s^{-1})	3.0±0.2	2.0±0.3*	2.4±0.4	2.3±0.6
V_{mean} (mm s^{-1})	1.9±0.2	0.8±0.3**	1.5±0.2	1.4±0.4
Shear rate (s^{-1})	557.8±61.3	360.2±61.5*	470.4±64.4	441.4±107.3
MABP (mm Hg)	119.4±11.2	123.9±10.2	106.7±6.7	90.0±10.0

*$P<0.05$ relative to control value (0 min). All values are mean±s.e.mean ($n=5$ animals per group).

cells min^{-1} at 60 min), adhesion (2.8±0.8 vs 0.0±0.0 cells 100 μm^{-1} at 60 min) and emigration (0.8±0.3 vs 0.0±0.0 cells field^{-1} at 60 min) versus buffer which was accompanied by a significant reduction in leukocyte rolling velocity (66.5±16.9 vs 122.8±8.4 μm s^{-1} at 60 min). However, pretreatment with losartan did not provoke any reduction in the leukocyte-endothelial cell interactions induced by CMP 48/80 (Figure 4). In addition, haemodynamic parameters were neither decreased after CMP 48/80 superfusion nor affected by losartan pretreatment (Table 3).

Discussion

Chronic NOS inhibition results in hypertension, which is known to be Ang-II-dependent (Pollock *et al*., 1993; Michel *et al*., 1996; Takemoto *et al*., 1997). In our study, losartan showed no effect on the increased blood pressure evoked by acute and systemic L-NAME administration. This is in agreement with the results obtained by Sigmond & Beierwaltes (1993), which found that losartan had no effect on MABP elevation induced during acute NOS inhibition. Conversely, Ang-II seems to be involved in the MABP elevation elicited by systemic COX inhibition. In chronic NOS inhibition an enhancement of angiotensin converting enzyme (ACE) activity and Ang-II receptors during the first week of treatment with L-NAME has been demonstrated (Takemoto *et al*., 1997). In contrast, in our study, 1 h of NOS inhibition was probably not enough to provoke an increase in ACE activity, and therefore, increase in MABP by acute L-NAME administration could not be effectively blocked by losartan pretreatment. Thus, the difference in ACE activity may explain the discrepancy between our results and those obtained in chronic NOS inhibition.

Despite these findings, in our study, we have clearly demonstrated a role for Ang-II in acute L-NAME and indomethacin-induced leukocyte–endothelial cell interactions. Losartan pretreatment inhibited the leukocyte rolling flux, adhesion and emigration which occurs after 60 min superfusion with L-NAME and returned leukocyte rolling velocity to basal levels. Similarly, losartan significantly reduced the leukocyte rolling and adhesion provoked by indomethacin superfusion at the same time point. In contrast, it showed no effect on the leukocyte recruitment which results from the acute mast cell activation evoked by CMP 48/80 superfusion. Therefore, these results indicate that the vasoconstrictor and proinflammatory effects of Ang-II are somehow disassociated. This is of considerable interest, since

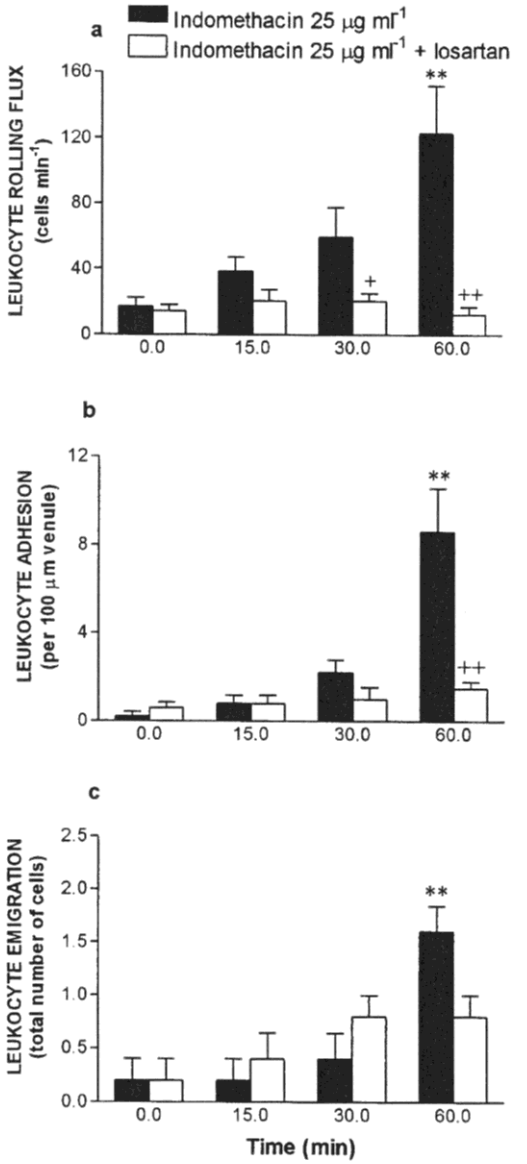

Figure 3 Effect of losartan pretreatment on indomethacin-induced leukocyte rolling flux (a), leukocyte adhesion (b) and leukocyte emigration (c) in the rat mesenteric postcapillary venules. Parameters were determined at 0, 15, 30 and 60 min after indomethacin (25 μg ml^{-1}) superfusion in animals untreated ($n=5$) or pretreated with losartan (10 mg kg^{-1}, i.v., $n=5$). Results are presented as mean±s.e.mean. *$P<0.05$ or **$P<0.01$ relative to the control value (0 min) in the untreated group. $^{+}P<0.05$ or $^{++}P<0.01$ relative to the untreated group.

it indicates that lack of NO or PGI_2 may result in the exposure of the vascular endothelium to the deleterious actions of vasoconstrictors. These vasoconstrictors could then trigger the subsequent leukocyte recruitment that may cause

Table 2 Haemodynamic parameters in untreated and losartan (10 mg kg^{-1}) treated animals before (0 min) and after (60 min) indomethacin superfusion (25 μg ml^{-1})

	Untreated animals		*Losartan-treated animals*	
	0 min	*60 min*	*0 min*	*60 min*
D_V (μm)	28.4±1.7	28.0±1.8	33.4±1.8	33.8±1.8
V_{rbc} (mm s^{-1})	2.7±0.3	1.9±0.2	3.6±0.3	3.4±0.4
V_{mean} (mm s^{-1})	1.7±0.2	1.1±0.1*	2.1±0.2	2.1±0.2
Shear rate (s^{-1})	511.5±51.5	326.1±21.9*	495.2±31.6	513.1±88.2
MABP (mm Hg)	120.0±4.5	118.3±3.1	126.3±8.5	109.2±12.8

*$P<0.05$ relative to control value (0 min). All values are mean±s.e.mean ($n=5$ animals per group).

the development of the vascular lesion detected in various disease states such as hypertension, atherosclerosis and myocardial ischemia-reperfusion injury (Mervaala *et al.*, 1999; Hernandez-Presa *et al.*, 1997; Ma *et al.*, 1993). In this context, it is well established that vasoconstrictors such as endothelin-1 (ET-1) or Ang-II can elicit leukocyte-endothelial cell interactions within the rat mesenteric microvasculature regardless of their vasoconstrictor activity (Sanz *et al.*, 1999; Piqueras *et al.*, 2000).

In addition, losartan administration significantly diminished the effect of L-NAME superfusion on haemodynamic parameters such as V_{mean} and shear rate in the rat mesenteric microvessels. Likewise, it significantly reversed the effect of indomethacin superfusion on V_{mean} and shear rate. This confirms previous reports in which it has been demonstrated that acute NOS inhibition increases blood pressure and decreases blood flow in visceral organs, and, that losartan has a tendency to attenuate the haemodynamic responses to L-NAME in all organ beds, including the intestine, without affecting the pressor response to L-NAME (Sigmon & Beierwaltes, 1993). These results, raise the question of whether leukocyte-endothelial cell interactions induced by COX or NOS inhibition are due to shear rate reductions, and whether restoration of vascular haemodynamics by losartan can explain the inhibition of leukocyte responses in these situations. Previous reports have shown that reduction of shear rate accounts for only a small fraction of the leukocyte adhesive response associated with inhibition of NO production (Kubes *et al.*, 1991). Moreover, 40% reduction in wall shear rate has been shown not to produce significant leukocyte adhesion (Perry & Granger, 1991). In our study, 60 min superfusion with either L-NAME or indomethacin caused significant reductions in shear rate of 35.5 and 36.2% respectively, while significant leukocyte-endothelial cell interactions were detected after 30 min superfusion. Therefore, we feel that the effects provoked by losartan on COX and NOS inhibition-induced leukocyte endothelial cell interactions are due to a direct effect on adhesion molecule expression, although a partial contribution of restoration of local haemodynamics cannot be discarded.

Among the different adhesion molecules involved in L-NAME-induced leukocyte-endothelial cell interactions, P-selectin seems to be the first to express itself on the vascular endothelium after acute NOS inhibition (Davenpeck *et al.*, 1994; Lefer *et al.*, 1999; Scalia *et al.*, 1999). Similarly, indomethacin superfusion or systemic administration causes significant vascular P-selectin expression within 1 h (Wallace

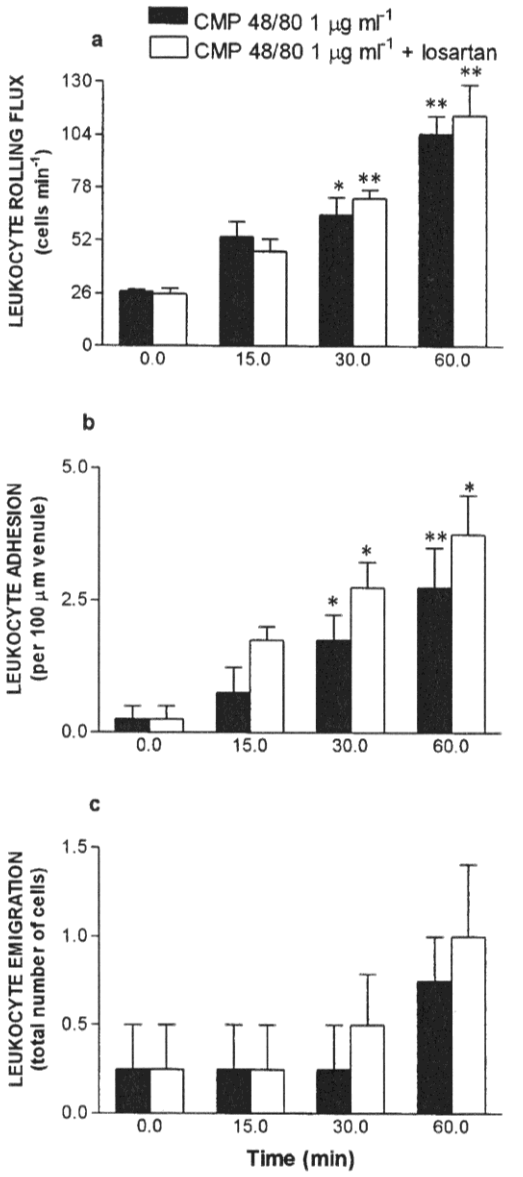

Figure 4 Effect of losartan treatment on CMP 48/80-induced leukocyte rolling flux (a), leukocyte adhesion (b) and leukocyte emigration (c) in rat mesenteric postcapillary venules. After the 30 min stabilization period, baseline values were determined (0 min). Parameters were measured 15, 30 and 60 min after superfusion with CMP 48/80 (1 μg ml^{-1}) in animals untreated ($n=4$) or pretreated with losartan (10 mg kg^{-1} i.v., $n=4$). Results are represented as mean±s.e.mean. *$P<0.05$ or **$P<0.01$ relative to the control value (0 min) in the untreated group.

et al., 1993; Kurose *et al.*, 1996; Morise *et al.*, 1998; 1999). Interestingly, we have recently demonstrated that leukocyte responses elicited by ET-1 and Ang-II are primarily mediated

Table 3 Haemodynamic parameters in untreated and losartan (10 mg kg^{-1}) treated animals before (0 min) and after (60 min) CMP 48/80 superfusion (1 μg ml^{-1})

	Untreated animals		*Losartan-treated animals*	
	0 min	*60 min*	*0 min*	*60 min*
D_V (μm)	26.0±0.6	26.0±0.6	30.0±2.9	29.0±3.3
V_{rbc} (mm s^{-1})	2.5±0.2	2.3±0.1	2.5±0.2	2.4±0.2
V_{mean} (mm s^{-1})	1.6±0.1	1.4±0.1	0.9±0.3	0.8±0.3
Shear rate (s^{-1})	482.1±44.5	438.2±28.6	420.4±23.2	399.3±22.4
MABP (mm Hg)	111.7±4.1	117.9±5.9	108.9±5.9	90.8±3.8

*$P<0.05$ relative to control value (0 min). All values are mean±s.e.mean ($n=4$ animals per group).

through increased endothelial P-selectin expression and that Ang-II causes subendothelial leukocyte infiltration which is Ang-II subtype AT_1 receptor dependent (Sanz *et al*., 1999; Piqueras *et al*., 2000). Indeed, the upregulation of P-selectin is understood to be a vital early step in leukocyte recruitment. Therefore, the degree of P-selectin expression determines the abundance of rolling leukocytes that may eventually adhere to the endothelium and extravasate into the tissue. Considered together, all these findings suggest that endothelial barrier dysfunction produced by lack of NO or PGI_2 results in leukocyte recruitment, which seems to be mediated through P-selectin upregulation. Therefore, it is likely that the attenuation of P-selectin expression caused by AT_1 Ang-II receptor blockade accounts for the potential inhibitory effect of losartan on both L-NAME and indomethacin-induced leukocyte-endothelial cell interactions.

On the other hand, previous reports have found that NOS inhibitors can cause mast cell degranulation (Kubes *et al*., 1993), and, as we found that L-NAME-induced leukocyte-endothelial cell interactions were inhibited by losartan, we hypothesized that the AT_1 receptor antagonist may also inhibit the leukocyte responses elicited by acute mast cell activation. Moreover, there is evidence that perivascular mast cells store and release chymotrypsin-like protease (chymase) which, in turn, promotes the conversion of Ang-I to Ang-II (Urata *et al*., 1996) which is considered to constitute an alternative Ang-II generating-system. In our study, we found no role for Ang-II in the leukocyte-endothelial cell interactions provoked by acute mast cell degranulation. Several explanations may account for the effects observed. Upon stimulation, mast cells are known to release a variety of fast acting mediators such as histamine, leukotrienes and PAF. Indeed, leukocyte rolling and adhesion evoked by CMP 48/80 can be inhibited by pretreatment with an H_1 and with PAF receptor antagonists respectively or by antibodies directed against P-selectin or β_2 integrin function (Gaboury *et al*., 1995). Therefore, upon acute mast cell activation, it is likely that preformed mediators such as histamine or PAF may constitute a much more powerful stimulus for the observed leukocyte accumulation than the newly synthesized Ang-II. Alternatively, it has recently been reported that chymase activity varies depending on the subclass of the mast cells and their tissue distribution within the same species (Akasu *et al*., 1998). Therefore, the absence of effect of losartan on leukocyte responses elicited by CMP 48/80 superfusion could also be explained by the low chymase activity in rat peritoneal mast cells compared to that found in mast cells from other organs such as the lung, heart or aorta or that encountered in mast cells under pathophysiological conditions.

In conclusion, the most striking observation in the present study is the fact that leukocyte-endothelial cell interactions elicited by both NOS and COX inhibition can be dramatically attenuated by pretreatment with an Ang-II antagonist directed against its AT_1 receptor subtype. Therefore, it is possible that losartan modulates leukocyte–endothelial cell interactions regardless of its anti-hypertensive activity. Consistent with this concept, we have previously found that leukocyte recruitment elicited by Ang-II occurs at doses that are devoid of vasoconstrictor activity (Piqueras *et al*., 2000). Thus, it can be postulated that the pro-adhesive and vasoconstrictor actions induced by Ang-II are not associated and that, therefore, vascular lesions occur at early stages when the hypertensive process has not yet fully developed. Hence, our data strongly suggest that the beneficial effects exerted by systemic administration of losartan when vascular balance is disrupted by reduced levels of vasodilators are due to a significant inhibition of leukocyte-endothelial cell interaction and restoration of vascular haemodynamics. This may be a key mechanism by which losartan contributes to the amelioration of the consequences of an endothelial disruption and may represent a new strategy for modulating the pathophysiology of leukocyte-induced endothelial dysfunction in circulatory disorders.

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III. RESUMEN GENERAL

III. 1. PROCESO INFLAMATORIO

BASES GENERALES DEL PROCESO INFLAMATORIO

La inflamación es una reacción de la microcirculación que se caracteriza por el movimiento de fluido y leucocitos desde el torrente sanguíneo hacia el tejido extravascular. Es un intento del huésped de localizar y eliminar células alteradas metabólicamente, partículas extrañas, microorganismos o antígenos.

El proceso inflamatorio normalmente es consecuencia de un daño tisular previo. Este daño inicia los mecanismos responsables de la localización y eliminación de los agentes extraños, seguidamente la respuesta inflamatoria se amplifica, activándose tanto la liberación de mediadores solubles como los sistemas de inflamación celular. Una vez ha tenido lugar la eliminación del agente patógeno, la finalización de la respuesta inflamatoria es llevada a cabo por inhibidores específicos de estos mediadores.

El inicio de la respuesta inflamatoria que sigue a un daño tisular tiene lugar dentro de la microvasculatura, concretamente en las vénulas capilares y postcapilares. Dentro de la red vascular se encuentran los componentes celulares de la respuesta inflamatoria: plasma, eritrocitos, plaquetas y leucocitos circulantes. Estas células están normalmente dentro del compartimento intravascular limitadas por una continua capa de células endoteliales las cuales están conectadas unas a otras por uniones tirantes y separadas del tejido por una membrana basal. Después de un daño tisular se producen cambios en la estructura de la pared vascular que llevan a:

- Pérdida de la integridad de las células endoteliales.
- Extravasación de fluido y componentes plasmáticos desde el espacio intravascular.
- Migración tanto de eritrocitos como de leucocitos hacia el tejido extravascular.

Esta respuesta de la vasculatura al daño está regulada por mediadores específicos de la inflamación producidos en los lugares donde éste se ha producido. Entre ellos están las **moléculas vasoactivas** que actúan directamente en los vasos produciendo alteraciones en el tono vascular y en el flujo sanguíneo intensificándose así la permeabilidad vascular a macromoléculas y los **factores quimiotácticos** que producen la extravasación de leucocitos desde el torrente sanguíneo hasta el foco inflamatorio (Fantone & Ward, 1994). De esta forma, los leucocitos una vez alcanzan el tejido dañado secretan otros mediadores que pueden aumentar o inhibir la respuesta inflamatoria.

Muchos de estos mediadores específicos de la inflamación son también dañinos para las células y las proteínas del huésped pero si tienen lugar durante períodos cortos son beneficiosas ya que estas células dañadas amplifican las señales que reclutan a los leucocitos hacia el lugar de la inflamación. Este mecanismo que daña a las células huésped está limitado por la eliminación del agente que causa el daño. En condiciones normales la respuesta inflamatoria elimina al agente patógeno, produciéndose una regeneración del tejido y de sus funciones fisiológicas; en otras ocasiones se produce un daño irreversible aunque se elimine al agente causal. Sin embargo, cuando no se elimina al agente causal esto da lugar a un daño tisular mayor o una respuesta inflamatoria exagerada lo cual puede conducir a la pérdida de la funcionalidad del órgano o tejido.

La naturaleza del estímulo inflamatorio determina la subclase de leucocito predominante (neutrófilo, eosinófilo, monocito o linfocito) mediante señales moleculares específicas que controlan el tráfico selectivo de estas células. Así, en enfermedades como asma o enfermedades alérgicas las principales células leucocitarias implicadas son los eosinófilos, en reacciones inflamatorias agudas son los neutrófilos y en procesos crónicos linfocitos y monocitos.

FACTORES QUIMIOTÁCTICOS

Una de las características de la respuesta inflamatoria es la acumulación local de leucocitos; la función esencial de este proceso es la acumulación de glóbulos blancos en los lugares de daño o infección con el fin de eliminar al agente patógeno. Los mecanismos que envuelven esta acumulación leucocitaria son complejos y dependientes de la interacción entre los leucocitos y las células endoteliales microvasculares. La acumulación leucocitaria *in vivo* se inicia por la generación de mediadores químicos que pueden ser generados por el fluido tisular (proteínas derivadas del complemento como el factor 5 del mismo C5a), liberadas de las células huésped (quimiocinas, factor activador de plaquetas (PAF) y leucotrieno B_4 (LTB_4)) o derivadas de los microorganismos invasores (péptidos quimiotácticos formilados y endotoxinas). Estos mediadores pueden actuar directamente sobre los leucocitos como el factor C5a y LTB_4, actuar sobre las células endoteliales haciéndolas adhesivas para los leucocitos como lipopolisacárido bacteriano (LPS) o citocinas como interleucina-1 (IL-1) o factor de necrosis tumoral (TNFα) o actuar indirectamente induciendo la liberación de nuevos mediadores, por ejemplo IL-1 y TNFα pueden causar la liberación de interleucina-8 (IL-8) de macrófagos, fibroblastos o células endoteliales. Estas señales químicas son factores quimiotácticos, es decir, son una familia de moléculas de diferente naturaleza química que comparten la capacidad de dirigir la movilidad de los leucocitos (Celi et al., 1997). Nos centraremos principalmente en los factores quimiotácticos que resultaron de interés para el desarrollo de esta tesis doctoral, concretamente PAF y metabolitos derivados del ácido araquidónico (AA).

PAF

PAF se forma a partir de fosfolípidos precursores denominados plasmalógenos que son constituyentes de las membranas celulares. Se sintetiza a través de dos vías: vía síntesis *de novo* y vía remodeladora.

La vía síntesis *de novo* es la que produce el PAF que es necesario en condiciones fisiológicas para la regulación funcional de las membranas plasmáticas. En ella, a partir de 1-alquil-2-liso-*sn*-glicero-3-fosfato se obtiene PAF después de una acetilación, una defosforilación y una transferencia de fosforilcolina a través de la fosfocolina transferasa.

La vía remodeladora es la que produce PAF en condiciones alérgicas o inflamatorias. En esta vía, las alquilacilglicerofosfocolinas se convierten en PAF pasando por un intermediario liso a través de la acción sucesiva de fosfolipasa A_2 (PLA_2) y de Acetil-CoA:1-alquil-2-liso-*sn*-glicero-3-fosforilcolina acetiltransferasa. Esta vía está asociada a la producción de AA.

La generación de PAF por la vía remodeladora está limitada únicamente a células inflamatorias tales como eosinófilos, macrófagos, células endoteliales, neutrófilos y plaquetas, aunque en estas últimas en muy pequeñas cantidades. En mastocitos, neutrófilos y en células endoteliales, una gran cantidad de PAF que se sintetiza es retenido intracelularmente.

El receptor de PAF se ha encontrado en plaquetas, neutrófilos, eosinófilos, macrófagos y tejido pulmonar, presenta un receptor con 7 dominios transmembrana típicos de receptor asociado a proteína G. Cuando PAF se une a su receptor se produce una serie de cambios bioquímicos:

- Activación de la fosfolipasa C (PLC) que produce la transformación de fosfoinositol 4,5 bifosfato en inosina trifosfato (IP_3) y diacilglicerol (DAG) que actúan como segundos mensajeros para las células.
- Aumento del calcio intracelular.
- Al menos en las plaquetas producen la inhibición de la formación de AMPc.

PAF es quimiotáctico para todo tipo de leucocitos y administrado exógenamente induce a adhesión leucocitaria (Bienvenu et al., 1993).

Derivados del metabolismo del AA

Los mediadores que se producen a través del metabolismo del AA son: prostaglandinas (PGs), tromboxanos (TXs), leucotrienos (LTs), hidroperoxiácidos y lipoxinas (LXs).

Estos mediadores no se encuentran almacenados en los tejidos, por tanto, para ser liberados previamente deben ser sintetizados. El paso limitante en la biosíntesis de estos mediadores es la formación del AA. La mayor fuente de AA son los fosfolípidos que son componentes estructurales de las membranas celulares. El AA se libera a partir de los fosfolípidos mayoritariamente a través de la acción de la enzima PLA_2 aunque también se puede liberar por la acción combinada de la PLC y la DAG lipasa.

PGs y TXs se generan a partir del AA por la acción de dos formas de la ciclooxigenasa (COX) (Mitchell et al, 1995). COX-1 es constitutiva y responsable de la liberación basal de PGs y TXs mientras que COX-2 es inducible por estímulos inflamatorios tales como citocinas o endotoxinas aunque recientemente se ha comprobado que puede ser constitutiva en algunos tejidos. La COX transforma al AA en endoperóxidos (PGG_2 y PGH_2) que son lábiles a pH y temperatura fisiológicos y se transforman en TXA_2 y PGI_2 (PG primarias). El metabolito formado varía de una célula a otra; así los

endoperóxidos se convierten casi exclusivamente en TXA_2 en las plaquetas sanguíneas pero en PGI_2 en el endotelio vascular.

PGE_2 y PGI_2 relajan el músculo liso vascular y son potentes vasodilatadores de las arteriolas precapilares. Esto conduce a características de la inflamación aguda como son la coloración rojiza y eritema. Por consiguiente, se produce un aumento del flujo sanguíneo en los tejidos inflamados que aumenta la pérdida de plasma causada por un aumento de la permeabilidad plasmática. Estos aumentos dan lugar a la formación de edema y además contibuyen a producir otros síntomas de la inflamación como son daño o fiebre; PGE_2 y PGI_2 son capaces de producir hiperalgesia frente a otros estímulos y PGE_2 es un potente agente pirético. Estas prostaglandinas tienen un efecto modulador en la acumulación leucocitaria y en la liberación de histamina por las células mastocitarias. Además, PGI_2 inhibe la agregación plaquetaria y la adhesión leucocitaria.

LTs e hidroperoxiácidos se obtienen a partir de la oxidación de AA por acción de la enzima lipooxigenasa (LO). El AA se convierte por la acción de la LO en ácidos hidroperoxieicosatetraenoicos (HPETEs) que se reducen rápidamente por la acción de la glutationperoxidasa a los correspondientes hidroxiácidos (HETEs). Se han descrito dos lipoxigenaciones; la primera ocurre en plaquetas sanguíneas a través de la 12-LO y da lugar a 12-HPETE; y la segunda está restringida principalmente a los neutrófilos, eosinófilos, monocitos, macrófagos y células mastocitarias y en ella a través de la 5-LO tiene lugar la formación de 5-HPETE. La formación de 5-HPETE tiene mayor interés ya que es el intermediario en la formación de los LTs. La reacción inicial es la conversión de HPETEs en un epóxido inestable, LTA_4. A partir del LTA_4 se forman el LTB_4 y el LTC_4 respectivamente. En este caso el metabolito formado también depende de la célula: así, los eosinófilos sintetizan fundamentalmente LTC_4 y los neutrófilos LTB_4. Además el LTC_4

puede ser metabolizado a LTD_4 y LTE_4. También a partir del LTA_4 pero no enzimáticamente se forman 5,12- y 5,6-dihidroxiácidos. Otros metabolitos del AA son los **ácidos trihidroxieicosatetraenoicos** (THETs), los **epoxieicosatrienoico**s (EETs), **epoxihidroxieicosatrienoicos** (EPHETs) y las **lipoxinas** que se forman por la acción combinada de 15- y 5-LO (Salmon & Higgs, 1994).

Los productos derivados de la acción de la LO tienen efectos débiles sobre el tono vascular pero LTB_4, LTC_4 y LTD_4 causan erupción en la piel por un mecanismo desconocido. **LTB_4** es uno de los agentes quimiotácticos más potentes para los leucocitos y, por tanto, produce migración de leucocitos al espacio extravascular. Tanto los neutrófilos (Nourshargh, 1993) como los eosinófilos (Sehmi et al., 1992) poseen receptores en su superficie para LTB_4. LTC_4, LTD_4, LTE_4 no activan directamente a los neutrófilos pero aumentan la adhesión de los neutrófilos a cultivos de células endoteliales.

Citocinas

Las citocinas son una familia de proteínas reguladoras que son sintetizadas por muchos tipos de células pero fundamentalmente son sintetizadas por los leucocitos como respuesta a varios estímulos. Las citocinas incluyen a ILs, interferones (IFNs), "colony-stimulating factors" (CSFs) y citotoxinas o TNFs (TNF-α y TNF-β). Las citocinas actúan como mensajeros intercelulares en el sistema inmunitario y conectan a éste con otros sistemas fisiológicos del organismo (Hamblin, 1994).

Dentro de la familia de las citocinas destacan las **quimiocinas** que son una familia de citocinas de bajo peso molecular (8-12 KD) que se caracterizan por la presencia en determinadas posiciones de cuatro cisteínas. La posición de los dos residuos cisteína más cercanos a la porción N-terminal determina las subfamilias: CXC quimiocinas, CC

quimiocinas, C quimiocinas y CX_3C quimiocinas. En las CXC quimiocinas las dos cisteínas cercanas a la porción N-terminal se encuentran separadas por un aminoácido mientras que en las CC quimiocinas las dos cisteínas son adyacentes. La C quimiocina representada únicamente por la linfotactina, presenta dos cisteínas en su estructura y sólo un residuo cisteína en la porción N-terminal y las CX_3C quimiocinas difieren del resto por la presencia de tres aminoácidos entre las dos primeras cisteínas (Rollins, 1997). De ellas destaca su selectividad en su actividad quimiotáctica para distintos subtipos de leucocitos, así las CXC quimiocinas son principalmente quimiotácticas para neutrófilos mientras que las CC quimiocinas son quimiotácticas para otros tipos de leucocitos como linfocitos, monocitos, eosinófilos o basófilos.

OTROS MEDIADORES O MODULADORES DE LA INFLAMACIÓN

Radicales libres

Un RLO es cualquier especie con existencia independiente que contiene uno o más electrones desapareados en los que el electrón desapareado ocupa un orbital atómico o molecular por sí mismo (Gillissen & Nowak, 1998). RLO es un término que también incluye a derivados del oxígeno que no contienen pares de electrones desapareados como peróxido de hidrógeno (H_2O_2), oxígeno molecular (O_2), ozono (O_3) y ácido hipocloroso (HOCl). Los radicales libres se forman constantemente en el pulmón y tienen un papel fisiológico. Una vez formados pueden destruir microorganismos, células normales o neoplásicas o modular la respuesta inflamatoria.

La formación de radicales libres por parte de las células inflamatorias juega un papel importante en la defensa natural de estas células. Los mecanismos microbicidas de los neutrófilos consisten en una combinación de procesos oxidativos y enzimáticos que parece que se activan simultáneamente cuando se inicia la fagocitosis. La fagocitosis consiste en el reconocimiento, internalización y digestión de sustancias extrañas o células dañadas. Estos procesos oxidativos dan lugar a una producción secuencial de RLO con propiedades microbicidas o microbioestáticas.

La primera especie que se produce es el anión superóxido (O_2^-) y se forma por la reducción del óxigeno molecular a través de oxidasas de la membrana plasmática y mitocondrial. Se convierte en peróxido de hidrógeno por dismutación espontánea o por la acción de la superóxido dismutasa (SOD). El peróxido de hidrógeno puede convertirse en oxígeno molecular por la acción de la catalasa aunque también puede dar lugar al radical

hidroxilo en presencia de metales de transición como el hierro a través de la reacción de Haber-Weiss o de Fenton. El peróxido de hidrógeno difunde fácilmente a través de las membranas celulares, por tanto, la formación de radical hidroxilo ocurre tanto intra como extracelularmente. El radical hidroxilo causa daño celular directo en el lugar en el que se forma. Por otro lado, el peróxido de hidrógeno puede también transformarse en ácido hipocloroso por acción de la mieloperoxidasa presente en los neutrófilos PMNs.

Normalmente existe un balance entre los niveles de oxidantes y los sistemas antioxidantes intra y extracelulares, de hecho, hay un extremado control del balance redox especialmente para proteger a las células sin bloquear el papel fisiológico de los oxidantes celulares. Cuando los RLO se generan en exceso o en lugares inapropiados o cuando la defensa antioxidante es insuficiente, los radicales pueden dañar ADN, lípidos, proteínas o carbohidratos. Los RLO derivados de los neutrófilos también pueden influir en algunas funciones celulares. Así, los RLO promueven la acumulación leucocitaria aumentando la expresión de moléculas de adhesión en las células endoteliales (Patel et al., 1991) y son capaces de promover la liberación de agentes quimiotácticos como PAF o LTB_4, ampliando así la respuesta inflamatoria.

NO

El NO es un radical libre de naturaleza gaseosa que es sintetizado por diversas células del sistema inmune e incluso neuronas a través de la oxidación de L-arginina a L-citrulina por la acción de NOS. Su principal efecto es la vasodilatación pero posee otras acciones tales como el mantenimiento del tono basal de los vasos o la inhibición de la activación, adhesión y agregación plaquetaria. Además, Kubes et al. (1991) descubrieron el efecto más interesante a nivel del proceso inflamatorio: el NO endógeno es capaz de

inhibir la adhesión leucocitaria. Así, la superfusión con inhibidores de NOS endotelial indujo a una acumulación leucocitaria en vénulas postcapilares a los 30 minutos de su administración. Este efecto del NO no podía explicarse como una acción de un simple vasodilatador. Una posible explicación basada en trabajos posteriores sería que los inhibidores de NOS produjeran una disminución de los niveles de GMPc y que este fenómeno indujera a la activación de moléculas de adhesión proadhesivas tales como P-selectina y así, la producción constitutiva de NO en condiciones basales actuaría como control de la adhesión leucocitaria (impidiendo el aumento de la expresión de P-selectina) (Kanwar & Kubes, 1995). Este mecanismo constituiría un proceso autocrino.

Estudios realizados in vivo sugieren que la principal diana del NO son los mastocitos, el NO es capaz de inhibir la activación mastocitaria y de esta forma indirectamente inhibir el proceso de extravasación leucocitaria (Gaboury et al., 1996). Esta inhibición de la activación mastocitaria se produce porque NO interacciona con el anión superóxido (que activa las células mastocitarias) inactivándolo. Este mecanismo constituiría un proceso paracrino.

Por otra parte, estudios realizados in vitro han demostrado que NO puede afectar la expresión genética de moléculas de adhesión en células endoteliales reduciendo los niveles de "vascular cell adhesion molecule-1" (VCAM-1) en su superficie celular tras activación con citocinas y de la expresión de integrinas β_2 en los leucocitos (De Caterina et al., 1995). De este modo, reduciría la adhesión y migración de neutrófilos y monocitos.

También se ha demostrado que NO reacciona con el anión superóxido y el producto de esta reacción es el peroxinitrito (Szabó, 1996). El peroxinitrito es una especie oxidante y tóxica que produce daño en las células endoteliales a través del inicio del daño del ADN. Este hecho tiene mucha importancia en condiciones de estrés oxidativo tales como

procesos de isquemia-reperfusión, shock endotóxico o incluso artritis reumatoide en las que esta combinación da lugar a daño endotelial (Elliot, 1996; Azma et al., 1996). El daño endotelial a su vez produce un aumento de la adhesión leucocitaria y una respuesta inflamatoria exacerbada (Ma et al., 1993).

De este modo, tanto los captadores de peroxinitrito como los donantes y agonistas de NO podrían utilizarse como fármacos antiflamatorios.

Agentes que aumentan los niveles de AMPc

La manipulación farmacológica que conduce a un aumento en los niveles de AMPc se puede llevar a cabo de dos formas: aumentando su síntesis a partir de ATP a través de la activación de la adenilato ciclasa (AC) (Torphy & Undem, 1991) o disminuyendo la hidrólisis del AMPc inhibiendo las fosfodiesterasas (PDE) (Beavo et al, 1994).

Recientemente se han implicado mecanismos de señalización intracelular en la regulación de la expresión de moléculas de adhesión como herramienta para modular la inflamación. En este sentido, se ha comprobado que AMPc es uno de esos mecanismos intracelulares implicados en una gran variedad de células y tejidos. Así, niveles elevados de este segundo mensajero dentro de las células activan la proteincinasa A (PKA) que fosforila otros sustratos y se ha demostrado que tiene efectos antiinflamatorios (Giembycz & Raeburn, 1991; Teixeira et al, 1997).

De este modo los agentes que elevan los niveles de AMPc serían moduladores efectivos de la acumulación leucocitaria in vivo inducida por varios estímulos y al modular la acumulación leucocitaria, estos agentes resultarían beneficiosos en la terapia de enfermedades que llevan asociado un proceso inflamatorio.

Estrógenos

Las mujeres, a diferencia de los hombres, poseen una protección natural frente a las enfermedades cardiovasculares hasta que comienza la menopausia. Multitud de estudios epidemiológicos han implicado a los estrógenos como mediadores clave de esta protección cardiovascular (Grodstein et al., 1997). Tanto las mujeres posmenopaúsicas que reciben un tratamiento de terapia hormonal sustitutiva como las mujeres premenopaúsicas presentan niveles bajos de lipoproteínas de baja densidad (LDL) y niveles elevados de lipoproteínas de alta densidad; pero además de este mecanismo parece que hay otro mecanismo implicado que es independiente de los niveles de lipoproteínas.

Éste es un mecanismo paracrino originado en el endotelio y que consiste en el aumento de la liberación de PGI_2 y NO. En este sentido, un posible mecanismo de la ateroprotección producida por estrógenos sería la capacidad de esta hormona de restaurar la integridad del endotelio dañado.

Se ha demostrado que hay niveles elevados de 6-Keto-prostaglandina$_{1\alpha}$ (producto en el que es metabolizada PGI_2) en arterias humanas uterinas de mujeres premenopaúsicas pero no en las posmenopaúsicas y que se produce una liberación de PGI_2 en células endoteliales tratadas con estrógenos en condiciones de hipoxia y de disminución de flujo sanguíneo (Steinleitner et al., 1989; Redmond et al., 1994). También se ha comprobado que los estrógenos pueden aumentar la expresión de la NOS constitutiva en células endoteliales humanas de cordón umbilical, de aorta y en miocardio de rata (Hishikawa et al, 1995; Nuedling et al., 1999; Huang et al., 2000). Recientemente se ha demostrado que los estrógenos pueden activar rápidamente la NOS sin inducir el aumento de la expresión de esta enzima (Chen et al., 1999; Stefano et al., 1999).

Además, los estrógenos tienen propiedades ateroprotectivas porque son capaces de interferir con las interacciones leucocito-endotelio, así se ha demostrado que inhiben la acumulación leucocitaria en varios estudios realizados in vivo (Nathan et al., 1999; Miyamoto et al., 1999; Squadrito et al., 1997). Por tanto, otro mecanismo de acción posible sería que los estrógenos produjeran una disminución de la expresión de diferentes moléculas de adhesión inducida por diversos estímulos.

MOLÉCULAS DE ADHESIÓN

Cuando se da una patología inflamatoria, los leucocitos como ya hemos comentado deben migrar desde el torrente sanguíneo hasta el foco inflamatorio. Este fenómeno implica la interacción específica entre ligandos presentes en la superficie leucocitaria y ligandos presentes en la célula endotelial. Las plaquetas, los leucocitos y las células endoteliales expresan moléculas en su superficie que se unen a ligandos presentes en otras células mediando así la interacción entre dos células o entre células y la matriz extracelular (Celi et al., 1997). Estas moléculas son las moléculas de adhesión. Basándose en su estructura homóloga, se han identificado cuatro familias de moléculas de adhesión: las selectinas, los ligandos de selectina tipo mucina, las integrinas y las moléculas de adhesión "tipo inmunoglobulinas" (Springer, 1994).

Las **selectinas** son proteínas dependientes de Calcio y tienen una estructura que se caracteriza por la presencia de un dominio NH_2 terminal tipo C-lectina, un dominio tipo "endothelial growth factor" (EGF), un número variable de estructuras homólogas a las proteínas reguladoras del complemento, un dominio transmembrana y un cola intracitoplásmica (Bevilacqua & Nelson, 1993). Las selectinas debido a su dominio tipo lectina se unen a estructuras carbohidratadas sialiladas (Lasky, 1992) y por tanto, comparten la habilidad de reconocer al tetrasacárido Sialil Lewis ^{x} (SLe^{x}) y a su isómero Sialil Lewis ^{a} (Sle^{a}). Hasta el momento se han identificado tres selectinas: L-selectina, E-selectina y P-selectina. **L-selectina** se expresa de forma constitutiva en los leucocitos pero no se haya ampliamente distribuida por la membrana plasmática sino que se concentra en los microvilli, en estructuras ricas en actina y miosina y en otros elementos del citoesqueleto; esta agrupación se piensa que es importante para la interacción de L-selectina con su/s ligando/s presentes en el endotelio (Konstantopoulus & McIntire, 1996;

Brown, 1997). Cuando los leucocitos se activan, su membrana plasmática se despoja de L-selectina, por tanto, la pérdida de L-selectina es un marcador muy fiable para distinguir leucocitos activados de los no activados (Brown, 1997). **E-selectina** es inducida en células endoteliales por citocinas o LPS (Bevilacqua & Nelson, 1993) y requiere síntesis de novo de proteína y de ARNm. Se ha identificado un ligando en los neutrófilos para E-selectina. "E-selectin ligand-1" (ESL-1) (Larsen et al., 1990). **P-selectina** se almacena preformada en plaquetas (Springer, 1994) siendo un componente integral de los gránulos α de las mismas en fase de reposo y también se almacena en células endoteliales (Carlos & Harlan, 1994) concretamente en las membranas de los cuerpos de Weibel-Palade de las células no estimuladas. Cuando estas células son activadas por mediadores de la inflamación tales como histamina o trombina, P-selectina es movilizada a la membrana externa (Springer, 1994) de plaquetas y de células endoteliales desde donde media su unión con neutrófilos, monocitos, eosinófilos, basófilos y T linfocitos. P-selectina en el endotelio interacciona con "P-selectin glicoprotein ligand-1" (PSGL-1) que es su ligando específico presente en los neutrófilos y con distintos carbohidratos.

Las **proteínas tipo mucina** son proteínas altamente glicosiladas ricas en serina y treonina y que tienen una extensa estructura. Estas proteínas son reconocidas por la familia de las selectinas. **PSGL-1** es el ligando mejor caracterizado de esta familia. Este polipéptido está compuesto por un dominio extracelular que contiene tres sitios potenciales para N-glicosilación, tres sitios potenciales para tirosín sulfatación y múltiples sitios para O-glicosilación. El resto de la secuencia contiene un dominio transmembrana y una cola intracitoplásmica. Esta proteína se expresa constitutivamente en monocitos, neutrófilos y ciertas clases de linfocitos (Larsen et al., 1989; Vachino et al., 1995). L-selectina reconoce dos mucinas presentes en grandes venas: glycosylation-dependent cell adhesion molecule 1

(GlyCAM-1) la cual es secretada (Lasky, 1992; Rosen, 1993) y **CD34** la cual se expresa en la superficie celular (Baumhueter et al., 1993).

Las **integrinas** son una familia de proteínas de superficie heterodímeras compuestas por dos subunidades, α y β, las cuales están unidas por enlaces no covalentes. De ellas destacaremos las integrinas β_2 y las integrinas α_4. Los leucocitos expresan tres integrinas que comparten la subunidad β_2 (CD18) pero tienen distintas subunidades α: LFA-1 (lymphocyte-function-associated-1 o CD11a/CD18 o $\alpha_L\beta_2$), Mac-1 (CD11b/CD18 o $\alpha_M\beta_2$) y la glicoproteína (gp) 150,95 (CD11c/CD18 o $\alpha_X\beta_2$) (Arnaout, 1990). **LFA-1** se expresa constitutivamente en la superficie de las células, pero su afinidad por unirse a los ligandos se aumenta mucho cuando sufre un cambio conformacional inducido por mediadores, entre ellos los factores quimiotácticos. Este proceso por el que una señal que se genera dentro de una célula y que al unirse a un agonista induce la activación de una integrina se conoce como "inside-out signaling". LFA-1 actúa como ligando tanto para ICAM-1 (intercellular adhesion molecule-1) como para ICAM-2 (intercellular adhesion molecule-2) (Larson & Springer, 1990; Springer, 1990). **Mac-1** también sufre el cambio conformacional que aumenta la unión al ligando. Mac-1 actúa como receptor de ICAM-1 (Diamond et al., 1991) y su expresión se aumenta por acción de distintos factores quimiotácticos. Las integrinas α_4 que nos interesan desde el punto de vista de la interacción leucocito-endotelio son principalmente dos: **VLA-4** (very-late-antigen-4 o $\alpha_4\beta_1$) y $\alpha_4\beta_7$. Se encuentran localizadas en algunos linfocitos, monocitos, eosinófilos y basófilos. Sus principales ligandos son otras moléculas de adhesión tipo inmunoglobulina: VCAM-1 para ambas y mucosal addressin cell adhesion molecule-1 (MadCAM-1) para $\alpha_4\beta_7$. También interaccionan con el fragmento CS-1 de fibronectina (Springer, 1994; Carlos & Harlan, 1994; Lobb & Hemler, 1994).

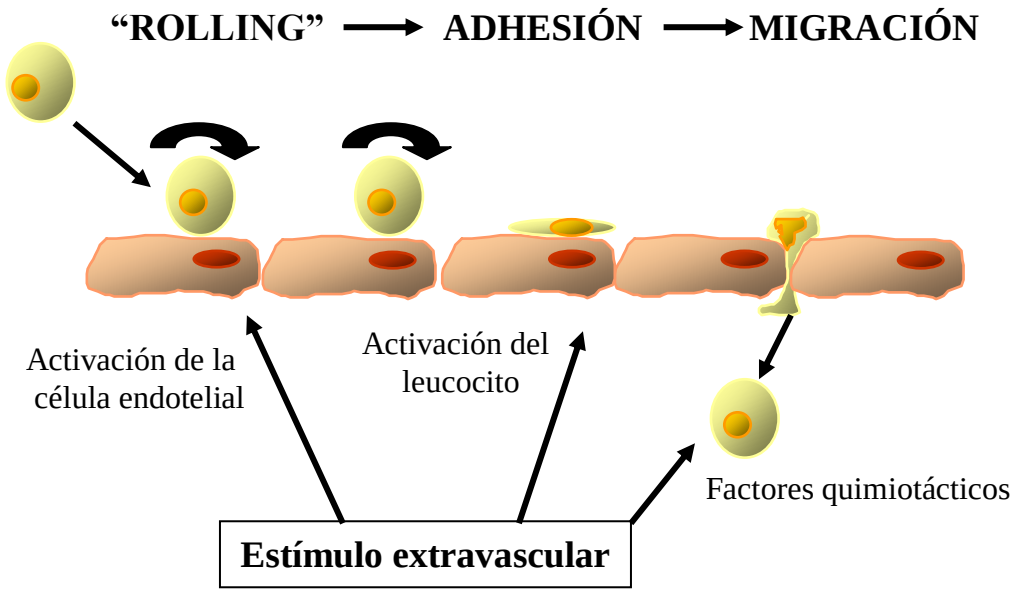
Las moléculas de adhesión **"tipo inmunoglobulinas"** se caracterizan por la presencia de un número variable de módulos similares a los observados en la región variable de las inmunoglobulinas plasmáticas. Se expresan en las células endoteliales normalmente, algunas de forma constitutiva y otras de forma inducible, y actúan como ligandos de integrinas. Entre las inmunoglobulinas destacan: ICAM-1, ICAM-2, VCAM-1, MadCAM-1 y platelet-endothelial cell adhesion molecule-1 o CD31 (PECAM-1). **ICAM-1** se expresa constitutivamente en las células endoteliales pero su síntesis se ve inducida por varias citocinas proinflamatorias (IL-1 o TNF) y endotoxinas (LPS) (Springer, 1990) e interacciona tanto con LFA-1 como con Mac-1 presentes en los leucocitos (Staunton et al., 1990; Diamond et al., 1991). **ICAM-2** se expresa constitutivamente por las células endoteliales y por las plaquetas y se une a LFA-1 (Carlos & Harlan, 1994). **VCAM-1** es el ligando para la integrina VLA-4 y $\alpha_4\beta_7$ (Rüegg et al., 1992; Chan et al., 1992) y se induce en células endoteliales estimuladas por diversas citocinas y endotoxinas (Bevilacqua, 1993). **MadCAM-1** se expresa en las placas de Peyer y venas de gran capacitancia. Contiene tres dominios tipo inmunoglobulina y una región tipo mucina entre los dominios dos y tres (Briskin et al., 1993); esta inusual estructura hace que actúe como ligando tanto para la integrina $\alpha_4\beta_7$ como para L-selectina (Berlin et al., 1993; Berg et al., 1993). **PECAM-1** es una glicoproteína expresada en leucocitos, plaquetas y células endoteliales (DeLisser et al., 1994), en estas últimas su expresión se concentra en las uniones entre las células. PECAM-1 puede mediar interacciones homotípicas o interacciones heterotípicas, las homotípicas tienen lugar entre PECAM-1 endotelial y PECAM-1 leucocitario y las heterotípicas entre PECAM-1 y la integrina $\alpha_v\beta_3$ (Newman et al., 1997) y media principalmente los procesos de migración leucocitaria.

INTERACCIONES LEUCOCITO-ENDOTELIO

La extravasación de leucocitos desde el torrente circulatorio hasta el foco inflamado tiene lugar sobretodo en las vénulas postcapilares y comprende varias etapas:

- Generación y liberación de mediadores químicos que actúan en el leucocito o en la célula endotelial.
- Activación del leucocito y/o de la célula endotelial, modificando la expresión o la afinidad de las moléculas de la superficie celular las cuales median la adhesión de los leucocitos a las células endoteliales venulares.
- Adhesión de los leucocitos a las células endoteliales activándose respuestas celulares tales como el cambio de forma de leucocitos.
- Regulación del paso de los leucocitos a través de las uniones existentes entre células endoteliales.
- Aumento de la expresión de receptores leucocitarios para proteínas de la matriz extracelular que median el movimiento de los leucocitos a través de la membrana basal perivascular y a través del intersticio.

La interacción estimulada de los leucocitos con las células endoteliales está mediada por una cascada secuencial de interacciones reversibles y transitorias entre estos dos tipos de células. Inicialmente, los leucocitos producen una deceleración en su tránsito, proceso que se conoce con el nombre de "rolling" leucocitario. Si el estímulo persiste, los leucocitos en fase de "rolling" se activan, se adhieren firmemente al endotelio, difunden a través de la superficie celular y migran a través de la monocapa de células endoteliales (Noursharg & Williams, 1990; Springer, 1994).



El primer paso de este proceso que conducirá a la extravasación leucocitaria es el **"rolling" leucocitario**. Este paso es iniciado por la actuación de mediadores que han sido generados en respuesta a un daño previo tales como histamina, trombina, PAF o LTD_4 los cuales producen una rápida activación de leucocitos y/o de células endoteliales que da como resultado el aumento de la expresión de las moléculas de adhesión responsables del "rolling" leucocitario. La familia de las selectinas (P-selectina, E-selectina y L-selectina) es la responsable de este proceso. Así, se ha demostrado que anticuerpos frente a P-, E- y L-selectina inhiben el "rolling" leucocitario en vénulas mesentéricas (von Andrian et al., 1991; Dore et al., 1993; Ley et al., 1991; Oloffson et al., 1994; Kubes & Kanwar, 1994; Ley, 1994; Asako et al., 1994). P-selectina y E-selectina en el endotelio interaccionan con ligandos presentes en los leucocitos y L-selectina en los leucocitos interacciona con ligandos que se encuentran en las células endoteliales. Estas moléculas actúan secuencialmente iniciando los enlaces entre los leucocitos y el endotelio, proceso que conduce a un enlentecimiento de la velocidad de los leucocitos y a un incremento de su

contacto con el endotelio (Brown, 1997). También se han implicado a las integrinas α_4 en el fenómeno de "rolling" leucocitario; en concreto se ha demostrado que $\alpha_4\beta_1$ (VLA-4) y $\alpha_4\beta_7$ median el "rolling" de eosinófilos y de linfocitos respectivamente (Sriramarao et al., 1994; Alon et al., 1995; Berlin et al., 1995) y que ambas son importantes en procesos inflamatorios crónicos. Los receptores leucocitarios envueltos en el proceso de "rolling" no se encuentran dispersos por toda la superficie celular sino que se encuentran concentrados en los microvilli o fuera de ellos. Así, L-selectina, PSGL-1 e integrinas α_4 se encuentran en los microvilli mientras que las integrinas β_2 se encuentran principalmente en el cuerpo celular de los leucocitos. Por tanto, la presencia de los receptores leucocitarios en los microvilli facilita la participación de éstos en los primeros pasos de la cascada debido a que la interacción entre las células endoteliales y los receptores presentes en los microvilli reducen el área inicial de contacto y por tanto, las fuerzas electrostáticas repulsivas (von Andrian et al., 1995). Además entre estas células se generan muchas uniones que son necesarias para dar suficiente firmeza a la unión como para resistir las fuerzas hemodinámicas que conducirían a la separación de las mismas. En estas uniones también es importante la cinética de asociación/disociación, la longitud y la flexibilidad de las uniones. Para enlentecer la velocidad de los leucocitos que viajan a gran velocidad es necesaria una velocidad de asociación extremadamente rápida. Así, los leucocitos entran en fase de "rolling" debido a que se dan simultáneamente una velocidad de formación y de disociación de los enlaces con la capacidad de la unión de aproximarse antes de que éstos se rompan debido a la fuerza del flujo sanguíneo (Bell, 1978).

El siguiente paso en la cascada es la transición de los leucocitos en fase de "rolling" a leucocitos adheridos. El "rolling" es paso limitante para que se de la adhesión leucocitaria ya que se ha demostrado que la adhesión firme de neutrófilos mediada por integrinas β_2 es inhibida cuando se bloquea el "rolling" leucocitario (von Andrian et al., 1992). La **adhesión firme** se produce por interacciones entre integrinas e inmunoglobulinas (Lawrence & Springer, 1991) y para ello, las integrinas deben estar activadas. Las integrinas leucocitarias pueden existir en dos estados conformacionales con respecto a la adhesión: en células circulantes inactivadas, las integrinas tienen baja afinidad y no median la adhesión aunque su ligando esté expresado en el endotelio; sin embargo, cuando se activan, la afinidad de la integrinas por sus ligandos aumenta y esto parece que es debido a que las integrinas sufren un cambio conformacional en su dominio extracelular. Este cambio conformacional puede ser producido por las tres selectinas y también por una gran variedad de factores quimiotácticos. Entre los factores quimiotácticos implicados en este proceso se pueden destacar péptidos n-formilados de origen bacteriano (f-MLP), quimiocinas, el factor C5a del complemento, PAF y LTB_4. Estas moléculas inducen la señal (inside-out signal) que activa a las integrinas leucocitarias (Springer, 1994). De igual forma, el endotelio debe ser activado por distintos estímulos como el LPS bacteriano o diversas citocinas que inducen la síntesis proteica de inmunoglobulinas inducibles (Celi et al., 1997) y así, aparecen en la superficie del endotelio inmunoglobulinas como ICAM-1, ICAM-2 o VCAM-1. Una vez activadas las integrinas, se produce la unión entre las integrinas leucocitarias que poseen elevada afinidad con las inmunoglobulinas expresadas en el endotelio vascular dando lugar a una adhesión célula-célula próxima y estable (Brown, 1997). En este proceso participan las integrinas α_4 (VLA-4 y $\alpha_4\beta_7$), las integrinas β_2 (CD11/CD18) y las inmunoglobulinas ICAM-1, ICAM-2 y VCAM-1. Así, la adhesión

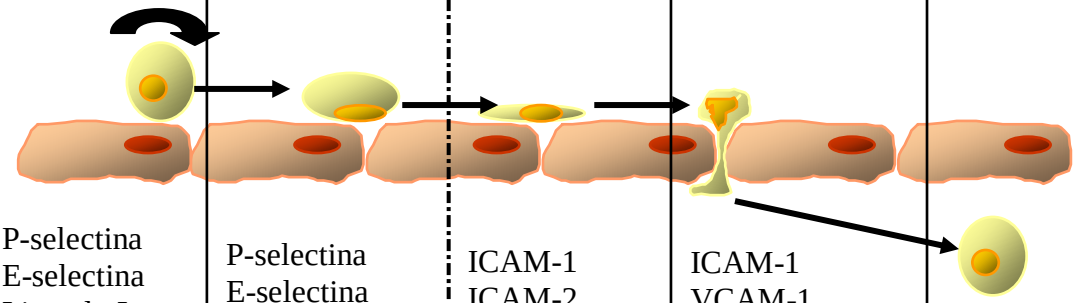
firme está mediada por los siguientes pares de moléculas de adhesión: CD11a/CD18-ICAM-1 y CD11a/CD18-ICAM-2 en todo tipo de leucocitos, CD11b/CD18-ICAM-1 y CD11c/CD18-ICAM-1 en neutrófilos y monocitos y VLA_4-VCAM-1 en linfocitos, monocitos, eosinófilos y basófilos (Panés & Granger, 1998).

El último paso en el trasvase de leucocitos a través de la pared vascular es la **migración leucocitaria** a través de las uniones entre células endoteliales y a través de la membrana basal perivascular. El proceso de transición de adhesión firme a extravasación también requiere la activación leucocitaria. Parece ser que las integrinas actúan como moléculas que emiten una señal que hace que los leucocitos se aplanen sobre el endotelio permitiéndoles atravesar las uniones entre células endoteliales (Hynes, 1992). Estas uniones son normalmente herméticas con una resistencia eléctrica que supera los 50 Ω (Graham et al., 1994). La transmigración leucocitaria no reduce esta resistencia eléctrica, sugiriendo que los leucocitos se van colocando entre las células endoteliales ya existentes (aposición) (Kansas, 1996). Entre las moléculas de adhesión implicadas en la extravasación leucocitaria tiene importancia el complejo formado por integrinas β_2 (CD11/CD18)/ICAM-1 después de la activación leucocitaria producida por agentes quimiotácticos o la activación de células endoteliales producida por citocinas (Smith, 1992; Furie et al., 1991; Ebisawa et al., 1992; Luscinskas et al., 1991). Otra molécula de adhesión que juega un papel relevante en la transmigración de neutrófilos es PECAM-1 (CD31) ya que anticuerpos que reconocen específicamente a esta molécula inhiben la acumulación leucocitaria en los lugares de inflamación *in vivo* (Muller et al., 1993; Vaporciyan et al., 1993) y bloquea selectivamente la extravasación leucocitaria en microvasos del mesenterio de rata activados con IL-1 sin alterar la respuesta al "rolling" y a la adhesión (Wakelin et al., 1996). Los niveles elevados de esta molécula en las uniones entre células hacen que

esté en una posición ideal para participar en el proceso de extravasación leucocitaria. Además, esta molécula también actúa como receptor capaz de producir la activación de integrinas leucocitarias y esta activación de integrinas leucocitarias sería el mecanismo por el cual PECAM-1 media y propaga el paso de leucocitos a través de la pared vascular. Además de las moléculas de adhesión, en este proceso también participan algunos factores quimiotácticos tales como PAF, IL-8, etc. Los leucocitos activados, una vez han migrado a través de la barrera endotelial, deben atravesar la matriz extracelular y dirigirse hacia el lugar de la infección o inflamación extravascular. Los leucocitos expresan en bajos niveles a las integrinas $a_2\beta_1$, $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_v\beta_3$ (Brown, 1997) y éstas se unen a un gran número de proteínas de la matriz extracelular como lamilina, fibronectina, vitronectina, etc. Los mecanismos moleculares que median el paso de leucocitos a través de las uniones entre células endoteliales activarían los mecanismos que regulan el paso de los leucocitos a través de la membrana basal. Estos mecanismos incluirían la activación y el aumento de la expresión de los receptores leucocitarios para las proteínas de la matriz extracelular.

Como se ha descrito en este capítulo la migración leucocitaria comprende varias fases y en cada una de estas fases participan componentes leucocitarios, endoteliales y tisulares específicos como se recoge en la siguiente tabla:

	"Rolling"	**Activación → Adhesión firme**		**Migración Transendotelial**	**Migración subendotelial**
Leucocito	PSGL-1 Carbohidratos Sialomucinas L-selectina Integrinas α_4 ($\alpha_4\beta_1$ y $\alpha_4\beta_7$)	L-selectina Factores quimiotácticos	Integrinas α_4 ($\alpha_4\beta_1$ y $\alpha_4\beta_7$) Integrinas β_2	Integrinas β_2 Integrinas α_4 PECAM-1	Integrinas β
Endotelio	P-selectina E-selectina Ligando L-selectina VCAM-1	P-selectina E-selectina Citocinas	ICAM-1 ICAM-2 VCAM-1	ICAM-1 VCAM-1 PECAM-1	
Tejido	Histamina Trombina PAF LTD_4 O_2^- H_2O_2	Factores quimiotácticos: (f-MLP, C5a, PAF,LTB_4, quimiocinas)	Citocinas	Agentes quimiotácticos (IL-8, PAF, ...) Citocinas	Componentes de la matriz extracelular Agentes quimiotácticos (IL-8, PAF, ...) Citocinas



ACCIÓN PROINFLAMATORIA DE ANGIOTENSINA II E IMPORTANCIA CLÍNICA DEL TEMA OBJETO DE ESTUDIO

Hoy en día no existe ninguna duda de que la principal causa en la patogénesis del infarto de miocardio, del infarto cerebral y de la pérdida de función de las extremidades es la aterosclerosis; de hecho, esta enfermedad es la responsable del 50% de la mortandad que acontece en Estados Unidos, Europa y Japón (Ross, 1993). En esta última década se ha hecho cada vez más evidente que la responsable de la formación de la placa aterosclerótica es una respuesta inflamatoria generalizada, tanto humoral como celular (Ross, 1993; Price & Loscalzo, 1999). Así, la acumulación leucocitaria en la pared vascular es una característica de los primeros estadíos de la lesión aterosclerótica, del infarto agudo de miocardio y de enfermedades renales de diversa etiología (Badimon et al., 1993; Ricevuti et al., 1990; Klahr et al., 1988). En este sentido, elevados niveles de vasoconstrictores, como vasopresina o endotelina-1 (ET-1), en ciertas patologías vasculares, pueden contribuir a las interacciones leucocito-endotelio observadas a través de un aumento de expresión de P-selectina endotelial (Kanwar et al., 1995; Sanz et al., 1999). Sin embargo, es la activación del sistema renina-angiotensina la que parece que pueda jugar un papel crucial en el desarrollo de la lesión vascular asociada a estas patologías cardiovasculares y renales (Alderman et al., 1991; Badimon et al., 1993; Lafayette et al., 1992; Kim & Iwao, 2000; Thaiss et al., 1996). En este sentido, Ang-II es el principal péptido efector de este sistema. Además de su claro papel como vasoconstrictor y regulador de la presión arterial, Ang-II parece que también pueda causar la activación de monocitos y de PMNs. De hecho, se ha demostrado la existencia de receptores para este péptido en monocitos humanos (Shimada & Yazaki, 1978). Además, estudios in vitro han demostrado que Ang-II puede promover la adhesión y activación de monocitos (Hahn et al., 1994; Kim et al., 1996;

Gräfe et al., 1997) y estimular la expresión de las quimiocinas MCP-1 (monocyte chemoattractant protein-1) y RANTES en diversos modelos animales in vivo (Hernández-Presa et al., 1997; Ruiz-Ortega et al., 1998; Wolf et al., 1997). Asímismo, también se ha comprobado que Ang-II puede estimular la liberación de un factor quimiotáctico para PMNs en cultivos celulares de células endoteliales arteriales tanto humanas como bovinas (Farber et al., 1990).

Todo esto resulta de gran interés ya que los estados hipertensivos están asociados con la migración de monocitos a través de la pared vascular, estadio crítico en el desarrollo de la lesión aterosclerótica, la cual se ha demostrado que puede ser atenuada mediante el empleo de inhibidores de la enzima convertidora de la Angiotensina I (IECA) (Shimada & Yazaki, 1978; Hernández-Presa et al., 1997). De este modo, la activación de monocitos por Ang-II podría iniciar o perpetuar el estado de daño endotelial que está asociado a aterosclerosis e hipertensión (Hahn et al., 1994).

Por tanto, un posible blanco de actuación para inhibir o detener el desarrollo de la lesión aterosclerótica pasa por entender el proceso inflamatorio asociado a esta patología. De esta forma, si se controla el proceso inflamatorio en los estadios iniciales de la enfermedad, impidiendo la acumulación de las principales células leucocitarias implicadas, se podrán llevar a cabo nuevas estrategias terapéuticas que induzcan a la regresión de la lesión o incluso a prevenir su formación.

III. 2. RESULTADOS

Mecanismos y mediadores implicados en las interacciones leucocito-endotelio inducidas por Ang-II en procesos agudos y subagudos

En primer lugar se realizaron los experimentos agudos y para ello se administró en superfusión Ang-II a diferentes dosis entre 0.1 y 100 nM y se evaluaron las respuestas leucocitarias transcurridos 15, 30 y 60 min desde su administración. Ang-II indujo un incremento significativo en el flujo de leucocitos en fase de "rolling" (art.1, fig 1A), en la adhesión leucocitaria (art.1, fig 2A) y en la migración leucocitaria (art.1, fig 2B) así como una concomitante disminución en la velocidad de leucocitos en fase de "rolling" (art.1, fig 1B) todo ello de forma concentración y tiempo dependiente. Las respuestas más consistentes fueron las que se obtuvieron a la dosis de Ang II 1 nM, concretamente y comparando con los valores que se obtuvieron superfundiendo tampón fueron: número de leucocitos en fase de "rolling" 83.8±20.7 frente a 16.4±3.1 células/min, número de leucocitos adheridos 11.4±1.0 frente a 0.8±0.5 células/100 μm de vaso, número de leucocitos emigrados 4.0±0.7 frente a 0.2±0.2 células/campo y velocidad de leucocitos en fase de "rolling" 37.3±3.5 frente a 143.1±42.9 μm/s a los 60 min de superfusión.

Ang-II a dosis de 0.1-10 nM no indujo ningún cambio significativo ni en la presión arterial media ni el diámetro venular o arteriolar (datos no mostrados). Sin embargo, Ang-II a dosis de 100 nM produjo una vasoconstricción significativa tanto en las vénulas como en las arteriolas. De esta forma, para el resto de los experimentos que realizamos se utilizó Ang-II a la dosis de 1 nM que fue la que produjo las respuestas leucocitarias más consistentes a una dosis 100 veces menor a aquella que causó vasocontricción.

Por otra parte y debido a que Ang-II provocó migración leucocitaria, se comprobó su posible comportamiento como agente quimiotáctico y comprobamos que Ang-II no

presenta efecto quimiotáctico por sí misma ya que la estimulación de PMNs y monocitos de rata con Ang-II no provocó aumento de la expresión de integrinas β_2 ni corte de L-selectina en la superficie celular (art.1, fig 3).

Asímismo, la acción proinflamatoria de Ang-II tampoco se debió a la activación de las células mastocitarias ya que el pretratamiento de los animales con cromolin, agente estabilizador de la membrana mastocitaria, no indujo a cambios en las respuestas leucocitarias causadas por Ang-II (art.1, fig 4).

Sin embargo, el efecto proinflamatorio de Ang-II se comprobó que estaba mediado a través de la interacción de ésta con sus receptores tanto AT_1 como AT_2, ya que el pretratamiento con cada uno de sus antagonistas específicos losartan y PD123,319 respectivamente redujo significativamente el "rolling", la adhesión y la migración leucocitaria inducidas por superfusión de Ang II a los 60 min aunque la inhibición únicamente fue completa en el caso de la administración simultánea de ambos antagonistas (art.1, fig 5). Además, estas respuestas producidas por Ang II son totalmente dependientes del aumento de la expresión de la molécula de adhesión P-selectina ya que el anticuerpo monoclonal frente a P-selectina bloqueó todas las respuestas leucocitarias inducidas por la superfusión de Ang II a los 60 min (art.1, fig 7) y se deben a un efecto directo sobre el endotelio a través del aumento de la expresión de P-selectina endotelial ya que la estimulación de plaquetas de rata con Ang-II no produjo un aumento de la expresión de P-selectina plaquetaria (art.1, fig 8).

Por otro lado, la hipertensión causada por niveles elevados de Ang-II está parcialmente mediada por la generación y liberación de anión superóxido. Por tanto, seguidamente investigamos si las interacciones leucocito-endotelio inducidas por Ang-II también son debidas a la generación de RLO. El tratamiento con SOD inhibió

significativamente el "rolling" leucocitario, la adhesión y la migración leucocitaria en un 80%, 57% y 46% respectivamente (art.2, fig 1). Cuando se administró catalasa, el "rolling" y la adhesión leucocitaria fueron inhibidas en un 50% y 37% respectivamente, sin afectar a la migración leucocitaria (art.2, fig 1). Sin embargo, la co-administración de ambas enzimas fue capaz de reducir las interacciones leucocito-endotelio inducidas por Ang-II casi a niveles basales (art.2, fig 1) así como de retornar la velocidad de los leucocitos en fase de "rolling" a niveles basales (art.2, tabla 2). Además, la oxidación de Dihidrorrodamina-123 (DHR-123) a Rodamina-123 (R-123) cuando fue superfundida con Ang-II 1 nM nos indicó que la generación de RLO se produce primeramente en la pared vascular ya que únicamente se detectaron cambios significativos en la fluorescencia tras 60 min de superfusión con Ang-II en el espacio vascular (art.2, fig 2). Finalmente, debido a que los RLO pueden liberar factores quimiotácticos tales como PAF o LTs, se comprobó que tanto un antagonista selectivo del receptor de PAF (WEB2086) como un inhibidor de la 5-LO (ICI 230,487), fueron capaces de reducir las interacciones leucocito-endotelio inducidas por Ang-II (art. 2, fig. 5). En concreto, el "rolling", la adhesión y la migración leucocitarias inducidas por Ang-II a los 60 min de superfusión fueron inhibidas en un 69%, 84% y 43% respectivamente tras tratamiento con WEB2086 y en un 69%, 96% y 55% respectivamente mediante co-superfusión con ICI 230,487. Además, ambos tratamientos produjeron un retorno de la velocidad de leucocitos en fase de "rolling" a niveles basales (art. 2, tabla 2). Ninguno de los tratamientos utilizados en el presente estudio indujo a cambios en los distintos parámetros hemodinámicos estudiados (art. 2, tabla 3). Asímismo, la superfusión de Ang-II no causó reducción del número de leucocitos circulantes ($82.5\pm7.8x10^5$ células/ml a tiempo 0 frente a $86.5\pm9.8x10^5$ células/ml tras 60 min de superfusión de Ang-II 1 nM).

Para la realización de los experimentos subagudos, Ang-II a una dosis de 1 nM o 0,1 nM fue administrada intraperitonealmente. Transcurridas 4 h desde su administración se procedió a la evaluación de las respuestas leucocitarias. A la dosis de 1 nM y a nivel venular, se produjo un incremento significativo en el flujo de leucocitos en fase de "rolling" (126.7±14.1 frente a 29.8±8.1 células/min), en la adhesión leucocitaria (18.5±1.7 frente a 3.0±0.9 células/100 μm de vaso) y en la migración leucocitaria (13.4±2.5 frente a 1.3±0.3 células/campo visual) así como una disminución significativa de la velocidad de leucocitos en fase de "rolling" (30.2±2.2 frente a 81.6±14.6 μm/s) comparado con los resultados obtenidos con aquellos detectados tras la administración intraperitoneal de suero fisiológico durante el mismo espacio de tiempo (art 3, fig 1A, 1B y 1C). Sin embargo, el hallazgo más importante en este estudio, fue la presencia de leucocitos adheridos en las arteriolas de la microcirculación mesentérica de la rata (3.6±1.0 frente a 0.1±0.1 células/100 μm de vaso) tras la administración de Ang-II a una dosis fisiológica de 1 nM (art. 3, fig. 1D y fig. 3B). Además, la administración intraperitoneal de Ang-II 1 nM no causó reducción del número de leucocitos circulantes ($83.7\pm5.6x10^5$ células/ml frente a $95.0\pm12.9x10^5$ células/ml) ni alteraciones en los parámetros hemodinámicos comparando con animales a los que se les administró suero fisiológico. La administración de una dosis inferior de Ang-II (0,1 nM), aunque produjo un claro efecto proinflamatorio a nivel venular, no indujo ninguna respuesta a nivel arteriolar; por ello la dosis de Ang-II 1 nM fue utilizada para la realización de todos los estudios posteriores.

La administración de un anticuerpo monoclonal frente a P-selectina de rata produjo una inhibición del "rolling", adhesión y migración leucocitaria inducidas por administración intraperitoneal de Ang-II de un 66%, 78% y 88% respectivamente. Cuando se empleó un anticuerpo monoclonal frente a E-selectina de rata, las inhibiciones de estas respuestas

fueron de un 13%, 10% y 4% no alcanzando diferencias significativas con respecto a los valores obtenidos con Ang-II. Sin embargo, cuando se combinaron los dos anticuerpos, se abolieron las interacciones leucocito-endotelio inducidas por Ang-II a nivel venular (art. 3, fig. 2A, 2B y 2C). La adhesión leucocitaria presente a nivel arteriolar también fue inhibida tras pretratamiento con el anticuerpo monoclonal frente a P-selectina (88%), sin embargo, no fue afectada por la administración de un anticuerpo frente a E-selectina. La co-administración de ambos anticuerpos, anti-P y anti-E-selectina, inhibió casi completamente la adhesión leucocitaria causada por Ang-II (art. 3, fig. 2D y fig. 3C), implicando a ambas moléculas de adhesión en el efecto proaterogénico de Ang-II. El flujo de leucocitos en fase de "rolling" y la migración leucocitaria presente a nivel venular y la adhesión leucocitaria presente a nivel arteriolar inducidas tras 4 h de la administración intraperitoneal de Ang-II 1nM no se vieron afectadas por la administración de un anticuerpo frente a integrina α_4 (art. 3, fig. 4A, 4C y 4D). No obstante, la adhesión leucocitaria presente a nivel venular fue inhibida en un 58.7% por la administración de este último anticuerpo (art. 3, fig. 4B). En el caso de la administración de un anticuerpo monoclonal frente a VCAM-1 ni los parámetros venulares ni arteriolares inducidos tras 4 h de la administración de Ang-II 1 nM se vieron modificados (art. 3, fig. 4A, 4B, 4C y 4D). Finalmente, el pretratamiento de los animales tanto con un anticuerpo monoclonal frente a integrinas β_2 como con un anticuerpo frente a ICAM-1 no produjeron ninguna modificación en el "rolling" leucocitario a nivel venular inducida tras 4 h de la inyección de Ang-II 1 nM (art.3, fig. 5A), aunque ambos tratamientos produjeron una inhibición significativa tanto de la adhesión (92.7% y 34.8% respectivamente) como de la migración leucocitaria (89.0% y 66.0% respectivamente) inducidas a nivel venular (art.3, fig. 5B y 5C). En lo que respecta a la adhesión inducida por Ang-II a nivel arteriolar, el anticuerpo monoclonal frente a integrinas β_2 redujo este

parámetro en un 82.8% mientras que el anticuerpo frente a ICAM-1, aunque produjo una inhibición del 42.8% en este parámetro, esta inhibición no llegó a ser significativa (art. 3, fig. 5D).

La disminución de la velocidad de leucocitos en fase de "rolling" inducida por administración intraperitoneal de Ang-II 1 nM retornó prácticamente a niveles basales en el caso del pretratamiento con los anticuerpos WT-3, RMP-1 y RMP-1 en combinación con RME-1 (art.3, tabla 1). Los parámetros hemodinámicos no se vieron modificados en ninguno de los casos en los que los animales fueron pretratados con anticuerpos monoclonales frente a moléculas de adhesión (art. 3, tabla 2), mientras que el número de leucocitos circulantes sufrió un aumento en los casos de pretratamiento con la combinación de anticuerpos frente a P- y E-selectinas y con los anticuerpos frente a integrinas α_4 y VCAM-1 (art. 3, tabla 3).

Estudio de fármacos moduladores de las interacciones leucocito-endotelio inducidas por Ang-II

Los resultados presentados anteriormente indican que Ang-II produce acumulación leucocitaria in vivo la cual es dependiente de la generación de RLO. En este sentido, también comprobamos que la administración del antioxidante intracelular, dimetiltiourea, o del quelante de hierro desferrioxamina, inhibieron significativamente las respuestas leucocitarias provocadas por superfusión de Ang-II (art. 2, fig. 3). Por otro lado, el pretratamiento de los animales con N-acetilcisteína (NAC) causó una inhibición significativa del "rolling", adhesión y migración leucocitarias inducidas por Ang-II de un 79%, 91% y 82% respectivamente (art. 2, fig. 4). Asímismo, los tres tratamientos produjeron un retorno de la velocidad de leucocitos en fase de "rolling" a niveles basales

(art. 2, tabla 2) y ninguno de los tres tratamientos utilizados indujo a cambios en los distintos parámetros hemodinámicos estudiados (art. 2, tabla 3). Por tanto, el uso de antioxidantes puede constituir una terapia alternativa en el control de la infiltración subendotelial de leucocitos que tiene lugar en hipertensión y aterosclerosis.

Debido a que agentes capaces de elevar los niveles de nucleótidos cíclicos intracelulares son capaces de modular la acumulación leucocitaria en diversos procesos inflamatorios, creímos de interés evaluar el efecto de estas sustancias en las interacciones leucocito-endotelio inducidas por Ang-II in vivo. El pretratamiento con el análogo de PGI_2, iloprost, inhibió significativamente el aumento sobre el flujo de leucocitos en fase de "rolling", la adhesión leucocitaria y la migración leucocitaria inducidas por Ang-II a los 60 min de superfusión en un 96%, 92% y 90% respectivamente (art. 4, fig. 1). Igualmente las repuestas leucocitarias causadas por Ang-II fueron inhibidas en un 83%, 86% y 62% respectivamente en animales tratados por el agonista del adrenoceptor β_2, salbutamol (art. 4, fig. 1). Este último fármaco también fue capaz de reducir significativamente la presión arterial media. Además, la co-superfusión con un agente que activa directamente la AC, forskolin, causó unos efectos semejantes a los obtenidos con salbutamol e iloprost (art. 4, fig. 2). Resultados semejantes fueron obtenidos cuando se administró el inhibidor inespecífico de PDEs, teofilina, la cual inhibió el flujo de leucocitos en fase de "rolling", la adhesión y la migración leucocitaria inducidas por Ang-II en un 81%, 89% y 71% respectivamente. Al igual que ocurre con teofilina, rolipram, inhibidor selectivo de PDE4, redujo de forma similar las respuestas leucocitarias causadas por Ang-II (art 4, fig. 3). Asímismo, la co-superfusion con Ang-II del donante de NO (spermina-NO) o el análogo de GMPc (8-Br-GMP) así como el pretratamiento de los animales con un parche de

nitroglicerina causaron una significativa reducción de las interacciones leucocito-endotelio causadas por Ang-II (art.4, fig. 4 y fig. 5).

Por otra parte, la velocidad de leucocitos en fase de "rolling" se mantuvo a niveles basales con estos agentes que elevan, a través de diferentes vías, los niveles de AMPc o de NO (art. 4, tabla 1 y tabla 2) y los diferentes parámetros hemodinámicos estudiados no se vieron alterados por los distintos tratamientos, excepto, como ya hemos comentado, con salbutamol el cual por sí mismo fue capaz de dismunuir la presión arterial sistémica sin afectar al "shear rate" y forskolin, que administrado en superfusión, causó una reducción en el "shear rate" (art. 4, tabla 3 y tabla 4).

En este estudio también se pudo comprobar que, la inhibición producida por salbutamol de las respuestas leucocitarias inducidas por Ang-II era debida al aumento de AMPc intracelular y a la posterior liberación de NO, ya que el bloqueo de la síntesis de NO por L-NAME revertió la respuesta inhibidora de salbutamol (art. 4, fig. 6).

Por consiguiente, tanto fármacos capaces de aumentar los niveles de AMPc intracelular como donantes de NO, son potentes inhibidores de la acción proinflamatoria de Ang-II y por tanto, también pueden constituir una excelente herramienta terapéutica en la prevención del daño vascular asociado a los primeros estadios del proceso aterosclerótico.

Finalmente, debido a que las mujeres en edad fértil presentan un riesgo menor en el desarrollo de enfermedades cardiovasculares que hombres de la misma edad o que mujeres posmenopáusicas, se procedió al estudio del efecto de los estrógenos en las respuestas leucocitarias causadas por Ang-II. Así, la co-superfusión de 17β-estradiol con Ang-II produjo una inhibición del "rolling", adhesión y migración leucocitaria inducidas por este péptido a los 60 min de su superfusión del 95%, 87% y 90% respectivamente (art. 5, fig.

2). Sin embargo, cuando el 17β-estradiol (100 nM) es administrado 30 min después de la superfusión de Ang-II, las respuestas leucocitarias evaluadas 30 min más tarde fueron reducidas en un 59%, 68% y 73% respectivamente (art. 5, fig. 1). Una dosis inferior de 17β-estradiol (10 nM) produjo un efecto similar, aunque menos consistente y una dosis superior de 17β-estradiol (1000 nM) careció de efecto. Además, a las diferentes dosis estudiadas, 17β-estradiol produjo un retorno de la velocidad de leucocitos en fase de "rolling" a niveles basales (art. 5, tabla 1). Estos efectos inhibidores mediados por 17β-estradiol, se comprobaron que eran receptor-dependientes, ya que la administración de tamoxifen, antagonista del receptor estrogénico, abolió su respuesta inhibitoria y además, tampoco fueron debidos a su posible efecto antioxidante ya que el 17α-estradiol no manifestó ningún efecto (art. 5, fig. 3, fig. 4 y tabla 1). Ninguno de los tratamientos utilizados en el presente estudio indujo a cambios en los distintos parámetros hemodinámicos estudiados (art. 5, tabla 2). Estos resultados sugieren que el efecto antiaterogénico de estas hormonas puede ser explicado, en parte, a través de la respuesta manifestada sobre la acción inflamatoria de Ang-II. Asímismo, en este estudio también queda patente la importancia de la dosis de 17β-estradiol utilizada, ya que el uso de dosis elevadas se traduce en una pérdida de efecto. Posteriormente y debido a que los estrógenos pueden provocar la liberación de NO y PGI_2 de la pared vascular y a que estos vasodilatadores tienen propiedades antiinflamatorias, estudiamos este mecanismo de acción. Así, cuando 17β-estradiol fue co-superfundido 30 min después de la superfusión de un inhibidor de la síntesis de NO o 30 min después de la superfusión de un inhibidor de la síntesis de PGI_2, las respuestas leucocitarias inducidas tras 30 min de superfusión con estos inhibidores no fueron modificadas, indicando un claro papel para ambos vasodilatadores en las propiedades antiinflamatorias ejercidas por el 17β-estradiol (art. 5, fig. 5). Además, el

NO liberado por los estrógenos tiene un origen endotelial ya que tras inhibición de la NOS neuronal con el inhibidor específico 7-nitroindazol, las respuestas causadas por 17β-estradiol sobre las interacciones leucocito-endotelio inducidas por Ang-II no se vieron modificadas (art. 5).

Papel de Ang-II en disfunciones endoteliales

Recientemente, estudiamos el papel de Ang-II en las alteraciones hemodinámicas y en las interacciones leucocito-endotelio inducidas por inhibición de la NOS y de la COX. En nuestro estudio, Ang-II no parece estar implicada en la hipertensión inducida por el tratamiento agudo y sistémico con un inhibidor de la síntesis de NO ya que el pretratamiento con el antagonista del receptor AT_1 de Ang-II (losartan) no redujo la hipertensión inducida por L-NAME (art.6, fig. 1A). Sin embargo, Ang-II sí que está envuelta en las interacciones leucocito-endotelio inducidas por L-NAME ya que el tratamiento con losartan inhibió el "rolling", la adhesión y migración leucocitaria inducidas por L-NAME tras 60 min de superfusión en un 83%, 80% y 70% respectivamente (art. 6, fig.2). Adicionalmente, produjo un retorno de la velocidad de leucocitos en fase de "rolling" a niveles basales. Paralelamente, el pretratamiento con losartan fue capaz de revertir el descenso de los parámetros hemodinámicos V_{rbc}, V_{mean} y "shear rate" producido por la superfusión durante 60 min de L-NAME (art. 6, tabla 1). Por otro lado, Ang-II está implicada en la hipertensión inducida por la inhibición aguda y sistémica de la síntesis de PGI_2 ya que el pretratamiento con losartan inhibió la hipertensión inducida por COX (art. 6, fig.1B). Además, losartan fue capaz de inhibir las respuestas leucocitarias inducidas por la superfusión de indometacina durante 60 min (art. 6, fig. 3) y también causó una reversión del descenso de la velocidad de leucocitos en fase de "rolling" inducido por la

superfusión de indometacina. El pretratamiento con losartan también fue capaz de revertir el descenso de los parámetros hemodinámicos V_{mean} y "shear rate" producido por la superfusión durante 60 min de indometacina (art. 6, tabla 2). Estos resultados indican que la falta de vasodilatadores como NO o PGI_2, expone al endotelio a la acción dañina de vasoconstrictores. Estos vasoconstrictores dan lugar a la acumulación leucocitaria que provoca el desarrollo de la lesión vascular asociada a diferentes enfermedades cardiovasculares como hipertensión, aterosclerosis o procesos de isquemia-reperfusión.

Posteriormente y debido a que los mastocitos, que pueden ser activados por L-NAME, expresan una quimasa que constituye una via alternativa para la generación local de Ang-II, estudiamos la posible participación de Ang-II en las respuestas leucocitarias inducidas por desgranulación mastocitaria con CMP 48/80. Sin embargo, el pretratamiento de los animales con losartan no causó ningún efecto sobre las interacciones leucocito-endotelio inducidas por CMP 48/80, demostrándose por tanto que en estas respuestas leucocitarias Ang-II no está implicada (art. 6, fig. 4).

Seguidamente, comprobamos que la falta de estrógenos debida a la administración de un antagonista selectivo de su receptor, tamoxifen, ocasiona una respuesta inflamatoria rápida (60 min) en la cual están implicadas tanto la P-selectina como las integrinas β_2, ya que la administración de anticuerpos monoclonales frente a estas moléculas de adhesión inhibieron significativamente las interacciones leucocito-endotelio debidas a falta de estrógenos (art. 5, fig. 6). Debido a que los estrógenos pueden liberar tanto NO como PGI_2, y a que la falta de ambos vasodilatadores provoca una respuesta inflamatoria dependiente de Ang-II, creímos que podría ser interesante estudiar el efecto de losartan en estas respuestas. Así, la administración de losartan inhibió significativamente el efecto inflamatorio inducido por tamoxifen (art. 5, fig. 7), reduciendo el flujo de leucocitos en

fase de “rolling” en un 85%, la adhesión leucocitaria en un 74% y la migración leucocitaria en un 61% a los 90 min de la administración de tamoxifen. De igual forma, la administración de tamoxifen produjo una reducción en la velocidad de leucocitos en fase de “rolling” que fue revertida por pretratamiento de los animales con los anticuerpos RMP-1 o WT-3 y con losartan (art. 5, tabla 3). Los parámetros hemodinámicos no se vieron modificados en ninguno de estos grupos experimentales (art. 5, tabla 4). Estos resultados implican directamente a Ang-II en la mayor incidencia de enfermedades cardiovasculares con proceso inflamatorio asociado en el hombre y en la mujer posmenopáusica sin tratamiento hormonal sustitutivo.

Por último, la administración subaguda de L-NAME intraperitoneal (4 h) produjo un significativo aumento de las interacciones leucocito-endotelio a nivel venular, las cuales fueron significativamente reducidas mediante pretratamiento con losartan (art. 3, fig. 6A, 6B y 6C) y una disminución en la velocidad de leucocitos en fase de “rolling” que también fue revertida en el caso del pretratamiento con losartan (art. 3, tabla 4). Pero quizás, el hallazgo más significativo fue la clara adhesión leucocitaria a nivel arteriolar tras la administración intraperitoneal de L-NAME frente a los animales a los que se les administró suero fisiológico por el mismo espacio de tiempo (9.6±1.3 frente a 0.1±0.1 células/100 µm de vaso), la cual fue inhibida por losartan en un 85% (art. 3, fig 6D). La administración subaguda de L-NAMW y el pretratamiento con losartan no causaron ninguna modificación ni en los parámetros hemodinámicas ni en el número de leucocitos circulantes (art. 5, tabla 4): Este hallazgo implica directamente a Ang-II en el inicio de la lesión aterosclerótica tras disfunción endotelial por falta de vasodilatadores.

III. 3. COMENTARIOS Y DISCUSIÓN

Mecanismos y mediadores implicados en las interacciones leucocito-endotelio inducidas por Ang-II en procesos agudos y subagudos

La acumulación leucocitaria en la pared vascular es un paso clave en los primeros estadíos de diversas patologías como aterosclerosis, infarto de miocardio y enfermedades renales de diversa etiología en las que Ang-II parece jugar un papel crucial. De hecho, la inhibición de la ECA provoca una reducción de la infiltración leucocitaria en estos desórdenes con proceso inflamatorio asociado (Mervaala et al., 1999; Hernández-Presa et al., 1997; Ruiz-Ortega et al., 1998). Por consiguiente, en el presente trabajo de investigación hemos demostrado, en primer lugar, que Ang-II a dosis fisiológicas y sub-vasoconstrictoras (0.1 a 1 nM) induce un rápido (1 h) y significativo incremento de las interacciones leucocito-endotelio en vénulas postcapilares de la microcirculación mesentérica de rata y que en estas interacciones están implicados tanto los receptores AT_1 como los AT_2 para Ang-II. Igualmente, se descartó la posible activación directa de mastocitos presentes en el mesenterio por Ang-II y la consiguiente liberación de diversos mediadores almacenados en sus gránulos, ya que el pretratamiento de los animales con cromolin no modificó las respuestas leucocitarias causadas por Ang-II. Estos resultados indican que Ang-II podría constituir el estímulo inductor de la acumulación leucocitaria que conllevaría al daño vascular observado en hipertensión, aterosclerosis o procesos de isquemia-reperfusión, sobre todo cuando estas patologías van asociadas con incrementos de los niveles plasmáticos de este péptido.

Otro hecho relevante de este estudio es que Ang-II, a diferencia de otros peptidos vasoconstrictores como vasopresina o ET-1 (Kanwar et al., 1995; Sanz et al., 1999), además de inducir adhesión leucocitaria también provoca migración leucocitaria. Esta

circunstancia indujo a pensar en su posible comportamiento como agente quimiotáctico. Sin embargo, los resultados obtenidos mediante el empleo de la citometría de flujo descartaron esta posibilidad ya que, Ang-II no fue capaz de provocar aumento de expresión de integrinas β_2 ni corte de L-selectina de la superficie de neutrófilos y monocitos de rata. Por consiguiente, este péptido debe causar la liberación endógena de diversos mediadores quimiotácticos preformados capaces de causar la infiltración leucocitaria observada. En este sentido, existen evidencias que indican que Ang-II puede causar acumulación leucocitaria a través de la síntesis de factores quimiotácticos como MCP-1 o RANTES. De hecho, la administración de inhibidores de la ECA o de antagonistas específicos de Ang-II son capaces de inhibir la producción de estas quimiocinas (Hernández-Presa et al., 1997; Ruiz-Ortega et al., 1998; Wolf et al., 1997; Farber et al., 1990). Asímismo, Mangat et al. (1997) también demostraron la capacidad de Ang-II de activar la $cPLA_2$, la cual es crucial para la síntesis y liberación de potentes factores quimiotácticos como PAF o LTB_4. Así, nosotros, como comentaremos posteriormente, comprobamos que las interacciones leucocito-endotelio inducidas por Ang-II podían ser atenuadas mediante tratamiento de los animales con un antagonista del PAF o con un inhibidor de la 5-LO.

Profundizando en el mecanismo de acción de las respuestas leucocitarias inducidas por la administración aguda de Ang-II, se comprobó mediante la administración de anticuerpos monoclonales, que la principal molécula de adhesión implicada era P-selectina y concretamente la de origen endotelial. Esta conclusión fue confirmada posteriormente tras la realización de una serie de ensayos llevados a cabo en plaquetas de rata estimuladas con Ang-II y a través del empleo de la citometría de flujo. De esta forma se observó Ang-II era incapaz de inducir el aumento de expresión de esta molécula de adhesión contenida en los gránulos α de las plaquetas.

Una vez determinados los efectos agudos (1 h) de Ang-II, decidimos llevar a cabo un estudio de las acciones subagudas (4 h) de Ang-II, es decir, comprobar el efecto de este péptido sobre síntesis proteica y, por consiguiente, sobre las moléculas de adhesión inducibles. Si bien fue impresionante la respuesta inflamatoria detectada a nivel venular tras 4 h de la administración Ang-II, el dato más relevante de este estudio fue la presencia de leucocitos adheridos en el territorio arteriolar sin observarse "rolling" leucocitario. Este hecho es de gran trascendencia puesto que implica directamente y por primera vez a Ang-II en el inicio de la lesión aterosclerótica. Es más, este fenómeno ocurre únicamente a la dosis de 1 nM, no teniendo lugar a una dosis diez veces menor.

Aunque en varios trabajos se han estudiado las posibles moléculas de adhesión implicadas en la respuesta proinflamatoria provocada por Ang-II, existen discrepancias entre los estudios previamente realizados. Algunos autores no han encontrado la posible participación de E-selectina, VCAM-1 o ICAM-1 en la adhesión de monocitos a cultivos de células endoteliales estimuladas con Ang-II (Kim et al., 1996). Otros han descrito que el tratamiento con inhibidores de la ECA causa la disminución de expresión de VCAM-1 e ICAM-1 en diversos modelos animales (Mervaala et al., 1999; Morrisey & Klahr, 1998). En un estudio diferente se comprobó que el tratamiento con enalapril en pacientes con septicemia fue capaz de disminuir los niveles plasmáticos de E-selectina e ICAM-1 (Boldt et al., 1998). Por otro lado, Gräfe et al. (1997) demostraron el claro efecto de Ang-II sobre el aumento de expresión de E-selectina en células endoteliales de origen coronario sin afectar a la expresión de VCAM-1 o ICAM-1. De igual forma, Pastore et al. (1999) mediante el empleo de cultivos de células endoteliales de vena de cordón umbilical humano (HUVECs), pusieron de manifiesto el aumento de expresión de ICAM-1 tras estimulación con Ang-II. En ese mismo año, Tummala et al. (1999), encontraron aumento

de expresión de VCAM-1 en aorta de ratas sometidas durante 6 días a infusión de Ang-II. Finalmente, nosotros en experimentos agudos realizados con Ang-II, hemos demostrado el claro papel de P-selectina en las interacciones leucocito-endotelio inducidas por este péptido.

El trabajo realizado en el presente estudio ha ayudado, de alguna manera, a clarificar las diferencias encontradas en los estudios anteriormente citados. Así, a través del empleo de anticuerpos monoclonales frente a distintas moléculas de adhesión hemos dilucidado los mecanismos adhesivos que median las interacciones leucocito-endotelio inducidas por Ang-II tanto a nivel arteriolar como a nivel venular. De este modo, la adhesión arteriolar inducida por Ang-II está principalmente mediada por aumento de expresión de P-selectina. Sin embargo, aunque la administración de un anticuerpo frente a E-selectina no modificó las respuestas leucocitarias frente a Ang-II, el tratamiento de los animales con ambos anticuerpos abolió la acumulación leucocitaria producida por este péptido. Por tanto, parece claro que la selectinas endoteliales juegan un relevante papel en este proceso. De igual forma, las interacciones leucocito-endotelio inducidas por Ang-II a nivel venular siguen un patrón semejante a las encontradas a nivel arteriolar, es decir, son tanto P- como E selectina-dependientes. Estos resultados sugieren que las respuestas observadas *in vivo* no siempre se pueden correlacionar con aquellas encontradas *in vitro*. De hecho, los cultivos celulares de células endoteliales pierden la capacidad de expresar P-selectina a los pocos pases y por ello Gräfe et al. (1997), únicamente detectaron aumento de expresión de E-selectina en cultivos celulares después de su estimulación con Ang-II pero no de P-selectina como nosotros hemos hallado.

Por otro lado, nuestros resultados concuerdan con los encontrados por Pastore et al. (1999) que descubrieron el aumento de expresión de ICAM-1 en HUVECs tras

estimulación con Ang-II. En nuestro trabajo encontramos un claro papel de esta inmunoglobulina en la adhesión y migración leucocitaria a nivel venular tras la exposición del mesenterio a Ang-II durante 4 h. Por el contrario, aunque a nivel arteriolar la administración de un anticuerpo monoclonal frente a ICAM-1 careció de efecto, si que lo presentó el tratamiento con un anticuerpo frente a integrinas β_2. Estas integrinas tienen como principales ligandos ICAM-1 e ICAM-2 y hay que añadir que ICAM-1 se expresa constitutivamente en el endotelio aunque su expresión se puede ver aumentada por diversos estímulos.

Respecto a la adhesión leucocitaria mediada por la interacción integrinas α_4/VCAM-1, aunque parecen estar envueltas en esta respuesta causada por Ang-II en vénulas postcapilares, no parecen jugar ningún papel en la que ocurre a nivel arteriolar. Estos resultados no parecen estar en consonancia con los encontrados por Tummala et al. (1999), sin embargo, el aumento de expresión de VCAM-1 inducido por Ang-II, que estos autores describen, se halló en la adventicia de la aorta de rata y en la musculatura lisa vascular, pero no en el endotelio. Estos resultados sugieren que esta interacción sea quizás más relevante a nivel de la progresión de la lesión aterosclerótica que en el comienzo de la misma. Por consiguiente, los resultados obtenidos sugieren que la falta de efecto de los anticuerpos frente a integrinas α_4 en la adhesión leucocitaria arteriolar provocada por Ang-II, indica que el endotelio venular y arteriolar responden de forma diferente a la estimulación con Ang-II, siendo este fenómeno la primera vez que se describe.

En conjunto, todos estos resultados sugieren que ante un aumento de los niveles plasmáticos de Ang-II se produce una infiltración leucocitaria a nivel vascular, la cual está primeramente mediada por aumento de expresión endotelial de P- y E-selectinas e indirectamente, a través de un incremento de la expresión de integrinas β_2 en la superficie

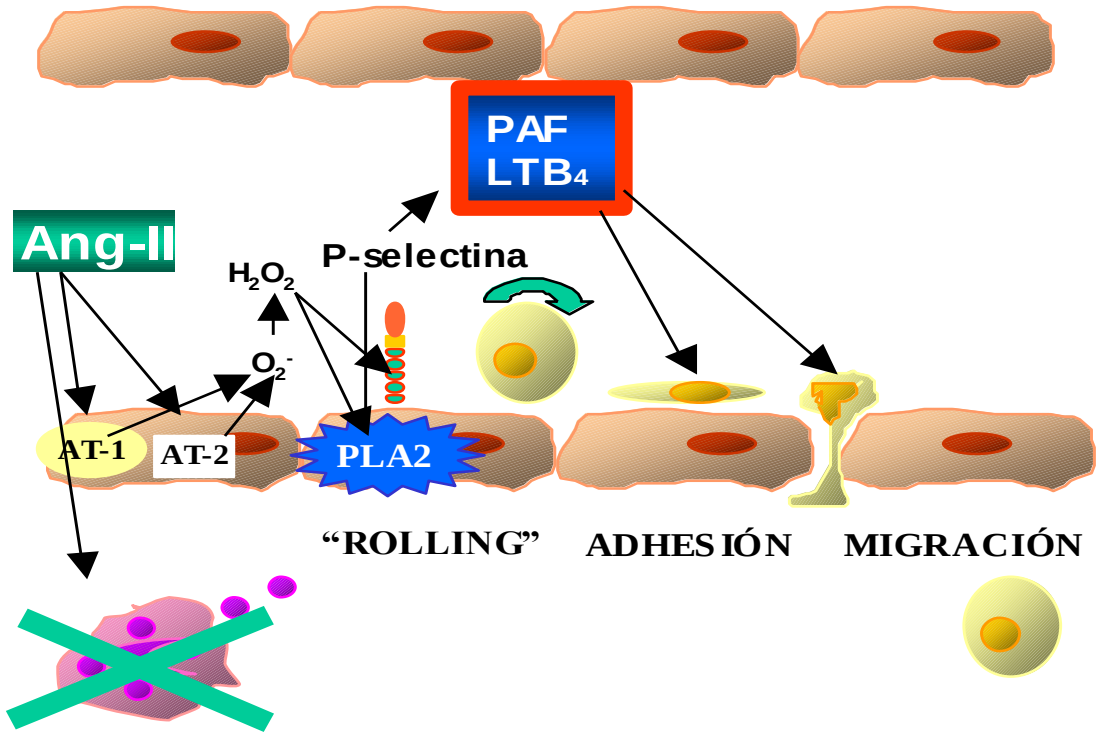
leucocitaria. A este respecto, se han detectado niveles incrementados de P-selectina y de E-selectina en pacientes hipercolesterolémicos o que padecen hipertensión esencial y maligna (Verhaar et al., 1998; Davi et al., 1998) y, además, se ha demostrado la implicación de ambas selectinas tanto en las primeras fases como en fases más avanzadas de la lesión aterosclerótica en distintos modelos ateroscleróticos en ratón (Dong et al., 1998; Eriksson et al., 2001). Por consiguiente, siendo la dosis de 1 nM fisiológica, parece que en estados hipertensivos que cursan con estos niveles de Ang-II, es posible que este péptido sea el desencadenante del inicio del desarrollo de la lesión aterosclerótica, la cual puede ocurrir en un corto espacio de tiempo.

La hipertension arterial debida a niveles elevados de Ang-II se comprobó que es dependiente de la liberación de anión superóxido (Laursen et al., 1997), por consiguiente se llevaron a cabo una serie de estudios con el fin de dilucidar si las interacciones leucocito-endotelio inducidas por Ang-II se debían igualmente a la liberación de RLO. Los resultados obtenidos indican que la acumulación leucocitaria causada por Ang-II *in vivo* es, efectivamente, dependiente de la generación de RLO tanto intra como extracelulares y que estas especies radicalarias se generan primeramente en la pared vascular, si bien por esta técnica no se pudo discernir si este fenómeno se producía a nivel de músculo liso vascular, de endotelio o de ambos ya que en ambos tipos celulares existen receptores para Ang-II y sistemas enzimáticos capaces de generar RLO (Griendling et al., 2000; Zweier et al., 1988). Estudios previos realizados *in vivo* demuestran que tanto el anión superóxido como el peróxido de hidrógeno provocan acumulación leucocitaria a través del aumento de expresión de P-selectina (Gaboury et al., 1994; Johnston et al., 1996), hecho que concuerda con nuestros resultados. Por otra parte es conocido que los RLO inducen la acumulación de leucocitos a través de la liberación de factores quimiotácticos como PAF y LTB_4.

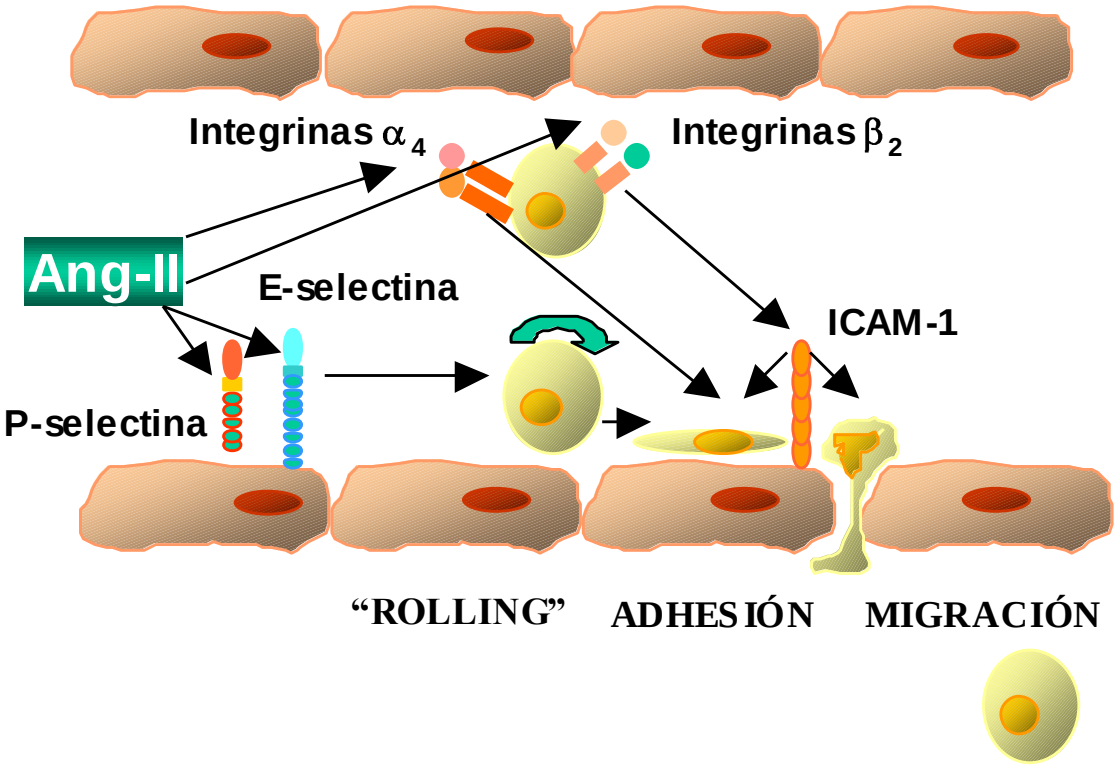
Asímismo, como ya hemos mencionado, en un estudio realizado recientemente se comprobó que Ang-II era capaz de activar la $cPLA_2$ (Mangat et al., 1997), enzima que produce la síntesis y liberación de PAF y de AA, y en el presente proyecto se ha demostrado igualmente la implicación de PAF y LTs, en las interacciones leucocito-endotelio causadas por Ang-II. Por tanto, el mecanismo propuesto es el siguiente: tras estimulación con Ang-II, tanto el anión superóxido como el peróxido de hidrógeno son rápidamente liberados y causan el aumento de expresión de P-selectina endotelial, la cual es responsable del aumento del flujo de leucocitos en fase de "rolling" observado. Paralelamente, los radicales libres liberados pueden estimular la $cPLA_2$, la cual provoca la síntesis y liberación de PAF y LTs que explica, en parte, la subsiguiente adhesión y migración leucocitaria detectada. Adicionalmente, nuevas especies radicalarias formadas a partir del metabolismo de la LO pueden ser las responsables de desencadenar un mecanismo amplificador que conlleva a un aumento de la adhesión leucocitaria, contribuyendo a la respuesta global observada por la administración de Ang-II exógena. Este hecho puede ser de gran trascendencia ya que en estados hipertensivos que cursan con niveles elevados de Ang-II, la liberación de RLO producida por este péptido también podría participar en la oxidación de las LDL, constituyendo ésta otra etapa clave en el desarrollo de la lesión aterosclerótica, además las LDL a su vez, también pueden producir por sí mismas adhesión y migración leucocitaria, provocando un efecto aditivo al causado por Ang-II (Liao et al., 1997).

Por tanto, los mecanismos por los que Ang-II induce interacciones leucocito-endotelio serían los siguientes:

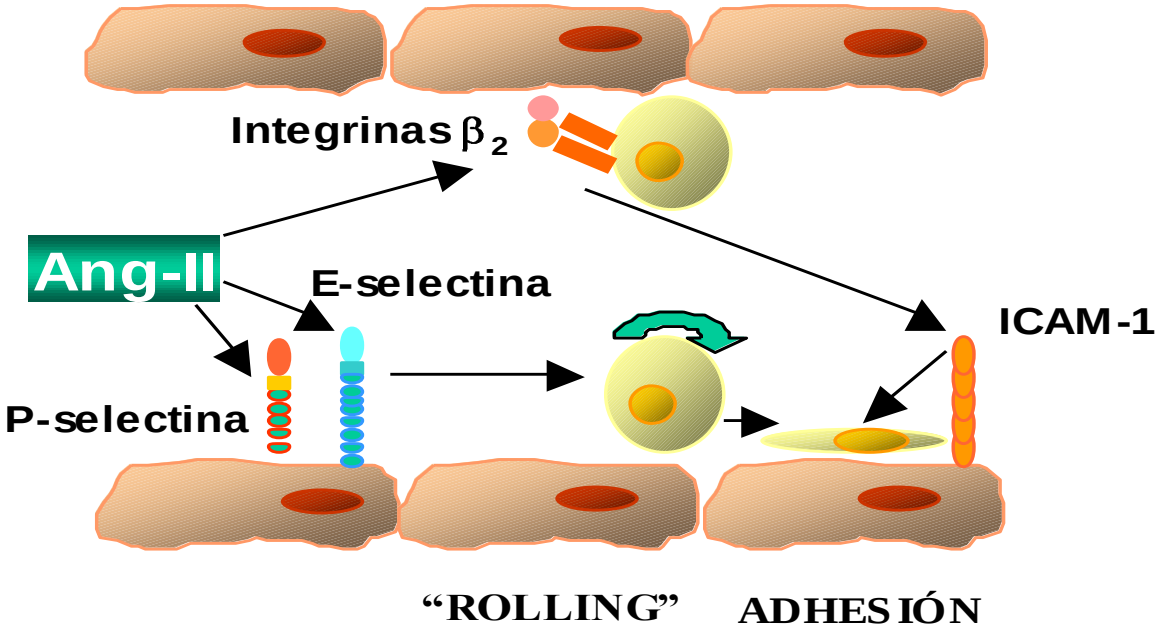
ANG-II EN VÉNULAS (1 h):



ANG-II EN VÉNULAS (4 h):



ANG-II EN ARTERIOLAS (4 h):



Estudio de fármacos moduladores de las interacciones leucocito-endotelio inducidas por Ang-II

Una vez estudiado parte del mecanismo de acción por el que Ang-II presenta actividad proinflamatoria se llevaron a cabo una serie de experimentos con diversos compuestos con el fin de modular estas respuestas. Los resultados realizados con fármacos moduladores indican que tanto los agentes antioxidantes como las sustancias capaces de aumentar los niveles de nucleótidos cíclicos, donantes de NO o los estrógenos son potentes inhibidores de la acción proinflamatoria de Ang-II *in vivo*.

En cuanto al mecanismo por el cual los agentes antioxidantes ejercen estos efectos inhibitorios parece que es debido a un mecanismo que actúa a nivel de la pared vascular previniendo la generación de radicales libres o captando aquellos que son liberados por acción de Ang-II y, por tanto, causando la disminución de la expresión de P-selectina

endotelial. Estos resultados están avalados por estudios previos realizados *in vitro* en los que se comprobó que agentes captadores de radicales libres y agentes quelantes del hierro pueden prevenir el aumento de expresión de moléculas de adhesión como P-selectina (Mannaioni et al., 1991). De entre todos los agentes antioxidantes ensayados en este estudio, NAC fue el que causó la inhibición más potente de las respuestas inflamatorias inducidas por Ang-II, este efecto quizás se deba a la gran variedad de mecanismos antiinflamatorios que posee este agente. En este sentido, sus propiedades antiadhesivas fueron claramente demostradas *in vivo* en procesos de endotoxemia y de isquemia-reperfusión a nivel hepático (Schmidt et al., 1998; Koeppel et al., 1996).

Por el contrario, todos los agentes que elevan los niveles de nucleótidos cíclicos tienen aproximadamente la misma potencia inhibitoria de las interacciones leucocito-endotelio inducidas por Ang-II. En el caso del agonista de los adrenoreceptores β_2 además de reducir estas interacciones, también fue capaz de reducir la presión arterial media, por tanto, podría tener especial interés en situaciones de hipertensión en las que existe el riesgo de desarrollar una patología aterosclerótica. Respecto al mecanismo inhibitorio por el cual actúan estos agentes en nuestro sistema, estudios realizados *in vitro* indican que estos agentes afectan a la expresión de moléculas de adhesión como VCAM-1 o E-selectina y además se requiere la combinación de varios de estos compuestos para que se produzca la inhibición de su expresión (Pober et al., 1993; Morandini et al., 1996; Blease et al., 1998). Sin embargo, los estudios realizados in *vitro* no tienen porque correlacionarse con los estudios que nosotros hemos realizado *in vivo* ya que estos últimos estudios apuntan hacia un efecto sobre otras moléculas de adhesión distintas a VCAM-1 y a E-selectina como la disminución de la expresión de P-selectina. Así, agentes que elevan los niveles de AMPc o donantes de NO pueden reducir la expresión y liberación de P-selectina en plaquetas

estimuladas (Konstantopoulos et al., 1998; Salas et al., 1994), además el tratamiento con prostaciclina en animales tratados con protamina produce una disminución de los niveles de E- y P-selectina (Katircioglu et al., 1999) y el tratamiento con donantes de NO atenúa el aumento de la expresión de P-selectina inducida por homocisteína en vénulas mesentéricas de rata (Pruefer et al., 1999).

Igualmente, y en este mismo estudio se demostró que las respuestas inhibitorias producidas por salbutamol estaban mediadas a través de la liberación de NO. De esta forma, nuestros resultados sugieren que los agentes que elevan los niveles de AMPc pueden reducir la expresión de moléculas de adhesión mediante la liberación de NO endotelial. Estos resultados están en consonancia con estudios *in vitro* en los que la vasorrelajación producida por el agonista del adrenoreceptor β_2 fue prevenida por la inhibición de la NOS (Ferro et al., 1999).

Por otra parte, en este proyecto hemos comprobado que hormonas como los estrógenos a dosis sustitutivas presentan un efecto anti-aterogénico que puede ser, en parte, debido a la inhibición de las interacciones leucocito-endotelio inducidas por Ang-II. Los estrógenos inhiben las acciones proinflamatorias inducidas por Ang-II de forma dosis-dependiente siendo este efecto rápido, específico y mediado por su interacción con su receptor tipo α. La rapidez de esta respuesta no requiere los mecanismos a nivel nuclear clásicos de esta hormona, sino que estos datos están en consonancia con un mecanismo, fisiológico y de tipo no genómico de los receptores estrogénicos presentes en la superficie de las células endoteliales; en este sentido, se ha demostrado que los estrógenos pueden ejercer gran variedad de acciones interaccionando con este subtipo de receptor y que este receptor está presente tanto en células endoteliales como en la superficie leucocitaria (Chen et al., 1999; Stefano et al., 1999). Igualmente, las dosis utilizadas también tienen una gran

importancia ya que bajas dosis de estrógenos, cercanas a las fisiológicas, provocan una mayor inhibición de estas interacciones leucocitarias inducidas por Ang-II que dosis superiores. Estos resultados resaltan la importancia de elegir la dosis adecuada en la terapia hormonal sustitutiva aplicada a mujeres postmenopaúsicas con el fin de obtener el efecto deseado. En cuanto al mecanismo de acción de esta respuesta inhibitoria, se comprobó que los efectos producidos se deben a una acción directa de los estrógenos sobre el endotelio y a la rápida liberación de NO y PGI_2. La posible participación de NO de origen neuronal quedó descartada, ya que la inhibición de la NOS neuronal por 7-nitroindazol no modificó la inhibición por estrógenos de la interacciones leucocito-endotelio causadas por Ang-II. Estos resultados concuerdan con estudios en los que el comportamiento de los estrógenos como ateroprotectores se explica a través de un mecanismo paracrino originado en el endotelio y mediado por el aumento de producción de vasodilatadores como el NO o PGI_2 (Selzman et al., 1998). Algunos de estos efectos inhibitorios ya se habían hecho patentes en estudios previos realizados *in vivo* (Nathan et al., 1999; Miyamoto et al., 1999; Squadrito et al., 1997) pero los mecanismos adhesivos implicados permanecen en controversia; mientras algunos autores han apuntado que actúan disminuyendo la expresión de ICAM-1 (Squadrito et al., 1997), otros piensan que disminuye la expresión de VCAM-1 (Nathan et al., 1999) y otros que actúan sobre la expresión E-selectina (Miyamoto et al., 1999). No obstante, la rapidez de estas respuestas nos hace descartar a estas moléculas de adhesión ya que son inducibles y nos hace apuntar hacia una disminución de la expresión de P-selectina endotelial, ya que las respuestas leucocitarias mediadas por Ang-II están inicialmente mediadas a través del aumento de la expresión de esta molécula de adhesión (Piqueras et al., 2000), además se detectaron niveles más bajos de P-selectina en mujeres premenopaúsicas que en hombres de la misma edad y la administración de una única dosis

de estradiol fue capaz de disminuir los niveles plasmáticos de esta selectina (Jilma et al., 1996).

En general, debido a que el grado de expresión de P-selectina determina la cantidad de leucocitos en fase de "rolling" que se adhieren al endotelio y se extravasan al tejido, todos estos agentes tendrán la capacidad de reducir la infiltración leucocitaria asociada al daño vascular detectado en diversas patologías cardiovasculares en las que Ang-II está implicada. Por tanto, pueden constituir nuevas alternativas terapéuticas en el tratamiento de hipertensión y aterosclerosis impidiendo el desarrollo de estas enfermedades.

Papel de Ang-II en disfunciones endoteliales

La inhibición crónica de NOS provoca un aumento de presión arterial dependiente de Ang-II (Takemoto et al., 1997), el cual se produce a través del aumento de la síntesis de la ECA. Sin embargo, como se ha demostrado en este trabajo, este peptido no está implicado en el aumento de la presión arterial inducida por la inhibición aguda de esta enzima, ya que 1 h no es suficiente para que se produzca un aumento de la síntesis de la ECA. Por contra, el rápido aumento de presión arterial inducido por la inhibición aguda de COX, sí que se demostró dependiente de Ang-II, ya que la administración de losartan redujo este parámetro a niveles basales.

A pesar de estos resultados, creímos de interés investigar si las interacciones leucocito-endotelio inducidas por inhibición de COX y NOS pudieran estar mediadas por Ang-II. De esta forma comprobamos que losartan fue capaz de inhibir significativamente tanto los cambios hemodinámicos como las interacciones leucocito-endotelio causados bien por inhibición aguda de NOS o de COX, sin afectar al aumento de presión arterial

causado por la inhibición aguda de NOS. Estos datos sugieren que las acciones vasoconstrictora y proinflamatoria de Ang-II están de alguna manera disociadas e incluso que las lesiones vasculares puedan ocasionarse aunque no exista un estado hipertensivo. En cuanto a la capacidad de losartan de reducir el aumento de flujo sanguíneo causado bien por inhibición de NOS o de COX, concuerdan con un trabajo previo realizado por Sigmon & Beierwaltes (1993), en el que la inhibición aguda de la NOS produce un aumento de la presión arterial y una disminución del flujo sanguíneo en órganos viscerales y en el que se comprobó que la administración de losartan aunque atenuó las respuestas hemodinámicas no afectó al aumento de presión arterial sistémica. La posibilidad de que las interacciones leucocito-endotelio inducidas por inhibición aguda de la NOS o la COX fuesen consecuencia de las reducciones en el "shear rate" queda descartada ya que, la reducción de este parámetro en un un 40% no causa adhesión leucocitaria (Kubes et al., 1991; Perry & Granger, 1991) y en nuestro estudio, las reducciones en el "shear rate" son inferiores a este valor a los 60 min. Por consiguiente, la respuesta causada por losartan parece que es debida a un efecto directo sobre el endotelio impidiendo el aumento de expresión de alguna molécula de adhesión. En consecuencia y de nuevo, entre las diferentes moléculas de adhesión que podrían estar involucradas, P-selectina vuelve a ser la candidata más firme ya que es la primera en expresarse tanto en inhibición aguda de NOS como en inhibición aguda de COX (Davenpeck et al., 1994; Wallace et al., 1993). Todos estos datos indican que la falta de vasodilatadores como NO o PGI_2, expone al endotelio a la acción dañina de vasoconstrictores que inducirían, a través del aumento de P-selectina, a acumulación leucocitaria responsable del desarrollo de la lesión vascular que se detecta en estados patológicos tales como hipertensión, aterosclerosis y procesos de isquemia-reperfusión (Mervaala et al., 1999; Hernández-Presa et al., 1997; Ma et al., 1993). De esta forma,

Ang-II, como ya hemos comentado, induce acumulación leucocitaria sin causar vasoconstricción (Piqueras et al., 2000), por consiguiente, estos resultados indican que los efectos beneficiosos de losartan cuando el equilibrio vascular está alterado debido a bajos niveles de vasodilatadores, se deben a un efecto sobre la expresión de P-selectina y, por tanto, al bloqueo de la acción inflamatoria de Ang-II.

Por otro lado, nuestros resultados apoyan totalmente la teoría "respuesta al daño" (Ross, 1993; Fuster et al., 1992). En esta teoría, se indica que la lesión aterosclerótica puede ser iniciada por diferentes agentes desencadenantes que disminuyan la función protectora del endotelio. Como hemos comprobado, la inhibición de la NOS durante 4 h provoca una respuesta inflamatoria a nivel de vénulas postcapilares, la cual, como era de esperar, es dependiente de Ang-II. No obstante, el hallazgo más significativo de este estudio subagudo fue la clara adhesión leucocitaria detectada a nivel arteriolar tras administración subaguda de L-NAME y la inhibición de esta respuestas por pretratamiento con losartan. Por consiguiente, la simple reducción de los niveles de NO vasculares puede desencadenar el proceso aterosclerótico y además, Ang-II está implicada directamente en el inicio de este proceso incluso cuando sus niveles plasmáticos no están necesariamente elevados.

Finalmente y como consecuencia de estos resultados, también comprobamos que la ausencia de estrógenos que tiene lugar en mujeres posmenopaúsicas provoca una carencia de vasodilatadores endoteliales (NO y PGI_2) dando lugar a una respuesta inflamatoria mediada por aumento de expresión de P-selectina e indirectamente de integrinas β_2. De hecho, tanto las respuestas leucocitarias inducidas por inhibición de NOS como las inducidas por inhibición de COX son dependientes tanto de P-selectina como de integrinas β_2 (Kubes et al., 1991; Davenpeck et al., 1994; Wallace et al., 1993; Kurose et al., 1996).

Esta respuesta, se comprobó que era igualmente dependiente de Ang-II, ya que la administración de losartan también atenuó de forma drástica las interacciones leucocito-endotelio inducidas por tamoxifen. Por tanto, la carencia de estrógenos provoca la ausencia de vasodilatadores y expone al endotelio a la acción dañina de Ang-II. Este fenómeno quizás pueda explicar el mayor riesgo de desarrollo de enfermedades cardiovasculares en la mujer postmenopaúsica no tratada con una terapia hormonal adecuada y en el hombre.

III. 4. CONCLUSIONES

Los resultados obtenidos en este trabajo nos permiten concluir:

1. Ang-II posee acción proinflamatoria in vivo a nivel agudo (induce interacciones leucocito-endotelio) a dosis subvasoconstrictoras y fisiológicas. Sin embargo, Ang- II no es quimiotáctica por sí misma y su acción proinflamatoria no se debe a la activación de células mastocitarias. Esta acción proinflamatoria de Ang-II está mediada tanto por receptores AT_1 como por receptores AT_2 y es totalmente dependiente del aumento de la expresión de P-selectina endotelial.

2. La acción proinflamatoria de Ang-II es dependiente de la formación de radicales libres tanto intra como extracelulares generados inicialmente en la pared vascular. Además, la adhesión y la migración leucocitaria causada por Ang-II están en parte mediadas por la liberación de PAF y LTB_4.

3. Ang-II provoca una potente respuesta inflamatoria a nivel venular a las 4 h de su administración: el flujo de leucocitos en fase de "rolling" es debido al aumento de expresión tanto de P- como E- selectina, la adhesión leucocitaria se debe al aumento de expresión de integrinas α_4, integrinas β_2 e ICAM-1 y la migración leucocitaria es debida al aumento de expresión de integrinas β_2 y de ICAM-1.

4. Asímismo, Ang-II provoca una respuesta inflamatoria a nivel arteriolar a las 4 h de su administración la cual se produce a dosis iguales o superiores a 1 nM. Este efecto es dependiente del aumento de expresión de P-selectina y E-selectina y de integrinas β_2 y,

al menos en parte de ICAM-1. Este hecho implica a Ang-II directamente con el inicio de la lesión aterosclerótica.

5. Agentes antioxidantes y captadores de radicales libres, agentes capaces de aumentar los niveles de AMPc intracelular, donantes de NO y estrógenos son capaces de atenuar la respuesta inflamatoria aguda inducida por Ang-II.

6. La inhibición aguda y subaguda de la NOS, la inhibición de la COX y la falta de estrógenos, provocan una respuesta inflamatoria que en todos los casos está mediada por la interacción de Ang-II con su receptor AT_1, ya que la administración de losartan disminuye todas estas respuestas.

III. 5. BIBLIOGRAFÍA

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