DEPARTAMENTO DE FARMACOLOGÍA

REGULACIÓN NITRÉRGICA DE LA FUNCIÓN MOTORA GASTROINTESTINAL EN LA ENDOTOXEMIA

ELSA QUINTANA FERNÁNDEZ

UNIVERSITAT DE VALENCIA
Servei de Publicacions
2004

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Depòsit legal:
I.S.B.N.:84-370-1444-1
Edita: Universitat de València
Servei de Publicacions
C/ Artes Gráficas, 13 bajo
46010 València
Spain
Telèfon: 963864115

UNIVERSITAT DE VALÈNCIA

FACULTAT DE FARMÀCIA

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"REGULACIÓN NITRÉRGICA DE LA FUNCIÓN MOTORA GASTROINTESTINAL EN LA ENDOTOXEMIA"

TESIS DOCTORAL

ELSA QUINTANA FERNÁNDEZ

Valencia, 2004

Tras una infección bacteriana se observan cambios agudos de la función gastrointestinal. En la presente Tesis hemos analizado en la rata los mecanismos involucrados en la alteración de la función motora gastrointestinal asociada a los estadios iniciales de la endotoxemia. En estas circunstancias se produce una inhibición de la motilidad gástrica caracterizada por hipocontractilidad del estómago proximal y enlentecimiento del vaciamiento gástrico; por el contrario, a nivel intestinal, se produce un incremento del tránsito acelerándose la excreción fecal. Este comportamiento dual podría interpretarse como un mecanismo puesto en marcha con la finalidad de disminuir la absorción de toxinas y facilitar su pronta expulsión. Estos cambios se producen rápidamente gracias a la actuación del sistema nervioso, tanto central como periférico. Así, los efectos de la endotoxina están mediados por la activación de las neuronas aferentes sensoriales, que envían la señal de "alerta" al sistema nervioso central donde rápidamente se procesa la información y posteriormente se activan fibras motoras eferentes vagales que están en íntimo contacto con el sistema nervioso entérico, modulando finalmente la motilidad gastrointestinal. Además, la regulación postraduccional de la enzima óxido nítrico sintasa neuronal tiene un papel protagonista en todo este proceso. Un incremento de la síntesis de óxido nítrico en el tronco del encéfalo y en neuronas postganglionares del plexo mientérico sucede en los primeros estadios de la endotoxemia. Pasado el efecto agudo de la endotoxina, empezaría a observarse lo que se considera parte de una respuesta inmune generalizada mediada por la expresión de las enzimas óxido nítrico sintasa inducible y ciclooxigenasa-2. En respuesta a una invasión bacteriana, parece que el organismo maximiza la actividad de sus recursos fisiológicos mientras se alcanza la plena actividad del sistema inmune.

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HACEN CONSTAR:

Que el trabajo titulado “Regulación nitrérgica de la función motora gastrointestinal en la endotoxemia”, presentado por la Licenciada Elsa Quintana Fernández 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, Febrero de 2004

Fdo. Dr. Juan V. Esplugues Mota Fdo. Dra. M. D. Barrachina Sancho

A mi familia

AGRADECIMIENTOS

Quiero expresar mi más sincero agradecimiento:

En primer lugar al que ha sido el fundador de este Departamento, el Profesor Juan Esplugues, gracias por sus entrañables consejos.

A mis directores, el Profesor Juan Vicente Esplugues, por su apoyo incondicional y preocupación por mi formación científica, y la Profesora María Dolores Barrachina, por su constante dedicación, entusiasmo, respaldo y amistad; a ella debo mi espíritu científico. A ambos, gracias por haber confiado en mí.

A Sara, por su enorme contribución al desarrollo de esta tesis, su rectitud en el trabajo ha sido un modelo para mí. A Mª Ángeles, por su tutela en el comienzo de mi carrera investigadora. A las dos, gracias por su inestimable ayuda diaria, por su amistad.

A mis compañeros de laboratorio con los que he compartido tantos buenos momentos. A Eugenia, Ángeles y Carlos, sin ellos todo habría sido mucho más difícil, gracias por su sincera amistad. A los más recientes, Alejandra, Nadezda, Reme, Sales, Cristina Núñez, Víctor, Marta, Susana, Jesús, Sol, Ana, Kenneth, Juan, Cristina Amézcua y por supuesto, a la eficiente Paqui. Al resto de compañeros del Departamento de Farmacología, especialmente a Inés, Amparo, Regina, Miguel, Dora y Maruja. Muchas gracias a todos por su ayuda, apoyo, ánimo y confianza que han hecho el día a día tan agradable.

A Raquel, Elvira, Pilar y Alberto, por haber hecho tan grata y especial la estancia en su laboratorio.

A Noelia, por su apoyo, paciencia y comprensión durante estos años de convivencia.

A todos, gracias por haber contribuido a la satisfacción con la que he vivido esta aventura.

ABREVIATURAS

2-DG	.....	2-deoxi-D-glucosa
7-NI	.....	7-nitroindazol
Ach	.....	acetilcolina
ATP	.....	adenosina trifosfato
BHE	.....	barrera hematoencefálica
canal $_{Ca}K^{+}$	.....	canal de potasio dependiente de calcio
CDV	.....	complejo dorsal vagal
CGRP	.....	péptido relacionado con el gen de la calcitonina
COX	.....	ciclooxigenasa
DAF-FM	.....	4-amino-5-metilamino-2',7'-difluorofluoresceína
DETA-NO	.....	(Z)-1-[2-(2-aminoetil)-N-(2-amonioetil)amino]diazon-1-io-1,2-diolato
GCs	.....	guanilato ciclasa soluble
GMPc	.....	guanosina monofosfato cíclica
IL	.....	interleucina
L-NAME	.....	N^{ω}-nitro-L-arginina metil éster
L-NIL	.....	N^{6}-(1-iminoetil)-L-lisina
L-NOARG	.....	N^{ω}-nitro-L-arginina
LPS	.....	lipopolisacárido
NANC	.....	no-adrenérgico no-colinérgico
NDV	.....	núcleo dorsal del vago
NO	.....	óxido nítrico
NOS	.....	óxido nítrico sintasa
NOSe	.....	óxido nítrico sintasa endotelial
NOSi	.....	óxido nítrico sintasa inducible
NOSn	.....	óxido nítrico sintasa neuronal
NTS	.....	núcleo del tracto solitario
ODQ	.....	1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-ona
PGs	.....	prostaglandinas
P_{IG}	.....	presión intragástrica
SNC	.....	sistema nervioso central
SNE	.....	sistema nervioso entérico
SP	.....	sustancia P
TNF-α	.....	factor de necrosis tumoral-α
TRIM	.....	1-[2-(trifluorometil)fenil]imidazol
TTX	.....	tetrodotoxina
VIP	.....	péptido intestinal vasoactivo

ÍNDICE

INTRODUCCIÓN **11**

I. Fase aguda de la infección bacteriana **12**

I.1. Señalización al sistema nervioso central 13

I.1.1. Vía humoral 14

I.1.2. Vía nerviosa 14

I.2. Alteración de la función gastrointestinal 15

II. Motilidad gastrointestinal **17**

II.1. Regulación nerviosa 17

II.1.1. Inervación intrínseca 17

II.1.2. Inervación extrínseca 20

II.2. Fisiología del vaciamiento gástrico 22

II.3. Fisiología del tránsito intestinal y excreción fecal 25

III. Óxido nítrico **27**

III.1. Síntesis 27

III.2. Mecanismo de acción 29

III.3. Óxido nítrico en el sistema nervioso 31

III.3.1. Sistema nervioso central 32

III.3.2. Sistema nervioso periférico 33

III.4. Regulación nitrérgica de la motilidad gastrointestinal 34

OBJETIVO **37**

ARTÍCULOS DE INVESTIGACIÓN 38

I. Efectos de la endotoxina sobre la motilidad gástrica **39**

Artículo 1: "A cerebral nitrergic pathway modulates endotoxin-induced changes in gastric motility". British Journal of Pharmacology (2001) 134: 325-332. 42

Artículo 2: "Transcriptional up-regulation of nNOS in the dorsal vagal complex during low endotoxemia". Neurogastroenterology and motility (2004, en revisión). 51

Artículo 3: "Synthesis of nitric oxide in post-ganglionic myenteric neurons during endotoxemia: implications for gastric motor function". The FASEB Journal express (2004) 18:531-533. DOI:10.1096/fj.03-0596fje. 70

Artículo 4: "Endotoxin inhibits gastric emptying in rats via a capsaicin-sensitive afferent pathway". Naunyn-Schmiedeberg's Archives of Pharmacology (2001) 363: 276-280. 100

Artículo 5: "Downregulation of nNOS and synthesis of PGs associated with endotoxin-induced delay in gastric emptying". American Journal of Physiology (2002) 283: G1360-G1367. 106

II. Efectos de la endotoxina sobre la motilidad colónica **115**

Artículo 6: "Endotoxin stimulates fecal pellet output in rats through a neural mechanism". Naunyn-Schmiedeberg's Archives of Pharmacology (2003) 367(1): 51-5. 116

III. Análisis global **122**

Artículo 7: "Nitrergic modulation of gastrointestinal function during early endotoxemia". Current Pharmaceutical Design (2004, en revisión). 122

RESUMEN DE RESULTADOS Y DISCUSIÓN GENERAL **156**

I. Efectos de la endotoxina sobre la motilidad gástrica 157

II. Efectos de la endotoxina sobre la motilidad colónica 169

CONCLUSIONES **172**

BIBLIOGRAFÍA **174**

INTRODUCCIÓN

I. FASE AGUDA DE LA INFECCIÓN BACTERIANA

Nuestro organismo está continuamente expuesto a agentes patógenos y por ello ha desarrollado mecanismos innatos que le permiten eliminar el agente invasor de una manera rápida y eficaz. Tras una infección, además de coordinar una respuesta inmunitaria periférica, el organismo reacciona señalizando al sistema nervioso central (SNC) y desencadenando una serie de eventos en cascada, principalmente cambios neuroendocrinos, metabólicos y del comportamiento, que se engloban bajo el nombre de "reacción de fase aguda". Los síntomas típicos de este estadio incluyen fiebre, confusión mental, hipotensión transitoria, pérdida del apetito y alteración de la función gastrointestinal caracterizada por vómito y diarrea. Esta fase constituye la primera reacción de defensa del organismo frente a una infección. Se trata de una respuesta inespecífica, rápida, intensa y eficaz que consigue, en la mayoría de los casos de infecciones leves, restaurar la homeostasis sin que se llegue a observar la sintomatología típica de la inflamación.

El principal componente bacteriano responsable de desencadenar dicha respuesta en el caso de las bacterias Gram-negativas es el lipopolisacárido (LPS), también llamado endotoxina. Éste es un componente constante de la membrana celular externa de estas bacterias (**figura 1**). Desde el punto de vista bioquímico, se trata de una macromolécula de carácter anfifílico compuesta por una parte lipídica y una parte azucarada. La parte lipídica, denominada "Lípido A", le sirve de anclaje a la membrana celular; su estructura está altamente conservada y es la responsable de la toxicidad biológica del LPS. La parte azucarada por el contrario queda expuesta al exterior celular y se compone de una cadena de oligosacáridos conocida como "Antígeno O", el cual es específico de cada bacteria y cepa, y un "corazón" oligosacárido que le sirve de unión entre el Lípido A y el antígeno O.

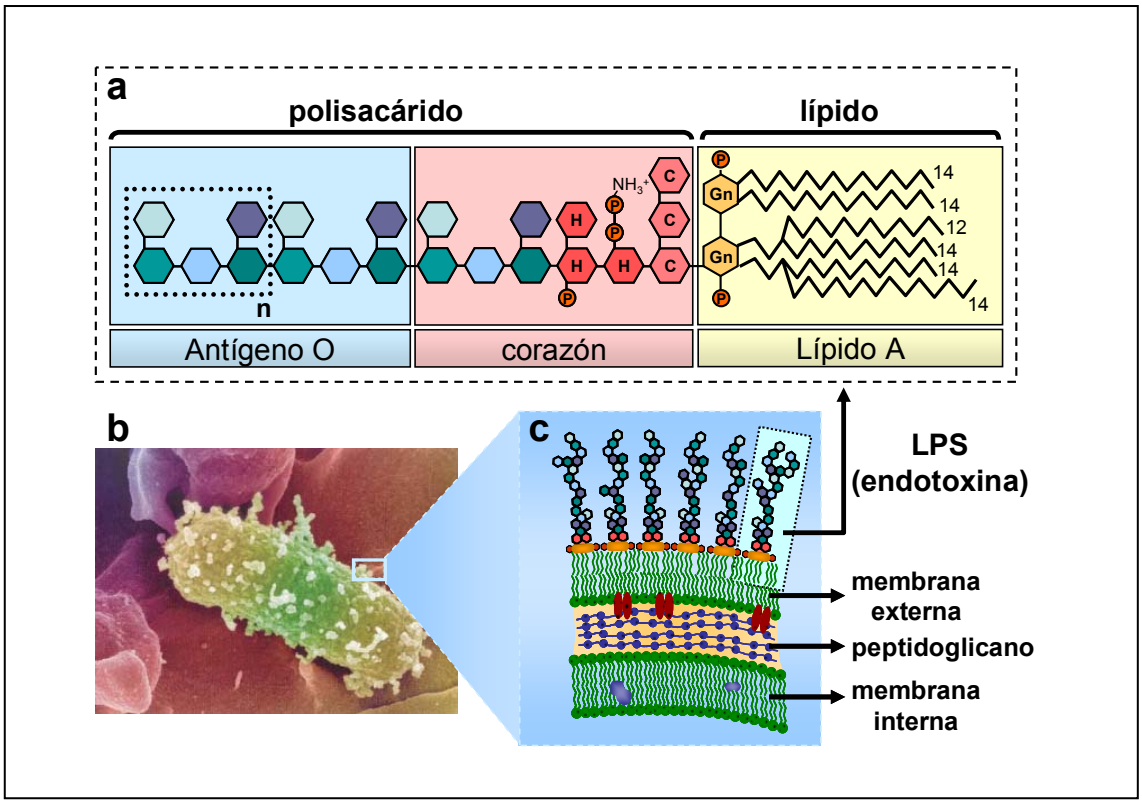

Figura 1. Lipopolisacárido de las bacterias Gram-negativas. Arquitectura del lipopolisacárido de *Escherichia coli* (a), junto con una microfotografía electrónica de la misma (b) (Beutler & Rietschel, 2003) y una representación esquemática de la localización del lipopolisacárido en la pared celular de la bacteria (c). Gn, D-glucosamina; H, L-glicero-D-mano-heptosa; C, ácido 2-ceto-3-deoxi-octulosónico; P, fosfato.

I.1. Señalización al sistema nervioso central

El mecanismo por el cual el LPS en la periferia señaliza al SNC para desencadenar la "reacción de fase aguda" ha sido tema de controversia en la última década (Blatteis *et al.*, 2004; Romanovsky, 2004). Los mensajeros propuestos encargados de informar al cerebro de la presencia periférica del LPS son determinadas citocinas liberadas a partir de macrófagos activados, principalmente las inlerleucinas 1 y 6 (IL-1, IL-6) y el factor de necrosis tumoral α (TNF-α). Este hecho está basado en las siguientes evidencias: a) estas

citocinas se detectan en sangre durante los 30-90 minutos tras la exposición al LPS (Givalois *et al.*, 1994; Jansky *et al.*, 1995) y b) la administración exógena de dichas citocinas mimetiza la mayoría de los efectos inducidos por el LPS en la fase aguda (Rothwell & Luheshi, 1994; Schobitz *et al.*, 1994).

I.1.1. Vía humoral

Clásicamente se había aceptado que las citocinas secretadas en respuesta al LPS acceden al SNC vía sanguínea; sin embargo, se hace difícil el paso a través de la barrera hematoencefálica (BHE) debido a su gran tamaño molecular y carácter hidrofílico. Se propusieron distintas teorías de incorporación al SNC: a) acceso directo por difusión simple a través de estructuras centrales carentes de BHE, como los órganos circunventriculares; b) acceso mediante sistemas de transporte específicos o c) unión a receptores específicos presentes en las células endoteliales de los vasos cerebrales y posterior liberación de otros mediadores en el parénquima cerebral. Existen evidencias de que todas estas situaciones se dan; sin embargo, hasta la fecha no se ha demostrado de forma directa que esta vía participe en los efectos mediados por el SNC, inducidos por el LPS.

I.1.2. Vía nerviosa

En 1987, se especuló sobre la posible participación de nervios periféricos en la fiebre inducida por el LPS (Morimoto *et al.*, 1987). La idea de la actuación de las neuronas aferentes en la señalización central de mediadores periféricos no era nueva en neurobiología ya que se había demostrado que estas fibras nerviosas mediaban los efectos de hormonas y péptidos periféricos sobre la función cerebral, incluida la adrenalina (Williams & McGaugh, 1993) o la colecistocinina (Anika *et al.*, 1977). Los primeros trabajos que demostraron de

una manera clara que el nervio vago estaba involucrado en los efectos centrales del LPS surgieron en 1994 (Bluthe *et al.*, 1994; Watkins *et al.*, 1994) y desde entonces, el número de publicaciones entorno a este tema incrementó considerablemente. Durante este tiempo los estudios se han centrado principalmente en la fiebre inducida por el LPS, utilizando como herramientas experimentales el bloqueo químico (aplicación local o sistémica de la capsaicina) o quirúrgico (vagotomía) de la transmisión vagal, y el estudio de la activación nerviosa mediante la detección del gen de expresión temprana *c-fos*. Actualmente, existe una elevada evidencia científica que apoya la teoría de la vía nerviosa en la que las neuronas aferentes vagales median el comienzo de los efectos centrales inducidos por el LPS (Blatteis *et al.*, 2000; Maier *et al.*, 1998; Romanovsky, 2000).

Sin embargo, todavía existe controversia sobre si la activación de las fibras aferentes vagales está mediada por las citocinas secretadas o si es una acción directa de la endotoxina (Blatteis *et al.*, 2000). Las citocinas no existen almacenadas en los macrófagos sino que requieren expresión de *novo*, lo cual necesita cierto tiempo. Existe una clara discrepancia temporal entre la primera detección de citocinas en sangre (30-90 min) (Givalois *et al.*, 1994; Jansky *et al.*, 1995) y la aparición de algunos de los efectos mediados por el SNC como por ejemplo la fiebre (15 min) (Li *et al.*, 1999). En este sentido, se ha propuesto que citocinas como el TNF-α no juegan un papel en la génesis sino en el mantenimiento de la fiebre (Roth *et al.*, 1998).

I.2. Alteración de la función gastrointestinal

En la fase aguda de una infección bacteriana se observan cambios en la función gastrointestinal. Por ejemplo, una endotoxemia moderada cursa en la clínica con una hiposecreción ácida gástrica (Hurley, 1995). Este hecho ha sido reproducido ampliamente en estudios experimentales. La administración de

dosis bajas de LPS produce una inhibición rápida (30 minutos) de la secreción ácida gástrica en la rata (Barrachina *et al.*, 1995b; Calatayud *et al.*, 1999; Esplugues *et al.*, 1996; Garcia-Zaragoza *et al.*, 2000; Martinez-Cuesta *et al.*, 1994). Por otra parte se ha visto que la endotoxina tiene un efecto dual sobre la integridad mucosa dependiendo de la dosis administrada. Durante el *shock* séptico se desarrollan importantes lesiones gástricas. Sin embargo, sorprendentemente, la administración de dosis bajas de endotoxina protege la mucosa gástrica frente a la agresión inducida por distintos estímulos ulcerogénicos como puede ser el estrés, el tratamiento con antiinflamatorios no esteroideos o la administración de etanol (Barrachina *et al.*, 1995a; Calatayud *et al.*, 2003; Tsuji *et al.*, 1993). Recientemente se ha sugerido que la prevención del daño se debe a un incremento del flujo sanguíneo de la microcirculación gástrica ya que se ha demostrado que el LPS previene la caída del flujo sanguíneo inducido por los antiinflamatorios no esteroideos (Calatayud *et al.*, 2003).

Nuestro grupo de investigación ha estudiado los mecanismos involucrados en este proceso. Así, sabemos que los cambios agudos de la función gastrointestinal inducidos por la endotoxina son consecuencia de un mecanismo rápido e independiente de la síntesis proteica característica de un estadio más tardío. Además, hemos visto que está regulado por los sistemas nerviosos autónomo y central y parece que la síntesis constitutiva de óxido nítrico (NO) juega un papel determinante.

De entre las distintas funciones gastrointestinales, la motilidad se altera de forma significativa en la fase aguda de la infección bacteriana observándose una inhibición del vaciamiento gástrico y un incremento del tránsito intestinal. Sin embargo, muy poco se sabe acerca del mecanismo por el que sucede este proceso.

II. MOTILIDAD GASTROINTESTINAL

La función motora gastrointestinal requiere del funcionamiento coordinado e integrado de las distintas capas musculares según la zona y el estado de cada momento. Esta función se ve modulada por diversas condiciones como son estado de ayuno, cualidades y componentes de la dieta, agentes nocivos, factores sensoriales y emocionales, etc. Una compleja red nerviosa distribuida a lo largo de todo el tracto digestivo (inervación intrínseca) y comunicada con el SNC (inervación extrínseca), desempeña un papel esencial en la coordinación motora.

II.1. Regulación nerviosa

II.1.1 Inervación intrínseca

En todas las culturas antiguas y modernas se ha tenido la conciencia, al menos popular, de que nuestro estómago es capaz de experimentar emociones. Tras décadas de trabajo, los científicos están en condiciones de afirmar que en el tracto gastrointestinal se aloja un "segundo cerebro" muy similar al que tenemos en la cabeza. El tubo digestivo está literalmente tapizado por más de 100 millones de células nerviosas (equivalente al contenido de la médula espinal) que constituyen el sistema nervioso entérico (SNE). Aunque está dotado de total autonomía, también mantiene estrechas conexiones con el SNC.

Los cuerpos celulares de las neuronas del SNE se encuentran aglutinados en estructuras ganglionares que a su vez se interconectan entre sí formando dos grandes plexos nerviosos: el plexo mientérico o plexo de Auerbach y el plexo submucoso o plexo de Meissner (**figura 2**). El plexo mientérico, ubicado entre las capas musculares longitudinal y circular, se extiende desde el tercio medio del esófago hasta el canal anal proporcionando inervación motora a ambas capas musculares e inervación secreto-motora a las células de la

mucosa. El plexo submucoso, ubicado por debajo de la *muscularis mucosae*, presenta su máximo desarrollo en el intestino delgado donde desempeña un importante papel en el control de la secreción. Ambos plexos mantienen estrechas conexiones entre sí.

Al igual que en el SNC, el SNE tiene tres clases de neuronas de acuerdo a su funcionalidad:

a) Neuronas sensoriales, cuyas áreas receptivas se encuentran en la musculatura de la pared y en la mucosa. Las terminaciones libres de estas neuronas tienen receptores capaces de responder a cambios de energía química, térmica o mecánica, transformando estos cambios en señales eléctricas. Son esenciales en el control de la digestión.

b) Interneuronas, que forman múltiples sinapsis entre sí dando lugar a circuitos integrados "lógicos". Estos circuitos son capaces de descifrar y procesar la información proveniente de las neuronas sensoriales y generar programas de respuestas reflejas motoras coherentes con la funcionalidad global del órgano. Constituyen la base de la propagación de las ondas peristálticas.

c) Neuronas motoras, que son el brazo efector del sistema que transmite las señales, estimulantes o inhibidoras, a los distintos efectores: vaso, músculo y epitelio.

La lista de neurotransmisores presentes en el SNE es larguísima y en general son prácticamente los mismos que se encuentran presentes en el SNC. Su naturaleza es muy diversa: aminas, aminoácidos, purinas, gases o péptidos. Los más importantes en la regulación de la función motora son acetilcolina (Ach) y sustancia P (SP), entre los neurotransmisores excitadores, y VIP, ATP y NO (Wood, 1994), entre los inhibidores.

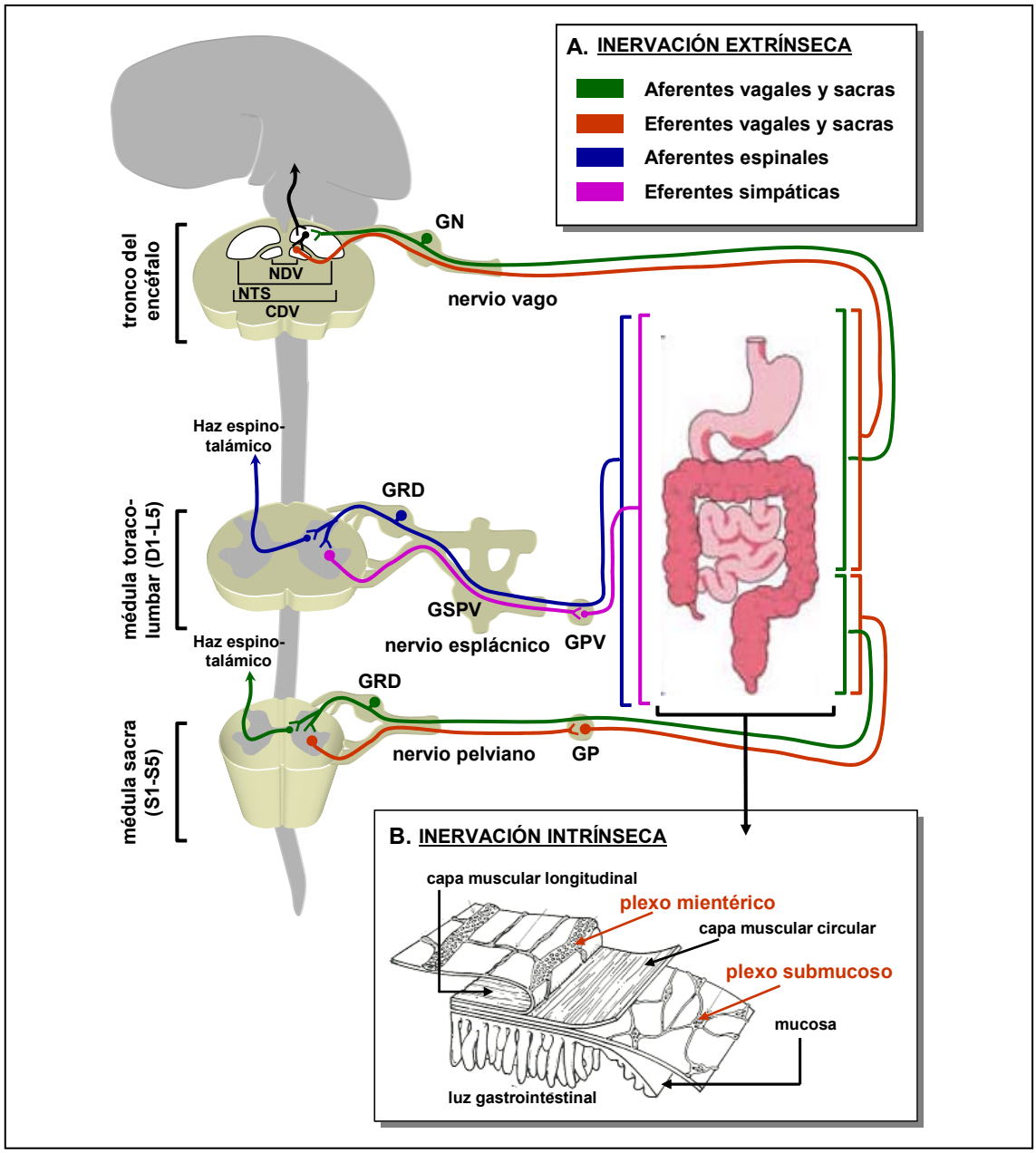

Figura 2. Inervación del tracto gastrointestinal. A: Vías aferentes y eferentes de los sistemas simpático y parasimpático que regulan la función gastrointestinal. B: Distribución de los plexos nerviosos entéricos en el tracto gastrointestinal. CDV, complejo dorsal vagal; CSPV, cadena simpática paravertebral; GN, ganglio nodoso; GP, ganglio pélvico; GPV, ganglios prevertebrales (celíaco, mesentérico superior e inferior, pélvico); GRD, ganglio de la raíz dorsal; NDV, núcleo dorsal del vago; NTS, núcleo del tracto solitario.

II.1.2 Inervación extrínseca

El tubo digestivo tiene una doble inervación extrínseca a través de los sistemas parasimpático y simpático. Ambos contienen fibras aferentes sensoriales que transmiten información al SNC, y fibras eferentes motoras que inervan los órganos efectores (**figura 2**).

a) Inervación vagal (parasimpático): El nervio vago es el principal nexo de unión entre el sistema nervioso central y entérico involucrado en la regulación de la función gastrointestinal. Inerva todo el tracto digestivo, aunque la densidad nerviosa disminuye conforme se avanza hacia el colon, siendo prácticamente inexistente en el colon distal.

- *Fibras aferentes vagales*: El flujo de mensajes desde el tubo digestivo a la cabeza supera con creces a las órdenes que llegan del cerebro al estómago. La vía aferente representa el 80-90% de todas las fibras del nervio vago. Son fibras amielínicas cuyos cuerpos celulares se encuentran en el ganglio nodoso y sus axones terminan en el núcleo del tracto solitario (NTS) situado en el tronco del encéfalo, núcleo al cual convergen impulsos somáticos y viscerales de otras partes del organismo, contribuyendo, junto con otras estructuras, a la integración de la información sensorial somática y visceral y a la respuesta autónoma. Una gran mayoría de estas fibras son sensibles a la acción de la capsaicina, una neurotoxina que bloquea selectivamente la transmisión de las fibras nerviosas aferentes amielínicas (tipo C) (Holzer, 1991).

- *Fibras eferentes vagales*: Estas fibras nacen en el núcleo motor dorsal del vago (NDV) situado en el tronco del encéfalo, íntimamente relacionado con el NTS. Son fibras colinérgicas que no inervan directamente los efectores sino que hacen sinapsis (a través de los receptores nicotínicos ganglionares) con los circuitos integrados del

plexo mientérico. Esto explica que un número relativamente bajo de fibras eferentes (10-20% del total de las fibras vagales) tenga efectos sobre un área tan extensa, influyendo simultáneamente en cambios de la motilidad, secreción y circulación. Estas fibras hacen sinapsis con neuronas postganglionares del plexo mientérico excitadoras (vía colinérgica) o inhibidoras (vía no-adrenérgica no-colinérgica, NANC).

- *Reflejos vago-vagales*: La función gastrointestinal está modulada por un número importante de reflejos vagales entre los que se encuentra el reflejo gastro-gástrico, entero-gástrico, hepato-pancreático o gastro-colónico. Estos reflejos se integran en el complejo dorsal vagal (CDV) que comprende los ya citados NTS y NDV. Estos núcleos no están compartimentalizados en unidades anatómicas distintas, sino que están compuestos de neuronas organizadas topográficamente en zonas solapadas. El NTS también manda información a núcleos nerviosos superiores del hipotálamo o de la corteza cerebral entre otros, los cuales a su vez están conectados con el NDV. De esta manera permite la integración de las funciones gastrointestinal, cardiovascular y respiratoria en reflejos autónomos como puede ser el reflejo del vómito.

b) Inervación sacra (parasimpático): Estas neuronas se prolongan por el nervio pelviano y controlan la motilidad del colon distal, recto y esfínter anal interno. Los somas están localizados en la porción sacra lumbar de la médula espinal (segmentos S1 a S5). Este sistema incluye neuronas aferentes y eferentes que forman el reflejo espinal que regula la motilidad colónica. Además, estas neuronas están moduladas por estructuras nerviosas superiores.

c) Inervación espinal (simpática):

- *Fibras aferentes espinales*: Estas fibras se originan en el ganglio de la raíz dorsal, en la porción torácica lumbar de la médula espinal, y alcanzan el tubo digestivo por el nervio esplácnico. La particularidad de estas neuronas es que no sólo transfieren la información visceral al SNC, sino que en ocasiones también actúan directamente sobre sistemas efectores gastrointestinales próximos, mediante reflejos axónicos que utilizan vías nerviosas bifurcantes. Estos reflejos axónicos, son responsables por ejemplo de la vasodilatación submucosa o la secreción duodenal de bicarbonato. Muchas de estas neuronas contienen el péptido relacionado con el gen de la calcitonina (CGRP) y la sustancia P (SP), y la mayoría de ellas son sensibles al efecto neurotóxico de la capsaicina (Holzer, 1991).

- *Fibras eferentes espinales*: Son fibras postganglionares cuyos somas se localizan en los ganglios prevertebrales (celíaco, mesentérico superior e inferior y pélvico). Son neuronas noradrenérgicas que principalmente ejercen una acción inhibidora de la función gastrointestinal; por una parte contraen los esfínteres y por otra inhiben la contractilidad de las regiones no esfintéricas al impedir la liberación de la Ach por parte de las neuronas excitadoras del plexo mientérico y submucoso. Además, también ejercen una acción inhibidora directa sobre el músculo liso.

II.2. Fisiología del vaciamiento gástrico

El estómago puede dividirse en tres regiones funcionales: el estómago proximal (cardias, fundus y cuerpo proximal), el estómago distal (cuerpo distal y antro) y el píloro. La acción motora coordinada de cada una de estas partes regula el vaciamiento del contenido gástrico. El vaciamiento de líquidos es un

proceso muy rápido que está principalmente regulado por la motilidad del estómago proximal. Por el contrario, el vaciamiento de sólidos nutrientes es un proceso mucho más lento y está principalmente regulado por la acción coordinada del estómago distal y esfínter pilórico (Mayer, 1994). El vaciamiento gástrico está controlado por tres tipos de regulación: paracrina, endocrina y nerviosa, tanto intrínseca como extrínseca.

a) Estómago proximal: La inervación extrínseca, en particular el nervio vago, ejerce un importante papel regulador del tono en esta zona. Mediante estudios experimentales se ha demostrado que el tono basal del fundus está regulado por el nervio vago (Azpiroz & Malagelada, 1987; Paterson *et al.*, 2000). El mantenimiento de la presión intragástrica está controlado por dos tipos de reflejos vago-vagales, la relajación receptiva y la adaptativa. El primero se pone en marcha tras la deglución mientras que el segundo se activa con la distensión gástrica. Ambos desencadenan la disminución del tono gástrico del fundus con la finalidad de albergar la comida ingerida. Además, existen reflejos entero-gástricos que se activan con la distensión duodenal o colónica y que también tienen como objetivo inhibir la actividad motora del estómago proximal. Hoy sabemos que los principales mediadores fisiológicos que median estos reflejos son NO, VIP o colecistocinina en el caso de los reflejos entero-gástricos. Por otra parte, las prostaglandinas (PGs) en general promueven la contracción tónica en esta región, favoreciendo así el vaciamiento gástrico de líquidos (Sanders, 1984).

b) Estómago distal: Esta porción del estómago tiene propiedades eléctricas y contráctiles muy distintas a la parte proximal, que le sirven para mezclar y triturar el contenido gástrico y para regular el vaciamiento de sólidos y, en menor medida, de líquidos. El nervio vago también regula de manera importante la motilidad de esta zona. La estimulación vagal de las fibras nerviosas de bajo umbral incrementa el tono, efecto mediado por la Ach, mientras que la

estimulación de fibras de elevado umbral inhibe el tono, efecto mediado principalmente por el NO y el VIP (Dickens *et al.*, 2000). Existe un reflejo vago-vagal mediante el cual la distensión del fundus provoca la contracción del antro. Este reflejo parece que es el que inicia el proceso de la digestión, al promover la fase de mezcla y trituración. A diferencia del estómago proximal, a nivel distal el efecto de las PGs es inhibidor ya que disminuye la amplitud de las contracciones y la capacidad del músculo liso de responder a estímulos excitadores. Como consecuencia, el vaciamiento gástrico de sólidos parece estar retrasado por las PGs endógenas (Sanders, 1984).

c) Esfínter pilórico: El píloro es el que permite la evacuación del contenido gástrico, regulando el volumen expulsado e impidiendo además la regurgitación del contenido duodenal. Posee unas propiedades musculares y nerviosas únicas que le distinguen de toda la zona de su alrededor. Así, se trata de un músculo grueso con elevada proporción de tejido conectivo. La densidad nerviosa es de 3 a 5 veces superior a la presente en el antro adyacente y la cantidad de neuronas extrínsecas aferentes que proyectan al NTS también es superior a la del duodeno (Daniel *et al.*, 1989). Comparativamente con el antro y el duodeno, existe un elevado número de neuronas que contienen VIP, NO, SP, encefalinas, neuropéptido Y o galanina, sugiriendo un papel regulador en el tono del esfínter para estas sustancias. La actividad pilórica motora se ha visto modulada por fibras vagales, excitadoras e inhibidoras, e inervación esplácnica que principalmente induce la contracción pilórica. Además, está regulada por vías nerviosas intrínsecas ascendentes (la contracción duodenal produce contracción pilórica) y descendentes (la despolarización del músculo antral relaja el esfínter), (Yuan *et al.*, 2001). La regulación nitrérgica es la principal responsable de la relajación del esfínter (Ishiguchi *et al.*, 2000). Además, ATP, VIP, galanina, prostaglandina E_1 y serotonina son sustancias que relajan el píloro; mientras que colecistocinina, secretina o histamina contraen el esfínter.

II.3. Fisiología del tránsito intestinal y excreción fecal

El peristaltismo es el principal mecanismo responsable del avance del bolo fecal a lo largo del intestino. El reflejo peristáltico básico es el resultado de una serie de reflejos locales cada uno de los cuales consiste en una primera contracción del músculo intestinal por encima de un estímulo intraluminal, que suele ser la distensión mecánica ejercida por el bolo, seguida de la relajación del músculo por debajo del estímulo (**figura 3**). La suma de estos efectos consigue la propulsión del contenido en dirección anterógrada y conforme el bolo avanza desencadena sucesivos reflejos. Este proceso está regulado principalmente por el SNE. La liberación controlada de los neurotransmisores excitadores e inhibidores determinará la velocidad de la propulsión del contenido intestinal. Estudios experimentales demuestran que el bloqueo de la síntesis de los neurotransmisores excitadores, Ach o SP, así como de los inhibidores, VIP o NO, retrasa el avance del bolo fecal (Foxx-Orenstein & Grider, 1996).

La excreción fecal es un proceso complejo regulado por mecanismos voluntarios e involuntarios. El incremento de la motilidad en el colon descendente y recto promueve el avance de las heces hacia el ano, donde la relajación de los esfínteres anales externo e interno facilita su expulsión. Este proceso está regulado por el reflejo entérico inhibidor recto-anal, mediante el cual la distensión del recto produce una marcada disminución, volumen-dependiente, en la presión del esfínter anal interno. Las fibras sensoriales intrínsecas son las responsables de la activación del reflejo mientras que VIP y NO son los principales mediadores de la relajación (Jones *et al.*, 2003; Sangwan & Solla, 1998).

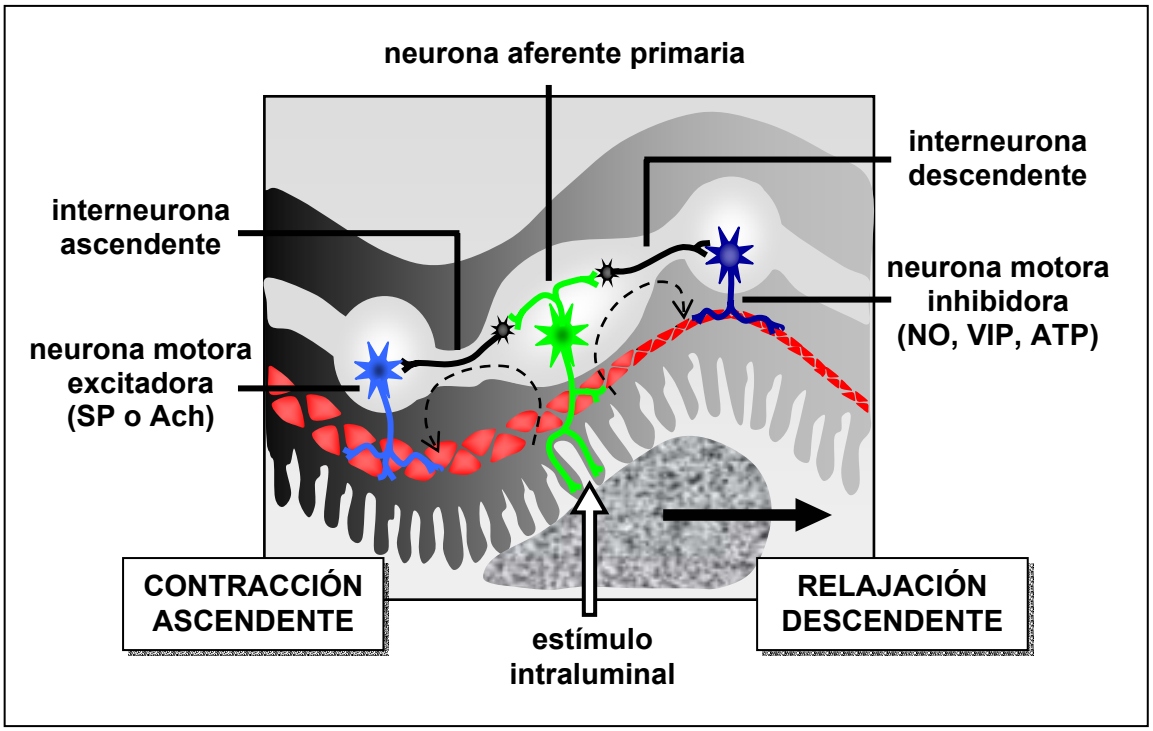

Figura 3. Reflejo peristáltico intestinal. El estímulo químico de la mucosa o la distensión mecánica de la luz intestinal dispara la actividad de las neuronas aferentes primarias. Éstas, activan a su vez interneuronas colinérgicas en sentido oral, las cuales estimulan neuronas motoras excitadoras que liberan Ach o SP, provocando así la contracción de la capa del músculo circular que está por encima del estímulo (contracción ascendente). Simultáneamente, por debajo del estímulo, las interneuronas colinérgicas descendentes activan neuronas motoras inhibidoras que contienen NO, VIP o ATP y producen la relajación muscular (relajación descendente). El conjunto de estos efectos permite el avance del bolo fecal.

III. ÓXIDO NÍTRICO

El NO fue identificado en 1987 como un mediador endógeno liberado por las células endoteliales que inducía la relajación vascular (Palmer *et al.*, 1987), e inhibía la agregación plaquetaria (Radomski *et al.*, 1987) y la adhesión de neutrófilos al endotelio vascular (Kubes *et al.*, 1991). Desde entonces una gran cantidad de estudios se han centrado en caracterizar esta particular molécula y actualmente sabemos que su papel fisiológico no sólo se circunscribe al sistema cardiovascular sino que está ampliamente distribuido y que posee una gran diversidad de funciones. En el SNC, actúa como un neurotransmisor implicado en procesos como el aprendizaje y la memoria, el desarrollo neuronal o la regulación neuroendocrina. En el sistema nervioso periférico, se le considera el mediador de la transmisión NANC, responsable de la relajación del músculo liso de los sistemas gastrointestinal, respiratorio y genitourinario. Además, el NO juega un importante papel en la respuesta inmunitaria no específica ya que su síntesis a partir de las células inflamatorias forma parte de los mecanismos de defensa inespecífica contra microorganismos y células tumorales (Moncada *et al.*, 1991).

III.1. Síntesis

El NO es una molécula gaseosa que se sintetiza a partir del aminoácido semiesencial L-arginina en una reacción catalizada por una flavoproteína denominada NO-sintasa (NOS). Se trata de una reacción de oxidación dependiente de nicotín adenín difosfato y que requiere de la presencia de determinados cofactores y de la unión del complejo Ca^{+2}/calmodulina para su correcto funcionamiento (**figura 4**). La NOS es una enzima compleja desde el punto de vista de su regulación, pudiendo estar controlada su expresión a nivel transcripcional así como su localización y actividad a nivel postraduccional.

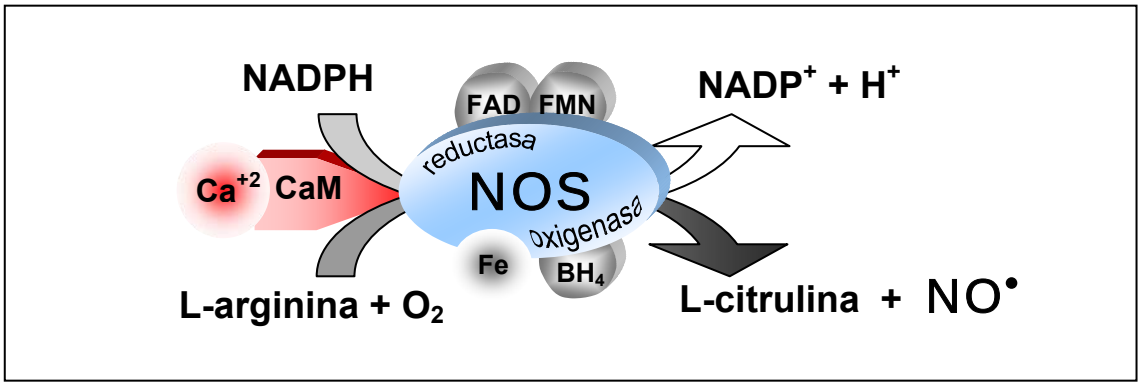

Figura 4. Reacción de síntesis del NO. El NADPH cede los electrones al dominio reductasa de la enzima los cuales, gracias a los cofactores FAD y FMN, se transfieren al dominio oxigenasa, donde los capta el átomo de Fe del grupo hemo (protoporfirina IX) junto con el cofactor BH_4, catalizando así la reacción del oxígeno con la L-arginina y generando L-citrulina y NO como productos. El flujo de electrones en el dominio reductasa requiere de la unión a la enzima del complejo calcio/calmodulina. BH_4: tetrahidrobiopterina; CaM: calmodulina; FAD: flavín adenín dinucleótido; FMN: flavín mononucleótido; NADPH: nicotín adenín difosfato; NOS: NO-sintasa.

Se han descrito tres isoformas de la enzima, cada una codificada por un gen distinto y expresada en diferentes condiciones y territorios (Alderton *et al.*, 2001). Dos de ellas están expresadas constitutivamente y son responsables de la síntesis de pequeñas cantidades de NO en respuesta a estímulos puntuales. Su actividad está principalmente regulada por el Ca^{+2} intracelular, ya que es el responsable de la unión de la calmodulina a la enzima. De acuerdo al lugar donde se caracterizaron por primera vez se han denominado NOS endotelial (NOSe), localizada preferentemente en células endoteliales, plaquetas y mesangiales renales, y NOS neuronal (NOSn), de localización nerviosa tanto central como periférica. Por otra parte se ha descrito la isoforma NOS inducible (NOSi), que se expresa únicamente cuando las células entran en contacto con endotoxinas o determinadas citocinas. Su acción requiere de un cierto tiempo para manifestarse (4-6 horas tras el estímulo inductor), pero una vez activada produce grandes cantidades de NO. Además, posee la calmodulina

fuertemente unida, a diferencia de las otras dos isoformas, lo cual hace que esta enzima sea insensible a las concentraciones de Ca^{+2} intracelular y la mantiene en un estado tónicamente activado dando lugar a la síntesis continua y prolongada de NO (horas/días). Inicialmente fue descrita en macrófagos y hepatocitos, si bien posteriormente se ha observado en numerosas células (músculo liso vascular, neutrófilos, células endoteliales,…). Sin embargo, hay que decir que la terminología constitutiva frente a inducible es una simplificación ya que se ha descrito que la expresión de las isoformas "constitutivas", tanto NOSe como NOSn, puede ser inducida en determinadas condiciones fisiológicas (Forstermann *et al.*, 1998) y, por el contrario, se ha descrito que la NOSi se expresa de forma constitutiva en determinadas células (Alderton *et al.*, 2001; Guo *et al.*, 1995).

III.2. Mecanismo de acción

La diversidad de funciones que tiene el NO se debe a su capacidad de reaccionar con un rango amplio de moléculas distintas. Este mediador interactúa con "dianas" moleculares específicas localizadas en la propia célula donde es generado (regulación autocrina) o en células vecinas (regulación paracrina).

La principal diana del NO caracterizada hasta la fecha es la enzima guanilato ciclasa soluble (GCs) (Southam & Garthwaite, 1993). El NO reacciona con el hierro del grupo hemo de esta enzima activándola y desencadenando así la liberación de guanosín monofosfato cíclico (GMPc), mediador final responsable de la mayoría de las acciones fisiológicas del NO. El GMPc, a través de la activación de una serie de cinasas, reduce la concentración de Ca^{+2} intracelular por distintos mecanismos; además, activa directamente los canales iónicos de K^+ hiperpolarizando a la célula (Carvajal *et al.*, 2000), (**figura 5**). Todas estas acciones conducen a la relajación de la célula del músculo liso. Por

otra parte, el NO también interacciona con el hierro del grupo hemo presente en la hemoglobina constituyendo este proceso la principal vía metabólica del NO.

Otra enzima con la que interacciona el NO es la citocromo c oxidasa presente en el complejo terminal de la cadena de transporte electrónico mitocondrial (Nisoli *et al.*, 2004). Se ha visto que el NO compite con el oxígeno inhibiendo de manera reversible la cadena respiratoria, por lo que se ha sugerido que pudiera estar actuando como un regulador fisiológico de la respiración celular (Brown, 2000). Apoyando esta teoría, existen evidencias que apuntan hacia la presencia de actividad NOS Ca^{+2}-dependiente en la membrana interna mitocondrial (Ghafourifar & Richter, 1997; Lopez-Figueroa *et al.*, 2000).

Además, debido a su condición de radical libre, el NO reacciona con el anión superóxido (O_2^-) para generar peroxinitritos ($ONOO^-$), sustancias que son altamente reactivas y que poseen un potente efecto oxidante. De esta manera se explica el efecto citotóxico del NO (Beckman & Koppenol, 1996). Esta reacción química se ve favorecida en circunstancias en las que existe una elevada concentración de NO como consecuencia de la inducción de la NOS.

El NO también reacciona con los grupos tiol (SH) de ciertas proteínas dando lugar a los nitrosotioles. Se ha propuesto que ésta podría ser una forma natural de almacén y transporte del NO (Muller *et al.*, 2002). Además, se ha demostrado en células vasculares aisladas que el NO puede activar directamente los canales de K^+ sensibles al Ca^{+2} gracias a este mecanismo (Bolotina *et al.*, 1994), desencadenando la relajación del músculo liso (**figura 5**). Sin embargo, la contribución de esta vía a la relajación vascular inducida por el NO *in vivo* está todavía por determinar.

Por último, también se ha visto que el NO puede combinarse con metales de transición (hierro, cobre o zinc) presentes en determinadas enzimas como la ciclooxigenasa, lipooxigenasa, citocromo P-450 y la aconitasa, modulando su función (Ortega Mateo & Amaya Aleixandre de Artinano, 2000).

III.3. Óxido nítrico en el sistema nervioso

El descubrimiento del NO como neurotransmisor cambió radicalmente el concepto clásico de sinapsis nerviosa. El NO es de naturaleza gaseosa y posee una vida media muy corta (segundos). A diferencia de los neurotransmisores típicos, no se almacena en vesículas sinápticas sino que se sintetiza cuando se necesita y no se libera por exocitosis ya que difunde libremente a través de las membranas. En 1997 se adoptó el término de nervios nitrérgicos (Moncada *et al.*, 1997) aplicados a aquéllos cuya función transmisora depende de la liberación de NO.

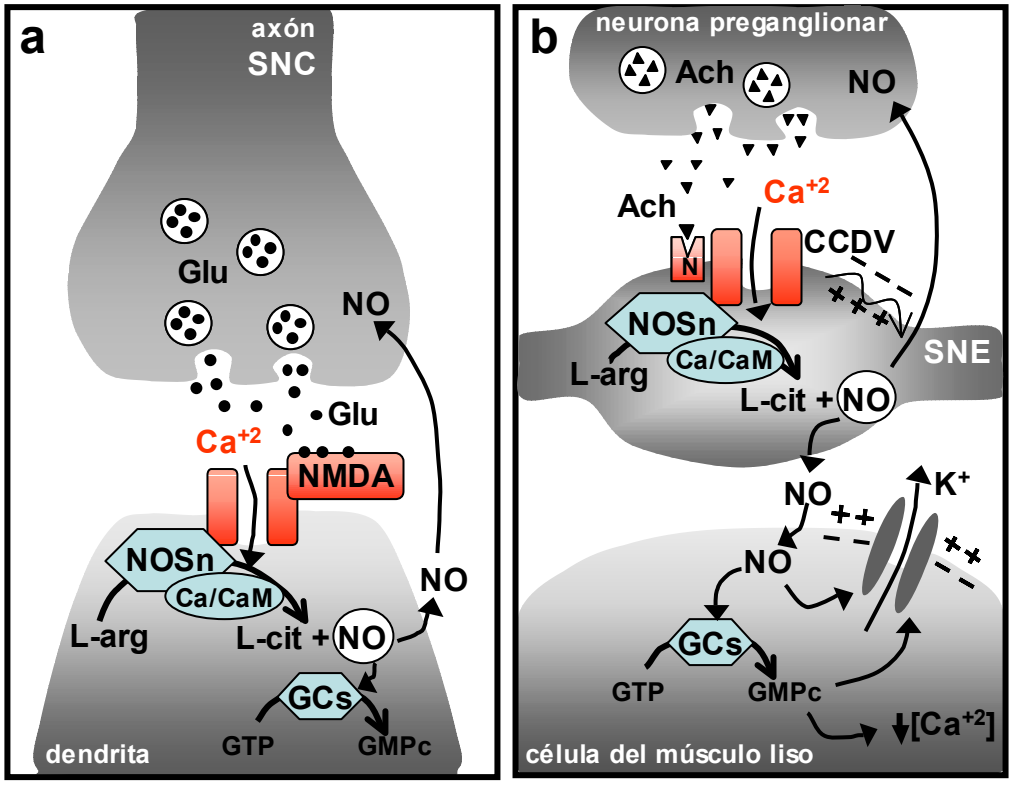

Figura 5. Regulación sináptica de la actividad NOSn. La apertura de los canales de Ca^{+2} dependientes de receptor NMDA (a) o dependientes de voltaje (CCDV) (b) permite la entrada de Ca^{+2} con la consecuente activación enzimática de la NOSn.

La principal isoforma responsable de la síntesis del NO en el sistema nervioso es la NOSn (Esplugues, 2002). Su activación está directamente regulada por la concentración de Ca^{+2} intracelular (**figura 5**). La llegada de un potencial de acción activa los canales de Ca^{+2} dependientes de voltaje y la entrada de este ión activa la enzima NOSn a través de la interacción con la calmodulina. Otra vía de activación de la enzima, muy frecuente en el cerebro, es la activación de los canales de Ca^{+2} dependientes de receptor. Concretamente, la estimulación postsináptica de receptores NMDA por el aminoácido excitador glutamato activa estos canales con la consecuente entrada de Ca^{+2} y activación de la NOSn.

III.3.1. Sistema nervioso central

La NOSn está ampliamente distribuida por todo el SNC. Principalmente se localiza en neuronas, aunque también se ha detectado su presencia en algunas células gliales y vasculares (Wiesinger, 2001). Se ha visto que el NO de origen neuronal interviene como neurotransmisor o neuromodulador en la memoria olfativa (Kendrick *et al.*, 1997), la nocicepción (Luo & Cizkova, 2000) y la visión (Cudeiro & Rivadulla, 1999), así como en procesos de control y desarrollo neuronal y formación de la memoria (Prast & Philippu, 2001). Además, el NO liberado en el SNC, principalmente en el complejo dorsal vagal (CDV), juega un papel importante en la regulación de una larga lista de funciones autónomas entre las que destacan el reflejo barorreceptor (Hironaga *et al.*, 1998), la presión arterial (Togashi *et al.*, 1992), el ritmo cardíaco (Sakuma *et al.*, 1992) o la función gastrointestinal (Barrachina *et al.*, 1995b; Beltran *et al.*, 1999; Esplugues *et al.*, 1996; Krowicki *et al.*, 1997).

Mientras que el NO media la transmisión sináptica en condiciones fisiológicas, niveles elevados de NO pueden ser neurotóxicos. Existen evidencias que demuestran que un exceso de la liberación de glutamato,

actuando sobre receptores NMDA, media la neurotoxicidad que contribuye a la generación de patologías neurodegenerativas como el Alzheimer o la enfermedad de Huntington (Boje, 2004).

III.3.2. Sistema nervioso periférico

En 1963 se demostró por primera vez que la activación de nervios periféricos en presencia de bloqueantes colinérgicos y adrenérgicos tenía un efecto relajante del músculo liso (Burnstock *et al.*, 1963), introduciéndose así el término de neurotransmisión no-adrenérgica no-colinérgica (NANC). Estos experimentos se desarrollaron en el tracto gastrointestinal, sin embargo rápidamente se observó que este fenómeno también se daba en los sistemas urogenital, respiratorio y cardiovascular (Burnstock, 1986). La identificación del neurotransmisor NANC ha sido objeto de discusión durante todos estos años. Inicialmente se habló del ATP o del VIP. Actualmente se acepta que es el NO el principal neurotransmisor NANC, aunque no se descarta la participación de los otros dos mediadores (Sanders & Ward, 1992).

Los efectos del NO como neurotransmisor NANC regulan procesos fisiológicos de especial relevancia. En el sistema respiratorio se cree que la inervación nitrérgica representa la principal vía broncodilatadora en los humanos, y su disfunción puede estar involucrada en el proceso asmático (Belvisi *et al.*, 1995). Además, la inhalación de NO constituye una importante herramienta terapéutica en el tratamiento de algunas enfermedades respiratorias. En el sistema urogenital, la neurotransmisión NANC contribuye a la erección del pene y la impotencia se ha relacionado con la alteración de la transmisión nitrérgica (Andersson, 2003). Además, la relajación NANC contribuye a la disminución de la presión intrauretral que precede a la micción (de Groat & Yoshimura, 2001). Por otra parte se ha visto que en los vasos sanguíneos, además de la importancia del NO derivado de la NOSe en la

regulación del tono vascular, la inervación nitrérgica también modula esta función en determinadas regiones como el mesenterio (Toda & Okamura, 2003). En el tracto gastrointestinal la regulación nitrérgica juega un papel fisiológico fundamental como se comenta a continuación.

III.4. Regulación nitrérgica de la motilidad gastrointestinal

Estudios de inmunohistoquímica revelan la amplia distribución de la enzima NOS en el tracto gastrointestinal. Se ha detectado actividad NOS en células endoteliales, secretoras, musculares y nerviosas (Salzman, 1995). Dicha actividad refleja en su conjunto la participación del NO como mediador endógeno en la regulación de funciones tales como motilidad, secreción, integridad tisular y microcirculación del tracto gastrointestinal.

La NOSn está ampliamente distribuida a lo largo del plexo mientérico (Aimi *et al.*, 1993). La mayoría de las fibras nerviosas nitrérgicas son neuronas intrínsecas cuyas varicosidades están situadas muy próximas a las células del músculo liso que contienen GCs (Ekblad *et al.*, 1994). La regulación nitrérgica NANC está implicada en muchos reflejos nerviosos fisiológicos tales como la relajación adaptativa (Desai *et al.*, 1991b) y receptiva (Desai *et al.*, 1991a) del estómago. También está implicada en la relajación de los esfínteres esofágico (Boeckxstaens GE & Pelckmans PA, 1997) y pilórico (Allescher *et al.*, 1992); a su vez, la ausencia de dicha regulación constituye la base de patologías tales como la acalasia (Mearin *et al.*, 1993) o la estenosis hipertrófica del píloro (Vanderwinden *et al.*, 1992). A nivel intestinal, la inervación NANC participa en el reflejo peristáltico (Foxx-Orenstein & Grider, 1996) y también permite el paso del contenido fecal al intestino grueso al participar en la relajación de la unión íleo-colónica (Boeckxstaens *et al.*, 1990). Por último, la regulación nitrérgica también es la responsable de la relajación del esfínter anal interno, siendo crucial en el proceso de la defecación (Chakder & Rattan, 1993).

El papel fisiológico de la inervación nitrérgica en la regulación de la motilidad gastrointestinal ha sido ampliamente estudiado. Sin embargo, poco se sabe acerca del papel de dicha inervación en las alteraciones motoras asociadas a circunstancias fisiopatológicas tales como la endotoxemia.

OBJETIVO

El objetivo planteado en el presente trabajo es caracterizar los mecanismos involucrados en la alteración de la función motora gastrointestinal asociada a los estadios tempranos de la endotoxemia.

ARTÍCULOS DE INVESTIGACIÓN

I. EFECTOS DE LA ENDOTOXINA SOBRE LA MOTILIDAD GÁSTRICA

Vía nerviosa y mediadores implicados

El primer objetivo que nos planteamos fue caracterizar la vía nerviosa implicada en la modulación rápida de la motilidad gástrica inducida por la endotoxina. Para ello realizamos todo un bloque experimental basado en la determinación de la presión intragástrica (P_{IG}) tras la administración de dosis bajas de la endotoxina de *Escherichia coli* en la rata anestesiada. En paralelo se analizó el papel neuromodulador del NO implicado en este proceso.

Las publicaciones derivadas de este bloque son:

"*A cerebral nitrergic pathway modulates endotoxin-induced changes in gastric motility*". British Journal of Pharmacology (2001) 134: 325-332.

"*Transcriptional up-regulation of nNOS in the dorsal vagal complex during low endotoxemia*". Neurogastroenterology and Motility (2004, en revisión).

En ellas demostramos que la inhibición de la P_{IG}, inducida por dosis bajas de endotoxina, está mediada por la activación de fibras nerviosas aferentes sensibles a la capsaicina y de centros nerviosos cerebrales situados en el CDV. Además, observamos que se incrementa la síntesis de NO derivada de la isoforma constitutiva NOSn, y no de la NOSi, en el tronco del encéfalo, y dicha síntesis media los efectos de la endotoxina. El análisis de la expresión génica de la enzima NOSn revela que la endotoxina incrementa el ARNm de esta enzima a las 2h, mientras carece de efecto a tiempos más cortos. Dicho incremento se localizó en el CDV, específicamente a nivel rostral.

Una vez confirmada la puesta en marcha de un reflejo nervioso que implica la participación del SNC durante la endotoxemia quisimos analizar la parte final de este reflejo. El segundo objetivo fue caracterizar los mediadores periféricos y

mensajeros intracelulares involucrados en los efectos de la endotoxina. Para ello realizamos un bloque experimental basado en la determinación de la contractilidad del fundus gástrico aislado de ratas pretratadas con la endotoxina mediante la técnica de baño de órganos.

Estos experimentos han dado lugar a la publicación:

"*Synthesis of nitric oxide in post-ganglionic myenteric neurons during endotoxemia: implications for gastric motor function*". The FASEB Journal express (2004) 18: 531-533. DOI: 10.1096/fj.03-0596fje.

En ella demostramos que la rápida inhibición de la contractilidad del fundus inducida por la endotoxina está mediada por la activación de neuronas postganglionares del plexo mientérico y por la posterior liberación de NO. Gracias a la microscopía confocal hemos podido observar por primera vez que la síntesis aguda de NO inducida por la endotoxina se da específicamente en neuronas postganglionares del plexo mientérico. Por último, demostramos que la relajación del fundus presente en estas circunstancias se debe a una acción del NO activando la vía GCs/canales de K^+ dependientes de Ca^{+2} de baja conductancia.

Modulación del vaciamiento gástrico

Tras caracterizar el mecanismo por el cual la endotoxina modula de forma aguda la motilidad gástrica quisimos analizar si este proceso tiene relevancia en la modulación de un proceso fisiológico como es el vaciamiento gástrico de sólidos nutrientes. Para ello analizamos dicha función tras la administración de la endotoxina en la rata consciente y mediante las herramientas farmacológicas adecuadas y con la ayuda de técnicas bioquímicas y de biología molecular hemos analizado la vía nerviosa y mediadores que participan en dicha respuesta.

Este bloque experimental ha dado lugar a las siguientes publicaciones:

"*Endotoxin inhibits gastric emptying in rats via a capsaicin- sensitive afferent pathway*". Naunyn-Schmiedeberg's Archives of Pharmacology (2001) 363: 276-280.

"*Downregulation of nNOS and synthesis of PGs associated with endotoxin-induced delay in gastric emptying*". American Journal of Physiology (2002) 283: G1360-G1367.

En ellas demostramos que la administración de endotoxina inhibe el vaciamiento gástrico de sólidos a través de la activación de fibras sensoriales sensibles a la capsaicina. Estas fibras, además de tener un efecto local sobre la motilidad gástrica, señalizan al SNC vía vagal y como consecuencia se activa una vía nerviosa eferente NANC. Una disminución de la expresión de la isoforma NOSn en la región antro-pilórica y un incremento de la síntesis de prostaglandinas derivada de la enzima COX-2 están mediando la inhibición del vaciamiento gástrico inducido por la endotoxina.

Artículo 1

"*A cerebral nitrergic pathway modulates endotoxin-induced changes in gastric motility*"

Elsa Quintana, Eugenia García-Zaragozá, M. Ángeles Martínez-Cuesta, Sara Calatayud, Juan V. Esplugues & María D. Barrachina

British Journal of Pharmacology (2001) 134: 325-332

British Journal of Pharmacology (2001) 134, 325–332

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A cerebral nitrergic pathway modulates endotoxin-induced changes in gastric motility

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1 This study analyses the neural pathway involved in the modulation of gastric motor function by stress.

2 Systemic administration of low doses of endotoxin (40 μg kg^{-1}, i.v.) prevents the increase in gastric tone induced by 2-deoxy-D-glucose (200 mg kg^{-1}, i.v., 2-DG) in urethane-anaesthetized rats.

3 Functional inhibition of afferent neurones by systemic administration of capsaicin (20 + 30 + 50 mg kg^{-1}, i.m.) in adult rats prevented the inhibitory effects of endotoxin.

4 Pre-treatment with the nitric oxide synthase (NOS) inhibitor, N^{G}-nitro-L-arginine methyl ester (L-NAME), both i.v. (10 mg kg^{-1}) and i.c. (200 μg rat^{-1}), prevented the inhibitory effects of endotoxin on gastric tone induced by 2-DG.

5 Immunohistochemical studies show Fos expression in the dorsal vagal complex (DVC) of the brainstem of 2-DG-treated animals. Peripheral administration of endotoxin (40 μg kg^{-1}, i.p.) increased the number of Fos-immunoreactive cells induced by 2-DG, both in the nucleus tractus solitarii (NTS) and in the dorsal motor nucleus (DMN) of the DVC. Pre-treatment with L-NAME prevented the increase in Fos expression induced by endotoxin in both nuclei.

6 Endotoxin (40 μg kg^{-1}, i.p.) increased Ca^{2+}-dependent nitric oxide synthase (cNOS) activity in the brainstem. Addition of 7-nitroindazole (600 μM, 7-NI) to the assay significantly inhibited the increase in cNOS activity caused by endotoxin. No change in NOS activity of any isoform was observed in the stomach of animals treated with endotoxin.

7 The present study suggests that inhibition of gastric motor function by low doses of endotoxin involves activation of capsaicin-sensitive afferent neurones and neuronal NOS in the brainstem.

British Journal of Pharmacology (2001) **134,** 325–332

Keywords: Nitric oxide; gastric motor function; brain-gut; capsaicin; NTS; DMN; DVC; central nervous system; endotoxin

Abbreviations: ABC, avidin biotin complex; cNOS, Ca^{2+}-dependent nitric oxide synthase; 2-DG, 2-deoxy-D-glucose; D-arg, D-arginine; DVC, dorsal vagal complex; DMN, dorsal motor nucleus; ecNOS endothelial constitutive nitric oxide synthase; L-arg, L-arginine; L-NAME, N^{G}-nitro-L-arginine methyl ester; L-NNA, N^{G}-nitro-L-arginine; LPS, lipopolisaccharide; NANC, non-adrenergic non-cholinergic; ncNOS neuronal constitutive nitric oxide synthase; 7-NI, 7-nitroindazole; NTS, nucleus tractus solitarius

Introduction

Changes in gastric function are associated with stress. Acute administration of endotoxin, in doses that do not modify systemic arterial blood pressure or rectal temperature, selectively decreases gastric acid secretion and increases mucosal resistance to damage through a mechanism that involves the central nervous system (Barrachina *et al*., 1995a, b; Esplugues *et al*., 1996). Similar doses of endotoxin have recently been reported to delay gastric emptying of a solid nutrient meal through activation of afferent fibres (Calatayud *et al*., 2001), suggesting that the superior neuronal network is also involved in the gastric motor inhibitory mechanism triggered by endotoxin.

The DVC of the brainstem is comprised of two interacting nuclei: the NTS, which receives primary afferent fibres and the DMN, where preganglionic motor neurones innervating the gastrointestinal tract are located (Kalia & Mesulam, 1980). Functional active pathways in the brain have been characterized using Fos immunohistochemistry as a neural tracing technique. Systemic administration of endotoxin has been shown to activate the early-appearing gene product c-*fos* in visceral afferents, neuroendocrine and autonomic regions of the brain (Hermann *et al*., 2001; Lin *et al*., 1998b). However, relatively high doses of endotoxin have been used and no relationship between brainstem neuronal activation and modulation of gastric motor function by endotoxin has been reported.

Nitric oxide (NO) acts as an intercellular messenger in the central nervous system (Garthwaite *et al*., 1988). Three different NOS have been characterized: two Ca^{2+}-dependent NOS, the neuronal (ncNOS) and endothelial (ecNOS) isoform, and a third Ca^{2+}-independent isoform (Barrachina *et al*., 2001; Moncada *et al*., 1991). Immunohistochemical studies have reported the presence of NOS in the DVC of the brainstem. In particular, vagal afferents and intrinsic neurones of the NTS as well as neurones of the DMN express NOS immunoreactivity (Lin *et al*., 1998a; Zheng *et al*., 1999). In addition, a role for NO in central brainstem

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circuits which control gastric secretory and motor functions has been reported (Esplugues *et al*., 1996; Krowicki *et al*., 1997). Thus, synthesis of NO is involved in the inhibition of gastric acid secretion by stress (Beltrán *et al*., 1999; Esplugues *et al*., 1996) or modulates the inhibition of gastric motor function by substance P (Krowicki & Hornby, 1996).

In the present study, in order to characterize the neural pathway and endogenous mediators involved in the changes in gastric motor function associated with stress, we evaluated the effects of low doses of endotoxin on: (1) intragastric pressure induced by the antimetabolic glucose analogue, 2-DG; (2) the pattern of brainstem neuronal activation and (3) the NOS activity both in the brainstem and the stomach.

Methods

Male Sprague-Dawley rats (220–250 g) were fasted for 16–20 h before the experiments but were allowed access to drinking water.

Determination of intragastric pressure

Rats were anaesthetized with urethane (1.5 g kg^{-1}, i.p.), the trachea intubated and a jugular vein cannulated. After performing a laparotomy, an intraluminal latex balloon was inserted in the stomach through an incision in the forestomach and held in place with a ligature. The balloon and catheter system was connected to a pressure transducer and intragastric pressure was registered on line by a multi-channel recorder (Power Lab.). After reaching an intragastric pressure of between 4–5 cm H_2O, by filling the balloon with 2–3 ml H_2O at 37°C, rats were allowed to stabilize for 1 h. Rectal temperature was monitored during the experiment and maintained at 36–37°C. Unless mentioned otherwise, data is expressed as Δ intragastric pressure, calculated as the difference between stimulated (the average measurement 30 to 60 min after 2-DG administration) and basal (the average in the last 10 min before 2-DG administration) intragastric pressure.

Experimental protocol

In a first group of experiments, endotoxin (40 μg kg^{-1}, i.v.) was administered 30 min prior to a bolus injection of 2-DG (200 mg kg^{-1}, i.v.) and intragastric pressure was monitored for 60 min. In order to analyse the role of sensory fibres, rats were administered with capsaicin (a selective neurotoxin on C-fibres; 20 + 30 + 50 mg kg^{-1}, i.m.) or its vehicle (10% ethanol + 10% Tween-80 + 80% saline, 1 ml kg^{-1}, i.m.) for 3 consecutive days, 15 days prior to the experiments. The role of NO in the effects of endotoxin was analysed by pretreatment (15 min) with the NO synthesis inhibitor, L-NAME (10 mg kg^{-1}, i.v. or 200 μg rat^{-1}, i.c.) or its vehicle (1 ml kg^{-1} or 10 μl rat^{-1}, respectively).

In order to analyse further the direct role of NO synthesis in the brain on intragastric pressure, in a second group of experiments some animals received an i.c. injection of L-arginine (200 μg rat^{-1}, L-arg), D-arginine (200 μg rat^{-1}, D-arg) or saline (10 μl rat^{-1}) and gastric contractility was monitored for 60 min.

Fos immunohistochemistry

Procedures were carried out as previously described (Barrachina *et al*., 1997). Ten minutes prior to the administration of 2-DG (200 mg kg^{-1}, i.p.), rats received two consecutive i.p. injections of either L-NAME (10 mg kg^{-1}) + endotoxin (40 μg kg^{-1}), L-NAME + saline (1 ml kg^{-1}), vehicle (1 ml kg^{-1}) + endotoxin or vehicle + saline. Additional groups of rats received a single i.p. injection of saline, endotoxin or L-NAME and were included as control groups. Two hours later, animals were anaesthetized with pentobarbitone (280 mg kg^{-1}, i.p.) and transcardially perfused with 0.9% saline followed by paraformaldehyde solution (4%). Brains were postfixed in the same fixative and cryoprotected overnight by immersion in 30% sucrose. Immunohistochemistry for Fos expression was processed on frozen brain coronal sections (50 μm thick) using the avidin–biotin complex (ABC) method (Hsu *et al*., 1981). Sections were incubated for 24 h at 4°C with the primary antibody c-*fos* (sheep policlonal, Genosys) diluted at 1 : 2000. Then, sections were incubated with biotynylated anti-sheep IgG (Vector Labs.), diluted at 1 : 200, followed by ABC (Vectastain ABC Kit, Vector Labs.), in both cases for 1 h at 25°C. Sections were incubated for 8 min in a substrate for peroxidase kit (Vector VIP, Vector Labs.) and were mounted, air-dried, dehydrated, cleared and coverslipped. The sections were observed and photographed using a brightfield microscope (Zeiss). The counting of Fos-immunoreactive cells was performed bilaterally in six sections per animal, regardless of the intensity of staining, for the NTS and DMN. The L.W. Swanson rat brain atlas was used to determine the anatomical locations of the nuclei.

Determination of NOS activity

Rats were administered with endotoxin (40 μg kg^{-1}, i.p.) or saline (1 ml kg^{-1}, i.p.) and sacrified by cervical dislocation 30 min later. Both, a section of the brainstem containing the DVC (+1 mm to −1.4 mm to the obex) and the stomach, were quickly introduced in liquid nitrogen and stored at −80°C. NO synthase activity was measured as the rate of conversion of L-[U-^{14}C]-arginine to L-[U-^{14}C]-citrulline (Salter *et al*., 1990). Briefly, the samples were homogenized (Ultra-Turrax) in an ice-cold buffer (330 mg ml^{-1}; pH 7.2) containing 320 mM sucrose, 20 mM HEPES, 1 mM EDTA, 1 mM DL-dithiothreitol, 10 μg ml^{-1} leupeptin, 10 μg ml^{-1} soybean trypsin inhibitor and 2 μg ml^{-1} aprotinin followed by centrifugation at 10,000 × *g* for 20 min at 4°C. Afterwards centrifugation, 40 μl of supernatant was incubated at 37°C for 20 min in assay buffer (pH 7.4) containing (mM) KH_2PO_4 50, $MgCl_2$ 1, $CaCl_2$ 0.2, L-valine 50, L-citrulline 1, L-arginine 0.02, DL-dithiothreitol 1, and 100 μM NADPH, 3 μM FAD, 3 μM FMN, 3 μM BH_4 and 950 nM L-[U-^{14}C]-arginine (348 mCi $mmol^{-1}$). The specificity of L-arginine conversion by NOS to L-citrulline was further confirmed using the NO synthesis inhibitors, N^G-nitro-L-arginine (L-NNA, 1 mM). Additionally, 1 mM EGTA, a calcium chelating agent was used to differentiate between Ca^{2+}-dependent and Ca^{2+}-independent isoform of NOS. The specific inhibitor of neuronal constitutive NOS (ncNOS), 7-nitroindazole (600 μM, 7-NI; a dose chosen from a previous dose-response curve; Babbedge *et al*., 1993) was used to differentiate

between ncNOS and endothelial constitutive NOS (ecNOS) isoform activity. All activities are expressed as picomol of product generated per minute per gram of tissue.

Drugs

Urethane, 2-deoxy-D-glucose, *Escherichia coli* endotoxin (serotype 026:B6), L-NAME, L-arginine, D-arginine, Tween 80 and all reagents used for determination of NOS activity were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). L-[^{14}C]-arginine was obtained from Amersham Life Science and Capsaicin was purchased from Fluka Chemic AG. Sodium pentobarbitone was used as a commercially available preparation (Abbott, Madrid). Unless mentioned otherwise all drugs have been dissolved in saline.

Statistical analysis

Data are expressed as mean ± s.e.mean. Comparisons between groups were performed by one-way analysis of variance followed by a Newman-Keuls test. Data are considered statistically significant when $P<0.05$.

Results

Effects of endotoxin on intragastric pressure

Urethane-anaesthetized rats fixed with a gastric balloon filled with 2.8 ± 0.2 ml of water exhibited an intragastric pressure of 5.4 ± 0.5 cm H_2O ($n=10$). A single i.v. injection of endotoxin (40 μg kg^{-1}) did not significantly modify intragastric pressure (5.3 ± 0.5 cm H_2O, $n=10$) in the 30 min following injection (Figure 1).

Administration of 2-DG (200 mg kg^{-1} i.v., $n=10$) induced a peak increase in gastric tone at 5 min (9.4 ± 0.9 cm H_2O) that was maintained above basal level for more than 60 min (6.7 ± 0.6 cm H_2O). Pre-treatment with endotoxin (40 μg kg^{-1}, i.v., $n=10$) significantly ($P<0.05$) impeded both the contraction peak (6.2 ± 0.7 cm H_2O) and the increase in gastric tone (5.1 ± 0.4 cm H_2O) induced by 2-DG (Figure 1).

As shown in Figure 2, functional inhibition of capsaicin-sensitive afferent neurones significantly prevented the inhibitory effects of endotoxin on 2-DG-increased intragastric pressure while it did not significantly alter gastric contractility in animals treated with 2-DG alone.

Basal gastric tone (5.4 ± 0.5 cm H_2O, $n=14$) was not significantly changed by inhibition of NO synthesis through systemic administration of L-NAME (4.9 ± 0.3 cm H_2O, $n=14$, measured 15 min after administration of L-NAME). Pre-treatment with i.v. L-NAME did not significantly modify the increase in intragastric pressure induced by 2-DG (Figure 3a). However, systemic inhibition of NO synthesis completely reversed the reduction by endotoxin of intragastric pressure

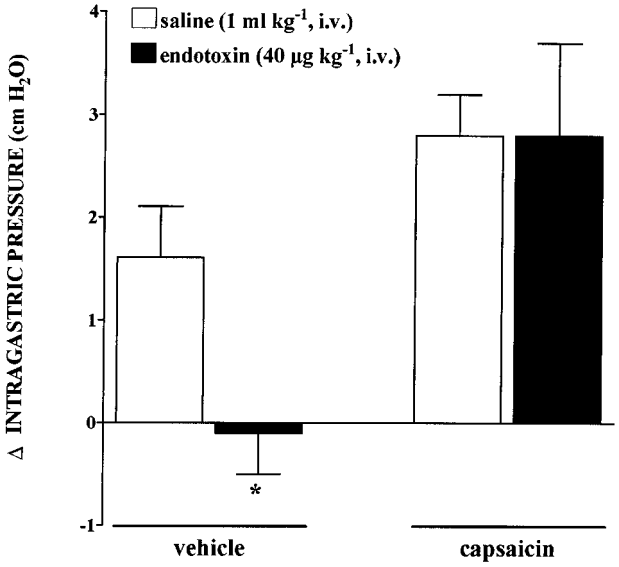

Figure 2 Effects of endotoxin or saline on intragastric pressure induced by 2-DG (200 mg kg^{-1}, i.v.) after pre-treatment (15 days) with capsaicin (125 mg kg^{-1}, i.m.) or its vehicle (ethanol, Tween 80 and saline, 10 : 10 : 80, 1 ml kg^{-1}, i.m.). Each bar represents mean ± s.e.mean of at least four animals. Significant difference from all other groups is shown by $*P<0.05$.

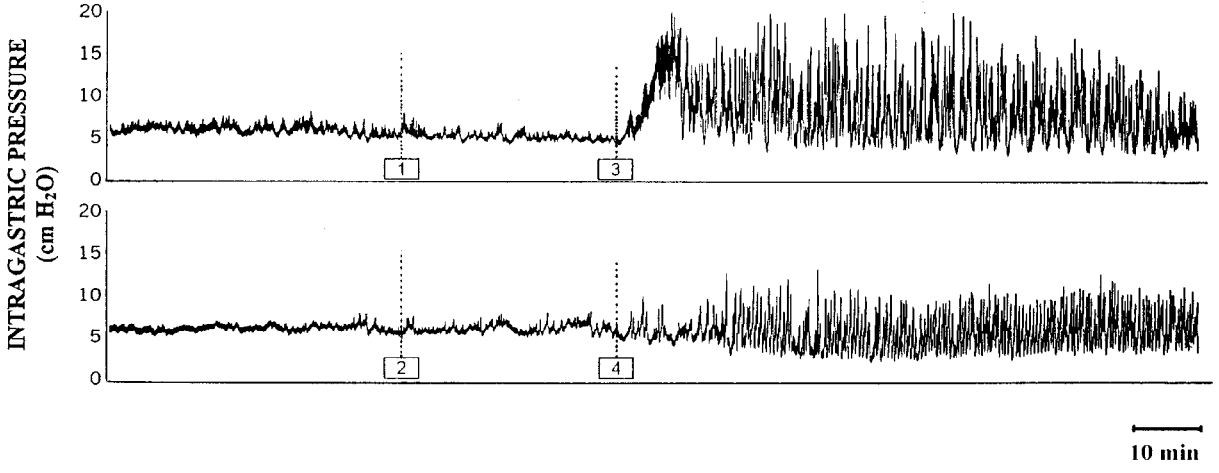

Figure 1 Original recording showing the effects of i.v. administration of vehicle (1 ml kg^{-1}, **1**) or endotoxin (40 μg kg^{-1}, **2**) on intragastric pressure induced by 2-Deoxy-D-Glucose (200 mg kg^{-1}, i.v.; **3, 4**).

induced by 2-DG (Figure 3a). Similar results were obtained when lower doses of L-NAME (200 μg rat^{-1}) were administered i.c. Central inhibition of NO synthesis did not significantly change basal or 2-DG stimulated intragastric pressure while it did prevent the inhibitory effects of endotoxin (Figure 3b).

Gastric tone in basal conditions (5.6$\pm$0.6 cm H_2O, $n=5$) was significantly ($P<0.05$) diminished by a single i.c. injection of L-arg (2.4$\pm$0.8 cm H_2O, $n=5$; 200 μg rat^{-1}). Neither an i.c. injection of vehicle (4.6$\pm$0.6 cm H_2O, $n=3$; 10 μl rat^{-1}), nor administration of D-arg (4.0$\pm$1.1 cm H_2O, $n=3$; 200 μg rat^{-1}) significantly modified basal intragastric pressure.

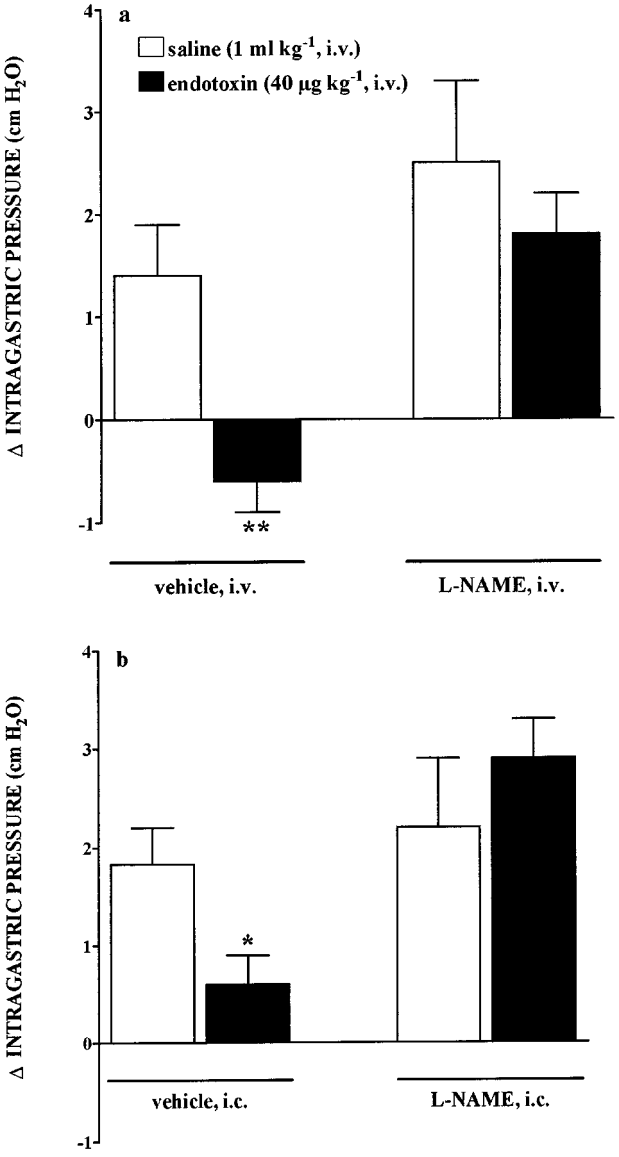

Figure 3 Effects of endotoxin or saline on intragastric pressure induced by 2-DG (200 mg kg^{-1}, i.v.) in animals pretreated: (a) with i.v. administration of L-NAME (10 mg kg^{-1}) or vehicle (1 ml kg^{-1}); (b) with i.c administration of L-NAME (200 μg rat^{-1}) or vehicle (10 μl rat^{-1}). Each bar represents mean$\pm$s.e.mean of at least six animals. Significant difference from all respective groups included in the same graph is shown by $^{**}P<0.01$ or $^{*}P<0.05$.

Effects of endotoxin on Fos expression

Fos expression was not observed either in the NTS or in the DMN of fasted rats injected i.p. with saline ($n=3$). Administration of 2-DG (200 mg kg^{-1}, i.p.) induced a pronounced staining for Fos immunoreactivity in the NTS and a slight Fos expression in the DMN (Figure 4a,d). Pre-treatment with endotoxin (40 μg kg^{-1}, i.p.) significantly increased the number of Fos-positive cells both in the NTS and the DMN of 2-DG-treated animals (Figure 4b,d). If administered alone, endotoxin induced a moderate increase in the number of Fos-immunoreactive cells in the NTS (9.3$\pm$0.1 nb/section, $n=3$) while having no effect on Fos expression in the DMN.

Pre-treatment with L-NAME (10 mg kg^{-1}, i.p.) significantly increased the number of Fos-positive neurones in both the NTS and the DMN of 2-DG-treated rats. Endotoxin did not augment the number of Fos-immunoreactive cells in animals treated with L-NAME + 2-DG (Figure 4d). L-NAME administered alone ($n=3$) increased Fos expression in the NTS (54.2$\pm$10.2 nb/section) and the DMN (1.8$\pm$1.2 nb/section), while vehicle-treated animals showed no Fos staining in both nuclei.

Effects of endotoxin on NOS activity

Brainstem NOS activity Ca^{2+}-independent NOS activity in the brainstem of vehicle-treated animals was almost non-apparent (4.5$\pm$0.9 pmol citrulline min^{-1} g tissue^{-1}), compared with cNOS activity (Figure 5a). Pre-treatment (30 min) with endotoxin (40 μg kg^{-1}, i.p., $n=4$) had no effect on Ca^{2+}-independent NOS activity (3.7$\pm$0.7 pmol citrulline min^{-1}g tissue^{-1}) while significantly ($P<0.05$) increased the activity of cNOS (Figure 5a).

cNOS activity in the brainstem of vehicle- or endotoxin-treated animals seems to be principally due to the ncNOS isoform, since addition of 7-NI (600 μM) to the assay significantly inhibited (77.8$\pm$1.6 or 82.9$\pm$2.8%, respectively) cNOS activity (Figure 5a).

Stomach NOS activity cNOS activity in the stomach of vehicle-treated animals was one-tenth than that exhibited in the brainstem of the same animals (Figure 5a,b). In a similar manner to that observed in the brainstem, Ca^{2+}-independent NOS exhibited low levels of activity (28.2$\pm$4.6 pmol citrulline min^{-1} g tissue^{-1}) in the stomach. Pre-treatment with endotoxin (30 min) did not significantly modify the Ca^{2+}-independent NOS activity (23.5$\pm$2.6 pmol citrulline min^{-1} g tissue^{-1}) or cNOS in the stomach (Figure 5b).

cNOS activity in the stomach of vehicle- or endotoxin-treated animals seems to be attributted to the ncNOS isoform, since addition of 7-NI (600 μM) to the assay significantly inhibited (66.6$\pm$6.5 or 74.0$\pm$2.1%, respectively) the cNOS activity (Figure 5b).

Discussion

Intravenous administration of 2-DG induced an increase in intragastric pressure that was rapid in onset, maintained itself for more than 60 min and was independent of both capsaicin-sensitive afferent neurones and NO synthesis in the brain-

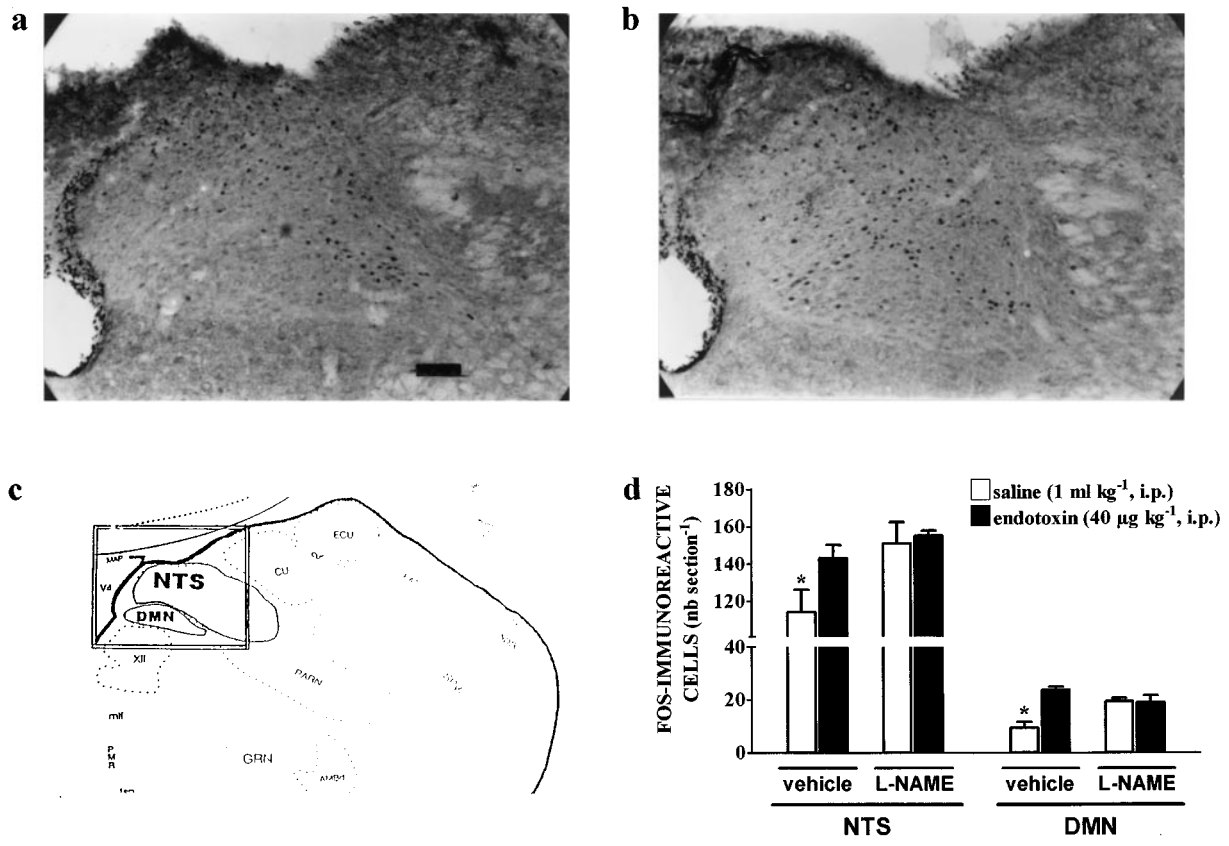

Figure 4 Fos immunoreactivity in the DVC of the brainstem after administration of endotoxin or saline in 2-DG-treated animals (200 mg kg^{-1}, i.p.). Representative microphotographs show Fos immunoreactivity in the NTS and DMN of the DVC, 2 h after injection of (a) vehicle + 2-DG, (b) endotoxin + 2-DG; Scale bar = 100 μm. (c) Coronal drawing section from brainstem adapted from Swanson rat brain atlas at the level of −13.6 mm from bregma, showing the localization of the NTS and DMN. (d) Graph showing the effects of endotoxin or saline on the number of Fos immunoreactive cells (nb/section, bilateral) induced by 2-DG in vehicle- (1 ml kg^{-1}, i.p.) or L-NAME- (10 mg kg^{-1}, i.p.) treated animals. Each bar represents mean ± s.e.mean of at least three animals. Significant difference from the rest of groups in the same nucleus is shown by $*P<0.05$.

stem. Pretreatment with doses of *E. coli* lipopolisaccharide (LPS) which do not modify arterial blood pressure or rectal temperature (Barrachina *et al*., 1995b; García-Zaragozá *et al*., 2000), both of which are associated with severe septicaemia, significantly prevented the increase in intragastric pressure induced by 2-DG. We have recently demonstrated that a similar dose of endotoxin significantly delays the 4 h rate of gastric emptying of a solid nutrient meal in conscious animals (Calatayud *et al*., 2001). The present results thus suggest that the rapid diminution of gastric tone induced by endotoxin is involved in the mechanism by which peripheral endotoxin delays gastric emptying. In accordance with this observation, in the present study, activation of capsaicin sensitive afferent neurones is shown to mediate the diminution of intragastric pressure by endotoxin, in a similar manner to that previously reported for the delay in gastric emptying by LPS (Calatayud *et al*., 2001). These sensory fibres are involved in the modulation of gastric acid secretion (Martínez-Cuesta *et al*., 1994) and resistance of the gastric mucosa to damage (Barrachina *et al*., 1995b) by peripheral endotoxin. However, the precise role of this activation is still unknown. Capsaicin-sensitive afferent neurones in the gastrointestinal tract appear to act through two mechanisms. One is related to the local effector function of these fibres, whereby several neuropeptides are released (Holzer, 1988); in this way, we have recently shown a role for endogenous release of CGRP in endotoxin-induced delay in gastric emptying (Calatayud *et al*., 2001). The second mechanism is related to sending information to the superior neuronal network in the brain and spinal cord, which initiates the efferent reflex loop (Holzer, 1998). In the present study, pretreatment with endotoxin, at doses that prevented the increase in intragastric pressure induced by 2-DG, significantly increased the number of Fos-immunoreactive neurones in the DVC of the brainstem of 2-DG-treated animals. This complex is comprised of the NTS and the DMN, and both nuclei exhibited Fos immunoreactivity after systemic administration of 2-DG. The increase in Fos expression observed reflects an interaction between 2-DG and endotoxin, since LPS injected alone had no influence on the number of Fos-positive neurones in the DVC of the brainstem. These observations may be significant in attempting to understand the diminution in intragastric pressure induced by peripheral endotoxin. The higher activity observed in neurones of the NTS in presence of endotoxin suggest that vagal primary afferents are excited by peripheral endotoxin, which would be in accordance with the functional part of the study. However, according to previous studies, activation of the NTS implies inhibition of the DMN, since many of the NTS neurones that project into the DMN are inhibitory (Zhang *et al*., 1998). In

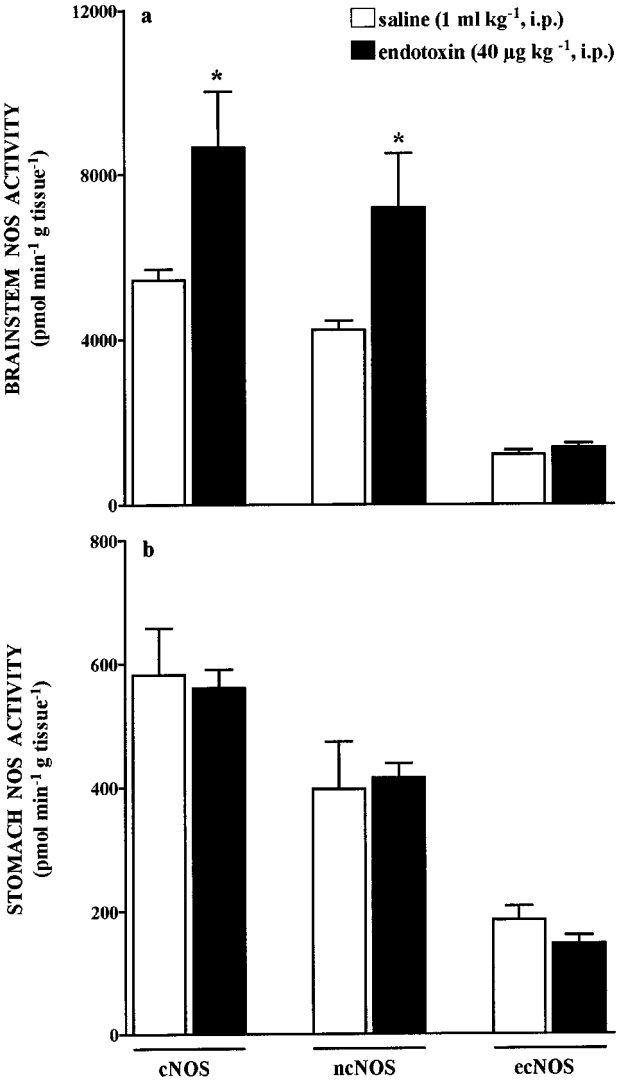

Figure 5 Ca^{2+}-dependent NOS (cNOS) activity in: (a) a section of the brainstem (+1 to −1.4 mm to the obex) and (b) the stomach of endotoxin or saline treated rats. Addition of 7-nitroindazole (600 μM) distinguishes the cNOS between neuronal NOS (ncNOS) and endothelial NOS (ecNOS) activity. Each bar represents mean ± s.e.mean of four animals. Significant difference from the respective saline treated group is shown by $^*P<0.05$.

this case we might expect that the DMN neurones would respond to peripheral endotoxin with a decrease in activity that would, thereby, mediate a decrease in gastric contractility. In contrast, the present results show an increase in Fos immunoreactivity of DMN neurones in response to peripheral endotoxin. Excitation of DMN neurones by different stimuli that inhibit gastric motor function has already been reported (Blackshaw *et al*., 1987; Miolan & Roman, 1984). Characterizing the nature of the vagal efferent fibres involved in the gastric effects of endotoxin would help us to better address this question.

It has been proposed that NO acts as an intercellular messenger in central brainstem circuits controlling gastric function (Esplugues *et al*., 1996; Krowicki *et al*., 1997). Pretreatment with L-NAME prevented the increase in Fos expression in the DVC induced by peripheral endotoxin, suggesting that Fos-immunoreactive cells are dependent on NO. However, results are not conclusive and the understanding of these observations is complicated since L-NAME alone significantly increased Fos expression in the DVC. Two basic assumptions can be made, each resulting in a different interpretation of our findings. One is that endotoxin and blockade of NO synthesis both activate the same population of neurones. If this were the case, results suggest that neurones activated by endotoxin are sensitive to the tonic inhibitor role of NO. The second possibility is that endotoxin and L-NAME activate a separate population of neurones. In this case, the inability of endotoxin to increase Fos expression in L-NAME-treated animals would suggest that NO, acting as a neurotransmitter or as second messenger, is responsible for the activation of these neurones. Previous functional studies support the second hypothesis (Barrachina *et al*., 1995b; Esplugues *et al*., 1996), although double immunostaining would be necessary to provide more conclusive results.

The present study demonstrates a role for cerebral NO synthesis in the diminution of intragastric pressure by peripheral endotoxin, since pre-treatment with i.c. L-NAME completely abolished the inhibitory effects of endotoxin. A role for NO in the brainstem in the modulation of gastric function has already been reported with exogenous administration of an NO-donor (Beltrán *et al*., 1999). This study extends previous observations and show a rapid decrease in intragastric pressure after i.c. administration of L-arg, an effect that seems specifically due to NO synthesis, since i.c. injection of the inactive enantiomer D-arg lacked a significant effect. Taken together results underline an important role for NO synthesis in the brainstem in the modulation of gastric function. In addition, the analysis of NOS activity in the brainstem shows that peripheral endotoxin significantly increases the activity of the enzyme, 30 min after administration. The NOS activity modulated by endotoxin was Ca^{2+}-dependent, which is in accordance with the present and previous studies (Barrachina *et al*., 1995a, b; Esplugues *et al*., 1996) showing that the effects of low doses of endotoxin on gastric function become apparent in less than 30 min. Furthermore, the use of an specific ncNOS inhibitor, 7-nitroindazole (Babbedge *et al*., 1993) significantly inhibited NOS activity in the brainstem, suggesting that the ncNOS isoform, rather than ecNOS, is responsible for the synthesis of NO induced by endotoxin. Taking into account that the ncNOS activity in the brainstem of control animals is responsible for most of the NOS activity, it would be reasonable to assume that this isoform is more sensitive to change. LPS has also been shown to stimulate the production of Ca^{2+}-independent NOS in the brain (Wong *et al*., 1996) but this change normally takes place several hours after the induction of endotoxaemia and, therefore, probably has little functional importance for the inhibition of gastric motor function shown in the present study.

The precise mechanism by which peripheral endotoxin increases the activity of ncNOS has not been evaluated in this study. Considering that the presence of NOS in vagal afferents which project into the NTS has been reported (Lin *et al*., 1998a), one possibility is the direct activation of ncNOS by stimulation of the afferent neurones. However, based mainly on the literature, we hypothesise a role for an intermediate neurotransmitter: (a) NO synthesis in the brain is predominantly regulated by Ca^{2+} influx through receptor operated channels, in particular following postsynaptic

stimulation by the excitatory neurotransmitter glutamate (Bredt & Snyder, 1989; Garthwaite *et al*., 1989). (b) Recent studies report that glutamate is the primary afferent neurotransmitter released in the NTS in the control of gastric function (Zheng *et al*., 1999). (c) The release of glutamate in the brainstem by peripheral endotoxin has been reported (Mascarucci *et al*., 1998) and we have recently shown that endogenous glutamate activates NO synthesis in the brain, which is in turn responsible for the inhibition of gastric acid secretion by peripheral endotoxin (García-Zaragozá *et al*., 2000). These observations, considered with a previous study reporting an inhibitory role on gastric motor function for glutamate acting in the NTS (Raybould *et al*., 1989) lead us to believe that endogenous release of this neurotransmitter is involved in the activation of ncNOS in the brainstem by peripheral endotoxin.

Irrespective of the afferent pathways involved, once synthesized in the brainstem, NO is involved in the diminution of intragastric pressure by endotoxin. NO in the DVC has been shown to modulate the firing rate of DMN neurons (Travagli & Gillis, 1994). Such a modulation implies activation of efferent vagal preganglionic fibres that would synapse with NANC inhibitory postganglionic neurones in the myenteric plexus. NO has also been consolidated as one of the non-adrenergic non-cholinergic (NANC) inhibitory neurotransmitters of the gastrointestinal tract. In the present study, systemic administration of L-NAME significantly prevented the reduction in intragastric pressure induced by endotoxin, suggesting a peripheral role for NO. However, taking into account that i.v. administration of L-NAME induces Fos expression in the DVC of the brainstem, and that similar doses of systemic L-NAME have been shown to affect the spontaneous discharge rate of NTS neurones (Ma *et al*., 1995), we cannot deduce the release of NO in the periphery. In addition, this role seems improbable since analysis of NOS activity in the stomach shows no change in the activity of any NOS isoform when evaluated 30 min after injection of endotoxin, a time interval in which changes in gastric function are already apparent. Regardless of whether or not activation of an inhibitory efferent neural pathway takes place in the inhibition of gastric motor function by endotoxin, the peripheral release of NO does not seem to be involved. Endogenous mediators such as VIP have also been reported as inhibitory neurotransmitters of the NANC innervation of the gastrointestinal tract. Further experiments are required to address this question.

In summary, the present results suggest that low doses of endotoxin reduce gastric contractility through activation of both capsaicin-sensitive afferent neurones and ncNOS in the brainstem. This inhibition may be part of the mechanism involved in the delay in gastric emptying associated with moderate endotoxaemia.

We would like to thank Prof C. Estrada and F. Portillo for their valuable help in the Fos immunohistochemistry. The present study has been supported by grants SAF 99-0114, SAF 98-0118 and 1FD97-1029 from Ministerio de Educación y Cultura. E. Quintana and E. García-Zaragozá are the recipients of a fellowship from Ministerio de Sanidad y Consumo and Ministerio de Educación y Cultura, respectively. Part of this study has been supported by the Royal Academy of Medicine of University of Valencia.

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(Received April 10, 2001
Revised June 19, 2001
Accepted June 26, 2001)

Artículo 2

"*Transcriptional up-regulation of nNOS in the dorsal vagal complex during low endotoxemia*"

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Neurogastroenterology and motility (2004, en revisión).

Transcriptional up-regulation of nNOS in the dorsal vagal complex during low endotoxemia

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Short title: LPS up-regulates nNOS in the brainstem

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ABSTRACT

The present study analyses neuronal nitric oxide synthase (nNOS) expression and distribution in the brainstem of animals pre-treated with *Escherichia coli* or *Helicobacter pylori* LPS, at doses that modulate gastric motor function. Systemic administration of *H. pylori* LPS prevented in a dose-dependent manner (5, 40 and 100 μg kg^{-1}, i.v) the increase in intragastric pressure induced by 2-deoxy-D-glucose (200 mg kg^{-1}, i.v.) in urethane-anaesthetized rats. Quantitative analysis showed a significant increase in the amount of nNOS mRNA induced by *E. coli* or *H. pylori* LPS (2 h later), in a section of the brainstem containing the dorsal vagal complex (DVC). Immunohistochemical studies showed nNOS presence in the DVC of vehicle-treated rats. Both *E. coli* (40 μg kg^{-1}, i.p.) and *H. pylori* LPS (100 μg kg^{-1}, i.p) significantly increased (2 h later) the number of nNOS immunoreactive cells in this area, mainly at the most rostral level. The present study shows that systemic administration of *E. coli* or *H. pylori* LPS induces a transcriptional up-regulation of the nNOS in the DVC of the brainstem and suggest a role for NO synthesis in this area in the control of gastric motor function under endotoxemia.

Keywords: brain-gut; dorsal motor nucleus; dorsal vagal complex; gastric motor function; neuronal nitric oxide synthase; nucleus of the solitary tract.

INTRODUCTION

Infection with Gram-negative bacteria is associated with acute changes in gastric motor function. Peripheral injection of *Helicobacter pylori* LPS has been related to a delay in gastric emptying in rats (1) while the effects of *Escherichia coli* on gastric motor function have been widely studied. Thus, low doses of *E. coli* LPS have been shown to attenuate intragastric pressure (2), decrease fundus contractility (3) and delay gastric emptying (4-6). These changes are rapid in onset, mediated by activation of capsaicin-sensitive vagal afferent neurones and dependent of NO synthesis in both the dorsal vagal complex (DVC) of the brainstem and the myenteric plexus of the stomach (4,5). Consistent evidence suggest a physiologic neural mechanism, mediated by the vagus nerve and involving NO as a neurotransmitter, responsible for the acute changes of gastrointestinal function during early endotoxemia.

Even the effects of endotoxin start fast after challenge, they last for several hours. Thus, inhibition of gastric acid secretion or delay in gastric emptying of a solid nutrient meal has been observed three-four hours after administration of the same doses of endotoxin (5,7). Although a non-trancriptional synthesis of NO from neuronal origin has been involved in the rapid modulation of gastrointestinal function induced by endotoxin, we hypothesise that additional mechanisms could be involved in the maintenance of the response. In the present study we aimed to characterize whether peripheral administration of *E. coli* or *H. pylori* LPS, at doses that inhibit gastric motor function, modulates nNOS expression in the DVC over time. In order to test this hypothesis, we have analysed by RT-PCR and immunohistochemical techniques, the expression and distribution of nNOS in the DVC of animals pre-treated with *E. coli* or *H. pylori* LPS.

MATERIAL AND METHODS

Animals

Male Sprague-Dawley rats (220-250 g) were housed under controlled temperature and lighting (12 h light-dark cycle with light on at 07:00 h) with food and water ad libitum

as standard light and feeding regimens, and were fasted with free access to drinking water 16-20 h before the experiments. All protocols comply with the European Community guidelines for the use of experimental animals and were approved by the ethics committee of the University of Valencia.

Determination of intragastric pressure

Procedures were carried out as previously described (2). Rats were anaesthetised with urethane (1.5 g kg^{-1}, i.p.), the trachea intubated and a jugular vein cannulated. After performing a laparotomy, an intraluminal latex balloon was inserted in the stomach through an incision in the fore-stomach and held in place with a ligature. The balloon and catheter system was connected to a pressure transducer and intragastric pressure was registered on line by a multi-channel recorder (Power Lab.). After reaching an intragastric pressure of between 4-5 cm H_2O, by filling the balloon with 2-3 ml H_2O at 37ºC, rats were allowed to stabilise for 1 h. Then, *H. pylori* LPS (5, 40 or 100 μg kg^{-1}, i.v.), *E. coli* LPS (40 μg kg^{-1}, i.v.) or saline (1 ml kg^{-1}, i.v.) were administered 30 min prior to a bolus injection of 2-deoxy-D-glucose (200 mg kg^{-1}, i.v., 2-DG) and intragastric pressure was recorded on line for 120 min. Rectal temperature was monitored during the experiment. Data is expressed as Δ intragastric pressure, calculated as the difference between stimulated (the average measurement during 5 minutes, at 15 or 90 min after 2-DG administration) and basal (the average in the 5 min before 2-DG administration) intragastric pressure (Figure 1).

Effects of LPS on nNOS expression

Neuronal NOS Immunohistochemistry

Conscious rats received a single i.p. injection of *E. coli* LPS (40 μg kg^{-1}) or saline (1 ml kg^{-1}) and 30, 60 or 120 min later were anaesthetised with pentobarbital (280 mg kg^{-1}, i.p.). Some rats received a single i.p. injection of *H. pylori* LPS (100 μg kg^{-1}) or saline (1 ml kg^{-1}) and 120 min later were anaesthetised with pentobarbital. Thereafter, animals were transcardially perfused with 0.9% saline followed by paraformaldehyde solution (4%). A section of the brainstem containing the DVC was postfixed in the same

fixative and cryoprotected by immersion in 30% sucrose for 2-3 days at 4ºC. Frozen coronal sections were cut at 50 µm throughout the length of the DVC, approximately 2 mm caudal to 2 mm rostral to the obex and processed as free-floating sections for nNOS immunohistochemistry. Sections were washed with PBS, treated with 1% H_2O_2, washed again with PBS, and then blocked with 10% sheep normal serum in PBS. Subsequently, sections were incubated with monoclonal nNOS antibody (BD Transduction Laboratories) diluted at 1:1000 for 24 h at 4ºC. Then, after being thoroughly washed in PBS, sections were incubated with biotinylated anti-mouse IgG (Amersham, from sheep), diluted at 1:200, followed by Vectastain ABC Kit (Vector Labs.), in both cases for 1 h at 25ºC. The final visualisation of nNOS immunoreactivity (nNOS-IR) was achieved by incubating sections with 0.05% 3',3'-diaminobenzidine tetrahydrochloride (DAB) in the presence of 0.033% H_2O_2 for 3 min. Following this, sections were transferred to slides, air-dried, dehydrated, cleared and coverslipped. Tissue processed without addition of primary antibody served as a negative control for nNOS-IR.

Data analysis

Sections throughout the DVC [-15.46 mm to –12.88 mm from the bregma (8)] were observed and photographed using a brightfield microscope (Zeiss). The counting of nNOS-IR cells was performed bilaterally in 2-3 sections per animal per region, analysed regardless of the intensity of staining. Taking into account the L.W. Swanson rat brain atlas we considered three regions within the DVC: the caudal region (-15.46 mm to –14.36 mm from the bregma), the intermediate (-14.16 mm to –13.76 mm from the bregma) and the rostral region (-13,60 mm to –12,88 mm from the bregma). A blinded observer performed all cell counts.

Quantification of nNOS mRNA by real-time quantitative RT-PCR:

Rats were administered with *E. coli* LPS (40 µg kg^{-1}, i.p.), *H. pylori* LPS (100 µg kg^{-1}, i.p.) or saline (1 ml kg^{-1}, i.p.). Thereafter, a section of the brainstem containing the DVC (approximately 2 mm caudal to 2 mm rostral to the obex) was frozen in liquid nitrogen and stored at –80ºC. Total RNA was isolated with TriPure Isolation Reagent (Roche Diagnostics) and treated with DNA-free (Ambion). cDNA was synthesized with SuperScript RT RNase H^- (Life Technologies), as described previously (6).

Quantitative PCR was carried out in a LightCycler instrument with the use of LightCycler-FastStart DNA Master SYBR Green I kit (Roche Diagnostics). Samples of 1 µl of cDNA were amplified with 0.5 µM primers, 2mM $MgCl_2$ and 5% DMSO in a final volume of 10 µl. Reactions were performed in duplicate and a negative control with water instead of cDNA was included in each run. Specific primers for nNOS (5'-AATGACCGAAGCTGGAAGAG, sense; 5'-GTGGAGACGCACGAAGATG, antisense) and Cyclophilin A (CyPA; 5'-CGTCTGCTTCGAGCTGTTTG, sense; 5'-GTAAAATGCCCGCAAGTCAA, antisense) were designed according to reported sequences with GenBank accession no. X59949 and NM_017101, respectively. Specificity was confirmed by melting curve analysis and agarose gel electrophoresis. For quantification, a standard curve was generated by amplification of serial dilutions of the purified PCR product for the corresponding primer pair from cerebellum cDNA. In order to normalize the results, interpolated values for each sample were divided by values for the housekeeping gene CyPA and results are expressed as nNOS:CyPA ratio.

Drugs used

Urethane, 2-deoxy-D-glucose and *E. coli* LPS (serotype 026:B6) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sodium pentobarbitone was obtained as a commercially available preparation (Abbott, Madrid). *H. pylori* LPS was prepared by hot phenol-water extraction and subsequent enzymatic treatments as described previously (9). Unless mentioned otherwise, all drugs were dissolved in saline.

Statistical analysis

Data are expressed as the mean ± standard error of the mean (SEM) values. Comparisons between groups were performed by unpaired Student's t-test or one-way analysis of variance (ANOVA) followed by a Newman-Keuls test where appropriate. Data were considered statistically significant when $P < 0.05$(*) or $P < 0.01$(**).

RESULTS

Effects of LPS on intragastric pressure

Urethane-anesthetized rats that were fixed with a gastric balloon filled with 2.6±0.1 ml of water exhibited an intragastric pressure of 5.9±0.4 cm H_2O (n=16). A single i.v. injection of *H. pylori* endotoxin (5, 40 or 100 μg kg^{-1}) did not significantly modify intragastric pressure (5.4±0.9, 6.5±0.7 or 5.2±0.8 cm H_2O, respectively, n≥4) in the 30 min period following injection (Figure 1a).

Administration of 2-DG (200 mg kg^{-1} i.v., n=4) induced a net increase in gastric tone within the first 15 min that was maintained above basal level for more than 90 min (Figure 1). Pre-treatment with *H. pylori* LPS (5, 40 or 100 μg kg^{-1}) prevented in a dose-dependent manner the contraction peak (Figure 1b). In addition the highest dose of *H. pylori* LPS induced a long-lasting inhibition of intragastric pressure that was observed 2 h after its administration. A similar pattern of inhibition was observed with *E. coli* LPS at the dose of 40 μg kg^{-1} (Figure 1).

Effects of LPS on nNOS-IR in the DVC

Analysis of nNOS-IR in the DVC of vehicle-treated animals exhibited different distribution of the enzyme depending on the region analysed. In the caudal DVC, the few positive cells observed were mainly present surrounding the central canal, lying at the border of the dorsal motor nucleus (DMN) and commissural nucleus of the solitary tract (NTS) (Figure 2a).

In the intermediate DVC very few nNOS-IR cells were observed. These cells were dispersed and moderately stained in commissural and lateralis subnucleus of the NTS and occasionally in lateral DMN (Figure 2b).

At the level of the rostral DVC (rostral DVC I) a high density of nNOS-IR cells was present and was markedly distributed along the different NTS subnuclei (medial, central and lateralis subnuclei) and the DMN (Figure 2c, 3a). At the level of the subnucleus centralis of the NTS, nNOS-IR positive cells are relatively small and densely packed (Figure 2c, 3a), making it difficult to count the stained nucleus.

At the level where the DVC is just beginning to migrate away from the fourth ventricle (rostral DVC II), there were numerous densely stained nNOS-IR cells throughout the DVC; however no apparent organisation of the positively stained cells was observed and we proceeded with counting in the whole area (Figure 2d,e; 3b).

Systemic pre-treatment with endotoxin from *E. coli* (40 μg kg^{-1}, i.p.) or *H. pylori* (100 μg kg^{-1}, i.p.) induced, 2 h later, a generalized increase in nNOS-IR in the DVC, and was the greatest at the most rostral level (Figure 3). Thirty or sixty min after *E. coli* or *H. pylori* LPS administration, there were not significant changes in nNOS-positive cells in the DVC, compared with control animals (Figure 3g).

Effects of LPS on nNOS mRNA expression in the DVC

Expression of nNOS mRNA was detected by real-time quantitative RT-PCR in the selected section of the brainstem containing the DVC. A single i.p. injection of endotoxin from both *E. coli* (40 μg kg^{-1}) or *H. pylori* (100 μg kg^{-1}) induced a significant increase 2 h later in the amount of nNOS mRNA in this section (Figure 4).

DISCUSSION

Lipopolysaccharides are a characteristic cell wall constituent of Gram-negative bacteria which are important in the structure and function of the outer membrane (10). Both *E. coli* and *H. pylori* are Gram-negative bacteria that cause pathophysiological disorders. However, *E. coli* is usually present in the intestine and just after access to blood triggers on a generalized systemic reaction, while *H. pylori* colonizes gastric mucosa and constitutes a ethiophatogenic factor in gastric ulcer (11). In the present study we demonstrate that both, *E. coli* and *H. pylori* LPS, at doses that inhibit gastric motor function, induce a transcriptional up-regulation of nNOS in the DVC of the brainstem.

Systemic administration of *H. pylori* LPS to anaesthetized rats prevented in a dose-dependent manner the increase in intragastric pressure induced by 2-DG. It seems likely that the rapid diminution of gastric tone observed in the present study is involved in the mechanism induced by *H. pylori* LPS to delay gastric emptying (1). Previous

studies have reported a similar effect with *E. coli* LPS (2). Although differences in the structure of *H. pylori* LPS from those of *E. coli* have been shown (12), in the present study slightly higher doses of *H. pylori* LPS have been necessary in order to get a similar degree of gastric inhibition. In line with these observations, the present study demonstrates that both *H. pylori* and *E. coli* LPS, at doses that inhibit gastric motor function induce a similar pattern of nNOS expression in the DVC of the brainstem. A detailed analysis of the distribution of the nNOS-IR along the DVC of control animals showed a majority of positive cells at the most rostral level. A single intraperitoneal injection of both *E. coli* and *H. pylori* LPS induced two hours later a significant increase in the number of nNOS-IR positive cells at the most rostral level, while it was not observed earlier. Such an increase was correlated with a higher amount of nNOS mRNA in the same area, suggesting that both *H. pylori* and *E. coli* LPS induce two hours later a transcriptional up-regulation of the nNOS in the brainstem.

The DVC comprises the two interacting nuclei NTS and DMN, and the increase in nNOS-IR induced by LPS has been observed in both. NTS constitutes the first point of synapse of the vagus, carrying sensory information from numerous peripheral structures (13) and strong evidence supports a role for the vagus nerve in brainstem modulation induced by peripheral LPS, since: (a) endotoxin-induced c-fos in central nuclei involved in autonomic functions is mediated by the vagus nerve (14,15); (b) primary afferent neurones of the vagus nerve express fos immunoreactivity in response to peripheral endotoxin (16); and (c) activation of capsaicin sensitive vagal afferent neurones has been involved in the inhibition of gastric tone induced by peripheral endotoxin (2). Although a direct effect of LPS or LPS-induced cytokines on nNOS gene expression can not be ruled out, the rapid up-regulation of nNOS observed in the present study led us to hypothesize that a nervous mechanism mediated by the vagus is involved in the regulation of nNOS expression in the NTS by peripheral LPS. In accordance with this hypothesis, a role for the vagus nerve in the regulation of nNOS expression in the myenteric plexus has been reported (17).

In the NTS, pre- and post-synaptic structures, as well as intrinsic neurones have been shown to express nNOS-IR (18). In the present study, the identity of the structures expressing nNOS in the NTS of endotoxin-treated rats has not been analysed. However, evidence suggests that these structures are not of vagal origin since: (a) pre-synaptic

nNOS-IR terminals in the NTS are not vagal afferent in origin (18); (b) strong evidence support a role for glutamate as the primary afferent neurotransmitter in the NTS (19); (c) endotoxin, at the doses used in the present study, has been shown to increase the release of glutamate in the brainstem (20) which in turn activates the nNOS (21); and (d) intracisternal injection of glutamate has been shown to modulate gastric function through an increased NO synthesis in the brainstem (21). Collectively, these results and previous studies suggest that nNOS-IR in the NTS of endotoxin-treated animals could be localized in other structures rather than vagal afferent terminals and modulation of nNOS expression and activity in the NTS could be due to glutamate receptor stimulation released from vagal afferent terminals (20).

In addition to the NTS, in the rostral DMN it was observed an increase in the number of nNOS-IR positive cells, two hours after LPS administration. An increased in nNOS protein amount in this nucleus has previously been related to changes in gastric function induced by hyperthermia (22). In line with these observations, and taking into account that inhibition of intragastric pressure is present two hours after challenge, a correlation between up-regulation of nNOS in the DVC and inhibition of intragastric pressure may be established. However it is important to note that even such a correlation exists, alternative mechanisms must also be involved since the present study demonstrates that diminution in intragastric pressure by LPS starts to be apparent thirty minutes after challenge, moment in which no changes in nNOS IR in the DVC are observed. Interestingly, functional and biochemical studies have demonstrated that a non-transcriptional regulation of NO synthesis in the brainstem is involved in the rapid (one hour) inhibition of intragastric pressure by systemic administration of *E. coli* LPS (2). Although the mechanism of the transcriptional regulation of the nNOS has not been analysed, it is possible that it is a consequence of the initial neural mechanism activated by LPS (17). Further studies are required to address this question.

In summary, the present study shows a transcriptional up-regulation of nNOS in the DVC of the brainstem induced by systemic administration of low doses of *E. coli* or *H. pylori* LPS. Modulation of the nNOS activity in these nuclei could be part of the mechanism activated by the host in order to eliminate the challenge and restore homeostasis.

ACKNOWLEDGMENTS

This study has been supported by grant FIS 01/1187 from the Ministerio de Sanidad y Consumo and grant C 03/02 from Instituto de Salud Carlos III. Elsa Quintana is the recipient of a fellowship from the Ministerio de Sanidad y Consumo.

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Figure 1. (a) Original recording showing the effects of *H. pylori* LPS, *E. coli* LPS or saline pre-treatment (30 min) on intragastric pressure induced by 2-DG. Graphs represent the effects of *H. pylori* or *E. coli* LPS on intragastric pressure induced by 2-DG (b) 45 min.- or (c) 120 min. after LPS treatment. Data is expressed as Δ intragastric pressure, calculated as the difference between stimulated (ii or iii, on recording) and basal (i, on recording) intragastric pressure (cm H_2O). Bars represent the mean±SEM (n≥4). Comparisons between groups are performed by ANOVA followed by a Newman-Keuls test. Significant difference from saline-treated rats is shown by **P<0.01 or *P<0.05.

Figure 2. Series of line drawings (adapted from Swanson rat brain atlas) of coronal sections from the brainstem of a control rat (saline, 1ml kg^{-1}, i.p.) showing the representative distribution of nNOS-IR cells (black triangles) along the rostro-caudal axis of the dorsal vagal complex. (i) Drawing of a longitudinal section of the rat brain showing the location of the coronal sections of the brainstem (grey area) represented. The distance from the bregma (β) of each plate is indicated in mm. (a) Caudal section; (b) intermediate section; (c) rostral I section; (d) and (e) rostral II section. AP, area postrema; cc, central canal; ce, subnucleus centralis of the NTS; co, commissural NTS; DMN, dorsal motor nucleus of the vagus; l, lateralis subnucleus of the NTS; m, medial subnucleus of the NTS; NTS, nucleus tractus solitarius; V4, forth ventricle.

Figure 3. Representative microphotographs of coronal sections of the brainstem at the rostral I (a,c,e; -13.60 mm from the bregma) and rostral II (b,d,f; -13.15 mm from the bregma) regions showing nNOS-immunoreactivity in the NTS and DMN of the DVC, 2h after injection of (a,b) saline (1 ml kg^{-1}, i.p.), (c,d) *E. coli* LPS (40 μg kg^{-1}, i.p.) or (e,f) *H. pylori* LPS (100 μg kg^{-1}, i.p.); Scale bar=100 μm. (g) Graph showing the effects of *E. coli* (30, 60 or 120 min before) or *H. pylori* (120 min before) LPS administration on the number of nNOS-IR cells (number/section, bilateral) present in the most rostral region (-13.28 to -13.15 mm from the bregma) of the DVC. Results are expressed as mean ± S.E.M. (n≥3). *P<0.05 vs respective saline-treated rats (Student's *t*-test).

Figure 4. Real-time quantitative RT-PCR for nNOS mRNA in brainstem from rats administered with saline, *E. coli* or *H. pylori* LPS, 2 hours beforehand. Relative amounts of nNOS or CyPA mRNA were obtained from their standard curves. Results are expressed as mean ± S.E.M. ($n \geq 4$). *$P<0.05$ or **$P<0.01$ vs saline-treated rats (one-way ANOVA followed by Newman-Keuls test).

figure 1

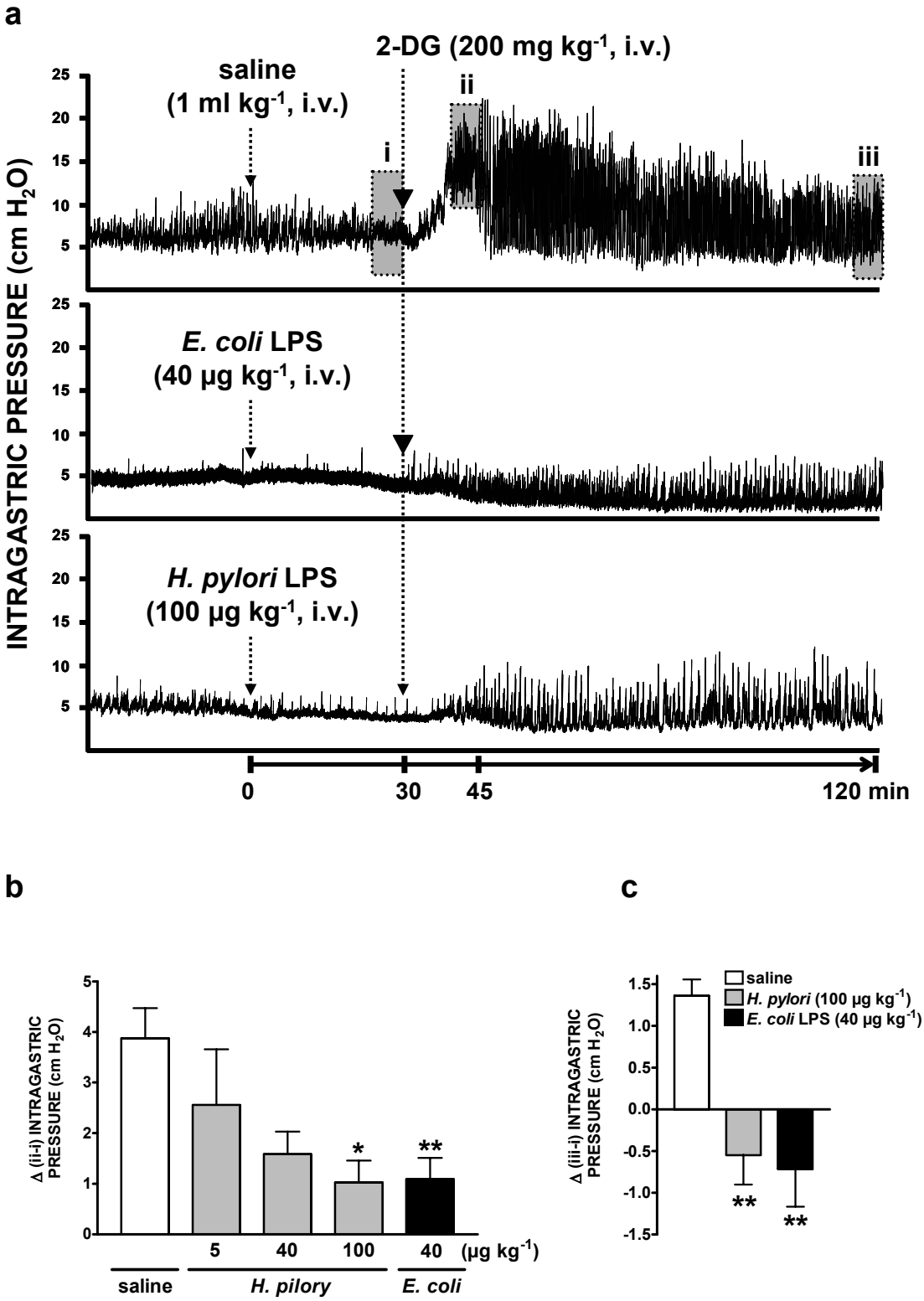

a
2-DG (200 mg kg-1, i.v.)
saline
(1 ml kg-1, i.v.)
i
ii
iii
INTRAGASTRIC PRESSURE (cm H2O)
E. coli LPS
(40 µg kg-1, i.v.)
H. pylori LPS
(100 µg kg-1, i.v.)
0
30
45
120 min
b
Δ (ii-i) INTRAGASTRIC PRESSURE (cm H2O)
5
40
100
40
(µg kg-1)
saline
H. pilory
E. coli
c
Δ (iii-i) INTRAGASTRIC PRESSURE (cm H2O)
saline
H. pylori (100 µg kg-1)
E. coli LPS (40 µg kg-1)

figure 2

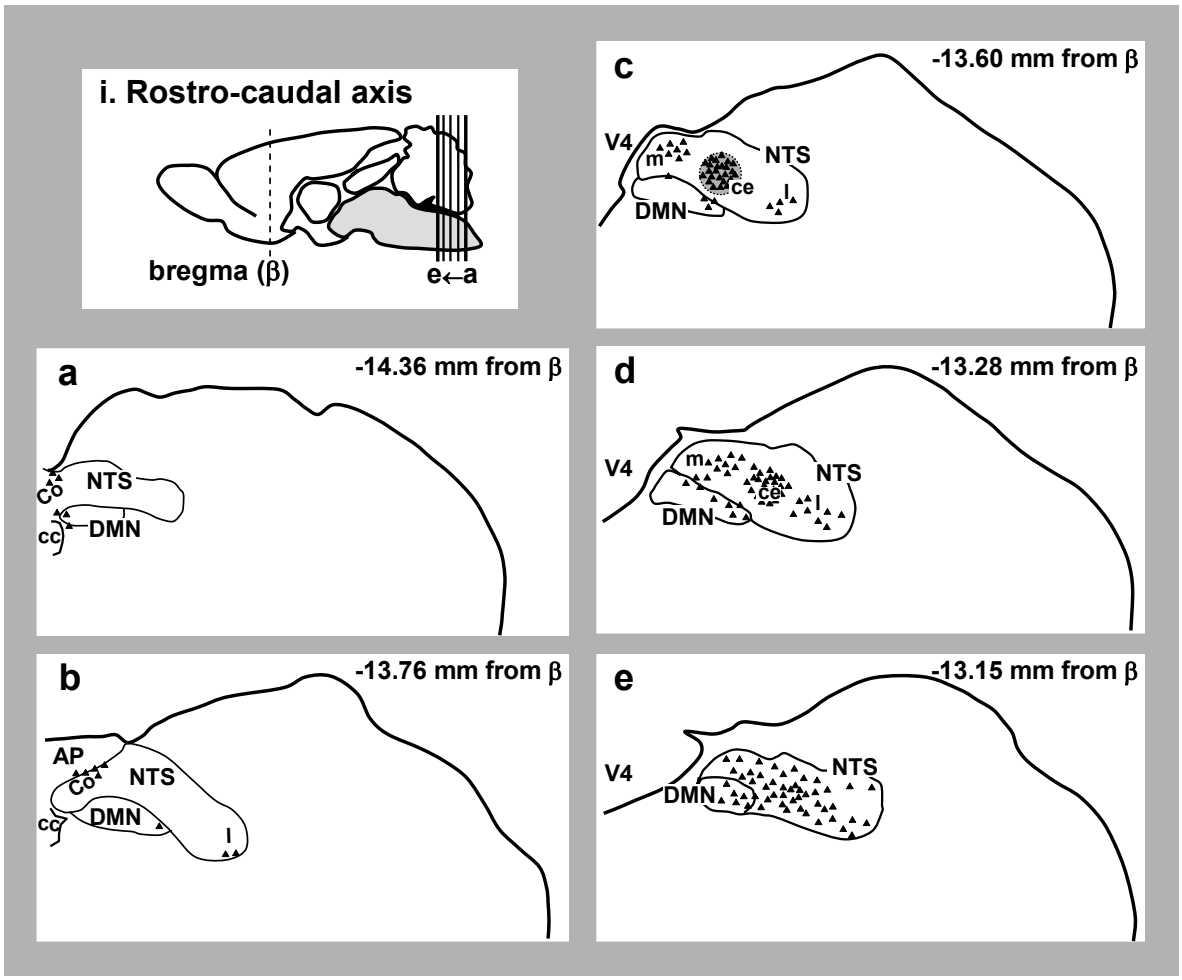

i. Rostro-caudal axis
bregma (β)
e←a
a
-14.36 mm from β
Co
NTS
cc
DMN
b
-13.76 mm from β
AP
Co
NTS
cc
DMN
l
c
-13.60 mm from β
V4
m
NTS
ce
l
DMN
d
-13.28 mm from β
V4
m
NTS
ce
l
DMN
e
-13.15 mm from β
V4
NTS
DMN

figure 3

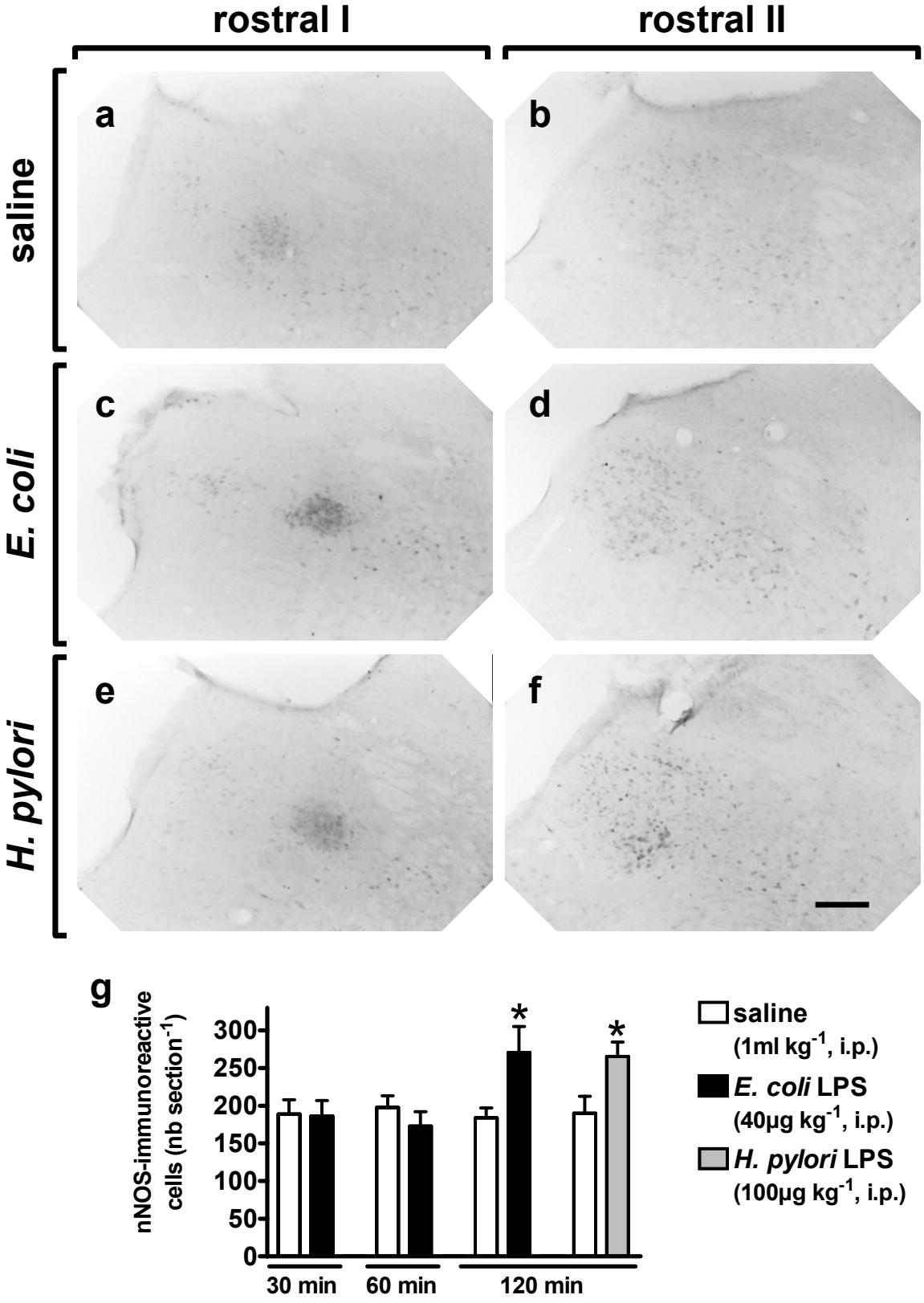
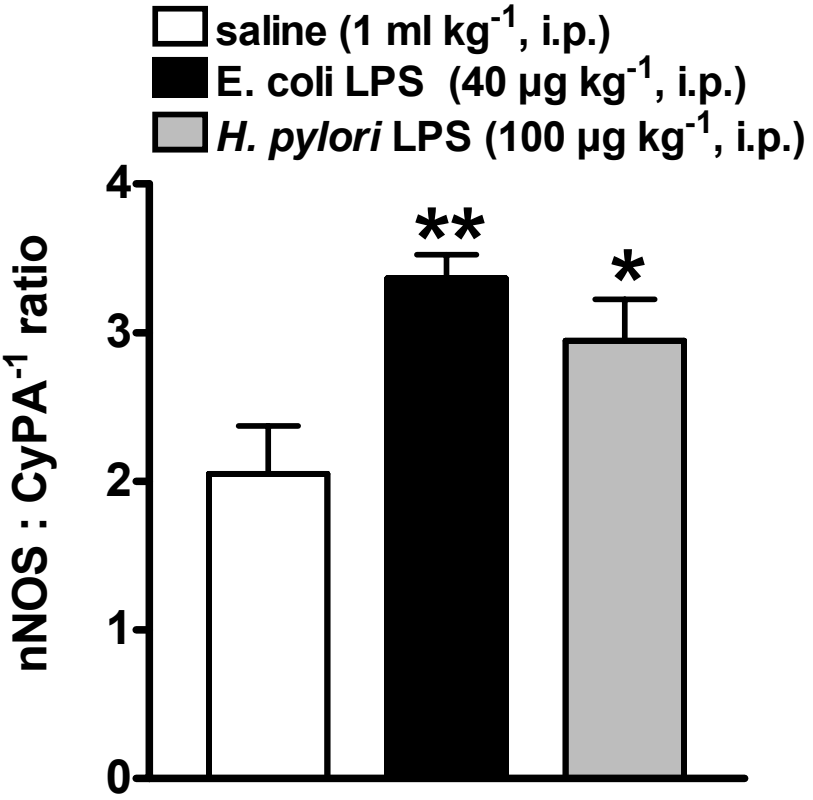

rostral I
rostral II
saline
E. coli
H. pylori
a
b
c
d
e
f
g
nNOS-immunoreactive cells (nb section-1)
300
250
200
150
100
50
0
*
*
30 min
60 min
120 min
saline (1ml kg-1, i.p.)
E. coli LPS (40µg kg-1, i.p.)
H. pylori LPS (100µg kg-1, i.p.)

figure 4


saline (1 ml kg-1, i.p.)
E. coli LPS (40 µg kg-1, i.p.)
H. pylori LPS (100 µg kg-1, i.p.)
nNOS : CyPA-1 ratio
0
1
2
3
4
**
*

Artículo 3

"*Synthesis of nitric oxide in post-ganglionic myenteric neurons during endotoxemia: implications for gastric motor function*"

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The FASEB Journal express article (2004)

a) ***Summary*: The FASEB Journal (2004) 18: 531-533.**

b) ***Full manuscript*: The FASEB Journal (2004) 10.1096/fj.03-0596fje**

Synthesis of nitric oxide in postganglionic myenteric neurons during endotoxemia: implications for gastric motor function in rats[1]

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SPECIFIC AIMS

NO synthesized by the enteric nervous system plays a physiological role in relaxation of the stomach, while the induction of iNOS by the immune system is involved in changes in gastrointestinal motor function associated with infection or injury. Following bacterial infection, the host responds acutely by activating neuroendocrine, metabolic, and behavioral processes that attempt to eliminate the challenge and restore homeostasis. Diminution in gastric motor function constitutes an early event in endotoxemia and we hypothesized that a neural mechanism mediated by nitric oxide (NO) synthesis in enteric motor neurones is involved in this event.

PRINCIPAL FINDINGS

1. Activation of inhibitory postganglionic myenteric neurons, by extrinsic neural control of the gut, mediates the decrease in gastric motor function during early endotoxemia

Thirty minutes after systemic administration to rats of low doses of endotoxin, the stomach was removed and gastric hypocontractility to carbachol observed in isolated fundal strips (**Fig. 1**). Local inhibition of neural transmission with the neurotoxin tetrodotoxin (TTX) or blockade of ganglia nicotinic receptors with hexamethonium significantly prevented the inhibitory effect of endotoxin (Fig. 1*A*). In vitro incubation with endotoxin (100 ng/mL or 1 μg/mL) for 30 min, 3 h, or 8 h failed to significantly modify ($P>0.05$) carbachol-induced fundus contraction.

2. nNOS-derived NO synthesis, but not VIP or ATP, is involved in the diminution in gastric motor function associated with early endotoxemia

Blockade of NO synthesis by incubation of fundal strips with L-NOARG or the selective nNOS inhibitor TRIM prevented the hypocontractility induced by endotoxin (Fig. 1*B*). However, the effect of endotoxin was not significantly modified ($P>0.05$) by incubation with the VIP antagonist VIP 10-28 (10 μM) or antagonism of P_2-purinoceptors with suramin (100 μM) plus PPADS (100 μM). No changes in nNOS mRNA or protein content were observed in gastric tissue 1 h after endotoxin.

3. During early endotoxemia, NO synthesis in the gastric wall is specifically localized in postganglionic myenteric neurons

We analyzed the cellular source of NO synthesis by confocal microscopy in living gastric tissue loaded with DAF-FM, a dye that upon binding to NO in the presence of oxygen results in irreversible fluorescence. Fundus whole mounts from endotoxin-treated rats exhibited an intense DAF-FM fluorescent signal in the myenteric plexus (**Fig. 2*D***). Moving along the Z axis of the fundus whole mount, DAF-FM fluorescence was observed localized in enteric nerve cell bodies and nerve fibers within myenteric ganglia (Fig. 2*D, J*) as well as in nerve fibers running parallel to smooth muscle cells (Fig. 2*E*). Image analysis of deep circular smooth muscle layer failed to show any DAF-FM fluorescent signal (Fig. 2*F*). Preincubation with L-NAME (1 mM, Fig. 2*G, J*), TTX (1 μM; Fig. 2*H, J*) or hexamethonium (100 μM; Fig. 2*I, J*) prevented the appearance of DAF-FM fluorescence in strips from endotoxin-treated rats, confirming the NO-specificity of the fluorochrome and neural cellular origin of DAF-FM fluorescence, and suggesting the location of NO synthesis in postganglionic myenteric neurons.

Fundus whole mount from saline-treated rats showed a weak DAF-FM signal (Fig. 2*B*) that was not modified by preincubation with L-NAME (Fig. 2*C*), TTX, or hexametonium; (Fig. 2*J*).

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[1] To read the full text of this article, go to http://www.fasebj.org/cgi/doi/10.1096/fj.03-0596fje; doi: 10.1096/fj.03-0596fje

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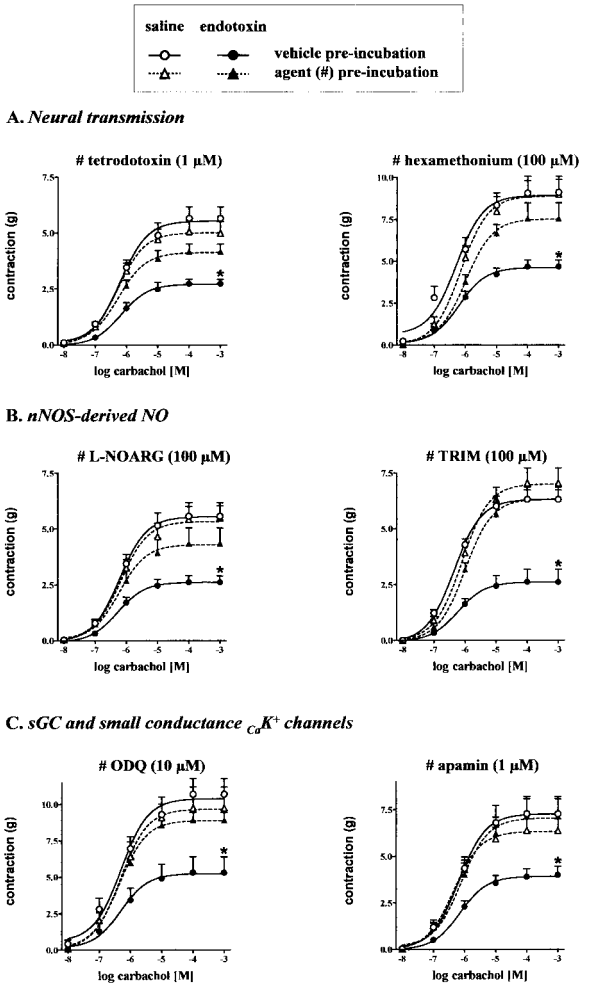

Figure 1. Concentration-response curves to carbachol in fundal strips from rats treated with saline (1 ml/kg, i.p.; open symbols) or endotoxin (40 μg/kg, i.p.; solid symbols). Data are expressed as means ± SE ($n \geq 5$). *$P < 0.05$ for Emax vs. all experimental groups in each graph.

4. NO-induced relaxation of gastric fundus is mediated via guanylyl cyclase and small conductance Ca^{2+}-sensitive K^+ channels

Inhibition of the activity of soluble guanylyl cyclase (sGC) with ODQ or blockade of the small conductance Ca^{2+} activated K^+ ($_{Ca}K^+$) channels by apamin, prevented the hypocontractility observed in strips from endotoxin-treated rats (Fig. 1*C*).

Under NANC conditions, nicotine (10 μM) -induced relaxation (96.6±3.6%) of 5-HT (3 μM) -precontracted strips was prevented ($P<0.05$) by preincubation with L-NOARG (27.1±8.3%; 100 μM), ODQ (17.0±4.9%; 10 μM), or apamin (56.4±14.5%; 1 μM).

Preincubation with the NO donor, DETA NONOate (100 μM) induced hypocontractility (52.3±4.3% of contraction) to carbachol (0.1 μM). This effect was prevented ($P<0.05$) in the presence of ODQ (116.2±9.0%) or apamin (79.3±7.6%). In a similar manner, addition of the NO-independent sGC activator BAY 41-2272 (3 μM) reduced the contraction to carbachol (32.8±4.4%), and this effect was prevented ($P<0.05$) by preincubation with ODQ (92.9±10.1%) or apamin (54.9±4.0%).

5. iNOS isoform is not involved in the decrease in gastric motor function during early endotoxemia

In vivo pretreatment with dexamethasone (5 mg/kg, s.c., twice) failed to significantly ($P>0.05$) modify the

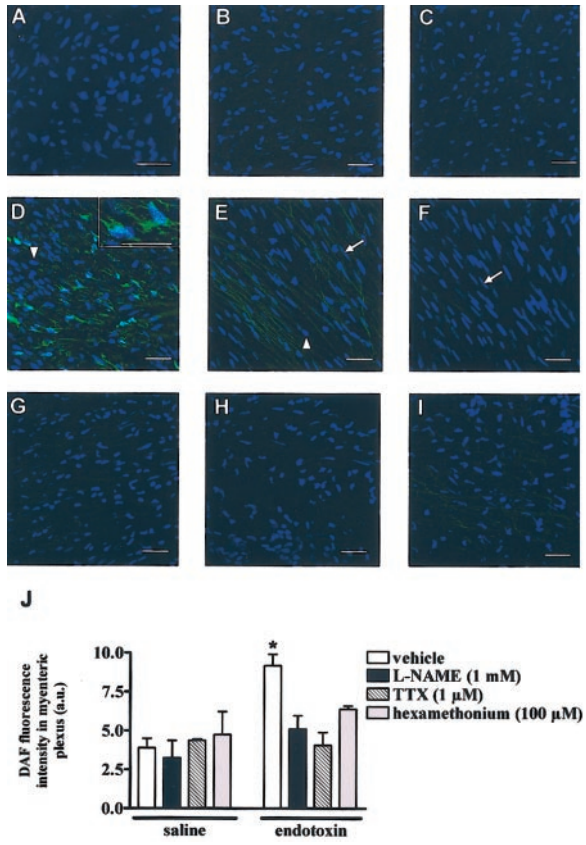

Figure 2. Confocal microscopy images of whole mount preparations of gastric fundus from saline (1 mL/kg, i.p.) *(B–C)* or endotoxin (40 μg/kg, i.p.) -treated rats *(D–I). A)* Only Hoechst 33342 staining (nuclear fluorochrome, blue) showing, almost undetectable, green auto fluorescence; *B–I)* DAF-FM staining (indicative of NO synthesis; green) and Hoechst 33342. Nuclei from smooth muscle cells (arrows) and nerve cells (arrowheads) have different shape. *(B–C)* DAF-FM fluorescence present in the myenteric plexus of a saline-treated rat (*B*); L-NAME preincubation failed to modify DAF-FM fluorescence *(C). D–F)* Three confocal images of whole mount Z projection from the myenteric plexus *(D)* to the circular muscle layer *(F)*. DAF-FM fluorescence is present in soma of the myenteric neurons *(D,* a higher magnification of a positive DAF-FM soma is shown in the insert), and nerve endings running parallel to the circular smooth muscle *(E),* while it is absent in the distal circular muscle layer *(F). (G–I)* Preincubation with L-NAME (*G*), TTX (*H*), or hexamethonium (*I*) significantly reduced DAF-FM fluorescence in the myenteric plexus of endotoxin-treated rats. *(J)* Graphic representation showing quantification of DAF-FM fluorescence present in the myenteric plexus. Results express means ± SE ($n \geq 3$). *$P < 0.05$ vs. all experimental groups. Scale bar = 25 μm.

response to carbachol in saline (Emax 5.3±0.7 g) or endotoxin-treated rats (2.4±0.4 g). In addition, no changes in iNOS mRNA expression, protein content and enzyme activity have been observed in gastric tissue one hour after the administration of endotoxin.

CONCLUSIONS AND SIGNIFICANCE

In the present study we have shown for the first time the synthesis of NO in rat gastric postganglionic myenteric neurons. This synthesis is observed in fundus from endotoxin-treated rats and seems to be involved in the associated hypocontractility.

Systemic administration of endotoxin to rats results in the hypocontractility to carbachol of isolated fundal strips. This effect is rapid in onset and independent of endotoxin-induced protein synthesis since in vivo pretreatment with dexamethasone did not prevent its effects, thus suggesting a neural mechanism. This is reinforced by the fact that both tetrodotoxin and hexamethonium prevented the hypocontractility. Failure of endotoxin to modify gastric contractility when directly added to the organ bath suggests that the effects of systemic endotoxin are a consequence of an extrinsic regulation of motor neurons rather than a direct action of endotoxin on enteric neurons or smooth muscle cells. These observations, together with previous studies showing activation of capsaicin-sensitive afferent vagal neurons and c-fos expression in the dorsal vagal complex of the brainstem by endotoxin, suggest a vago-vagal reflex involved in the diminution of gastric motor function observed in early endotoxemia (**Fig. 3**). Such a mechanism could be involved in the associated delay in gastric emptying.

NO synthesis mediates physiological relaxation of the fundus. However, the present study is the first to demonstrate a nontranscriptional regulation of NO synthesis via nNOS in the acute diminution of gastric motor function induced by endotoxin. In parallel confocal microscopy studies, we also demonstrate that in vivo pretreatment with endotoxin induces an intense DAF-FM fluorescence in the fundal myenteric plexus, indicative of NO synthesis. In addition, this fluorescence seems to be specifically localized in postganglionic myenteric neurons since pretreatment with TTX or hexamethonium significantly prevented it. Taking into account that nNOS is primarily expressed in neurons, these results suggest that NO synthesis localized in postganglionic myenteric neurones is involved in fundus relaxation induced by endotoxin (Fig. 3).

Further analysis of intracellular mechanisms showed that activation of sGC and small conductance $_{Ca}K^+$ channels mediates endotoxin-induced fundal relaxation. Furthermore, activation of these channels seems to be cGMP-dependent because gastric relaxation induced by an NO-independent sGC activator depends on their activity. Since a direct action of endotoxin on these targets has been ruled out, our results suggest that NO activates sGC, and subsequent increase in cGMP levels is involved in the activation of these channels and relaxation of the tissue (Fig. 3).

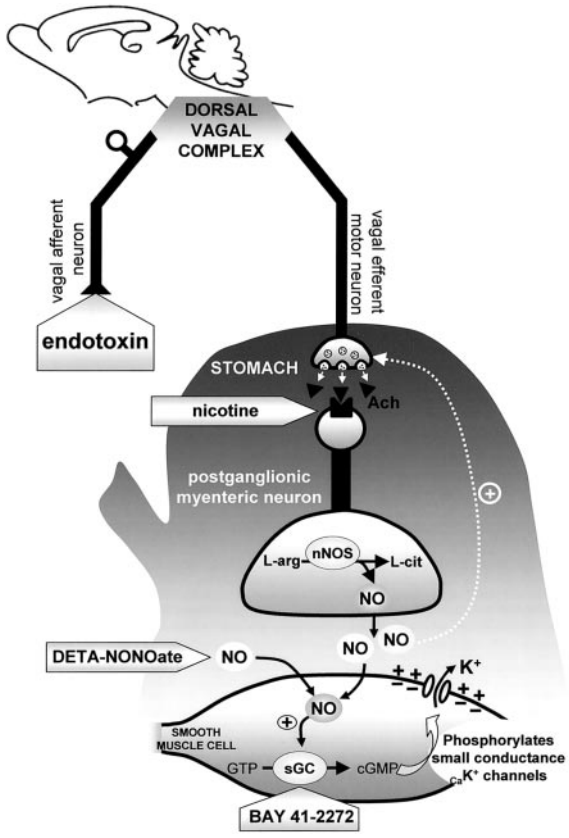

Figure 3. Schematic model of the neural pathway, endogenous mediators, and intracellular mechanisms involved in the diminution of gastric fundal tone in early endotoxemia. Systemic administration of endotoxin induces activation of nitrergic postganglionic myenteric neurons, via an extrinsic neural control of the gut. This triggers an increase in nNOS-derived NO synthesis in the myenteric plexus. Once released, NO acting on the sGC in smooth muscle cells leads to phosphorylation of small conductance $_{Ca}K^+$ channels. Increased K^+ conductance would tend to hyperpolarize membrane potential and to relax the fundus. This and previous studies allow us to hypothesize that activation of the enteric pathway is a consequence of a vago-vagal reflex.

Changes in gastrointestinal motor function during endotoxemia have been linked to iNOS-induction. However, our results do not support a role for iNOS-derived NO synthesis in the decrease in gastric tone induced by endotoxin, since in vivo pretreatment with dexamethasone failed to modify its effects. In addition, we observed no significant changes in iNOS mRNA expression, iNOS protein content or Ca^{2+}-independent NOS activity in strips from endotoxin-treated rats.

We hypothesize that in early endotoxemia the host tries to maintain homeostasis by activating neural mechanisms which maximize the activity of its constitutive resources (nNOS), before the immune response is fully functioning (iNOS). FJ

The FASEB Journal express article 10.1096/fj.03-0596fje. Published online January 8, 2004.

Synthesis of nitric oxide in post-ganglionic myenteric neurons during endotoxemia: implications for gastric motor function

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ABSTRACT

We have investigated the mechanisms underlying acute changes in gastric motor function triggered by endotoxemia. In fundal strips from rats pre-treated with endotoxin (40 μg/kg, i.p. 30 min), mechanical activity was analyzed and the source of nitric oxide (NO) was visualized by confocal microscopy of tissue loaded with the fluorescent dye DAF-FM. NOS expression was determined by quantitative RT-PCR and Western blot, and enzyme activity by the citrulline assay. Strips from endotoxin-treated rats were hypo-contractile. This was prevented by pre-incubation with the neurotoxin tetrodotoxin, the gangliar blocker hexamethonium, or non-selective and neuronal-specific NOS inhibitors (L-NOARG and TRIM, respectively). The soluble guanylyl cyclase (sGC) inhibitor ODQ and the inhibitor of small conductance Ca^{2+}-activated K^+ channels apamin prevented relaxation induced by endotoxin, nicotine, exogenous NO (DETA-NONOate), and the NO-independent sGC activator BAY 41-2272. NO synthesis was observed in neuronal soma, axons, and nerve endings of the myenteric plexus in the fundus of endotoxin-treated rats and was prevented by L-NAME, tetrodotoxin, and hexamethonium. nNOS and iNOS mRNA and protein contents were unchanged. Our findings demonstrate synthesis of NO in post-ganglionic myenteric neurons during early endotoxemia that mediates gastric hypo-contractility. The effect of NO is mediated via sGC and small conductance Ca^{2+}-activated K^+channels.

Key words: myenteric plexus • motility • confocal microscopy • DAF • neuronal NOS

Following infection by endotoxin-producing bacteria, the host reacts by coordinating the peripheral immune response and by signaling to the central nervous system, where a cascade of events are orchestrated in what is collectively termed the acute-phase response. A wide array of autonomic, endocrine, and behavioral changes occurs in this phase that is interpreted as an attempt to eliminate the challenge and restore homeostasis. Prominent among these is a substantial alteration in gastrointestinal motor function (1–3). In experimental animals,

an attenuated intragastric pressure and a delay in gastric emptying have been observed after administration of endotoxin (2, 4, 5). These changes are rapid in onset and seem to be mediated by mechanisms that involve both the autonomic and the central nervous systems. However, the local mechanism in the stomach that inhibits motor function in early endotoxemia remains unknown.

Non-adrenergic non-cholinergic (NANC) nerves in the myenteric plexus conduct the main inhibitory neurotransmission of the gut (6), and nitric oxide (NO) is postulated as a potential neurotransmitter (7, 8). Immunohistochemical studies report the presence of the neuronal nitric oxide synthase (nNOS) in inhibitory motor neurons that course through the gastrointestinal muscle layers (9, 10). The importance of nNOS-derived NO in the physiological relaxation of the stomach is clearly demonstrated by pharmacological studies (11, 12) and the use of nNOS knockout animals (13). However, the involvement of this neurotransmitter in the modulation of gastric motility under pathophysiological circumstances is less clear.

We hypothesized that the acute changes in gastric function associated with early endotoxemia result from the activation of enteric nitrergic motor neurons. To test this, we have analyzed the cellular localization of NO synthesis in the gastric fundus of animals pre-treated with endotoxin. These measurements were performed in real time by the use of NO-reactive fluorescence indicators in conjunction with confocal laser scanning microscopy. In parallel, we have determined the role of NO on muscle contractility as well as NOS expression and activity in the gastric wall during endotoxemia.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (220–250 g; Harlan Laboratories, Barcelona) were maintained on standard Purina laboratory chow and tap water ad libitum and were housed under conditions of controlled temperature (21±1°C) and light (07:00–19:00 h). Rats were fasted, with free access to drinking water, for 16–20 h before the experiments. All protocols comply with the European Community guidelines for the use of experimental animals and were approved by the ethics committee of the University of Valencia.

Measurement of the mechanical activity of isolated gastric fundus

Rats were killed by cervical dislocation, the stomachs were removed, and two strips from the fundus (15×3 mm) were cut in the direction of the circular muscle layer right next to the corpus region. Strips were mounted in organ baths (5 ml, 37°C) under a load of 1 g and bubbled with carbogen gas (95% O_2 + 5% CO_2) in Krebs solution [(mM): NaCl 118.5, KCl 4.8, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 1.9, $NaHCO_3$ 25.0, and glucose 10.1]. The tension was recorded isometrically by using Grass FT03-type force displacement transducers and was displayed on a multi-channel recorder (Power Lab.). All strips were equilibrated for 1 h, with rinsing every 15 min, and thereafter were exposed to K^+ (60 mM) as a test of muscle contractility.

In vivo pre-treatment with endotoxin

Rats were injected intraperitoneally with *Escherichia coli* endotoxin (40 μg/kg) or saline (1 ml/kg) and were killed 30 min later. Cumulative concentration-response curves for carbachol (10^{-8} to 10^{-3} M) were conducted in two parallel strips from endotoxin- or saline-treated rats, 15 min after incubation of the strips with pharmacological agents or vehicles.

The role of the enteric nervous system was analyzed by using the non-selective neurotoxin tetrodotoxin (TTX, 1 μM) and the autonomic ganglia nicotinic receptor antagonist hexamethonium (100 μM).

The role of peripheral endogenous mediators was analyzed by incubation with: a) a non-selective inhibitor of NOS, N^G-nitro-L-arginine (L-NOARG, 100 μM); b) a selective inhibitor of nNOS 1-(2-trifluoromethylphenyl)imidazole (TRIM, 100 μM); c) the VIP antagonist, VIP 10-28 (10 μM); d) a combination of the non-selective P_2 receptor antagonist suramin (100 μM, incubated for 50 min) and pyridoxal-phosphate-6-azophenyl-2',4'-disulphonic acid (PPADS, 100 μM).

The intracellular mechanisms involved in endotoxin-induced fundus relaxation were determined by incubation with: a) the soluble guanylyl cyclase (sGC) inhibitor 1H-(1,2,4,)oxadiazolo(4,3-a)quinoxalin-1-one (ODQ, 10 μM); b) the adenylyl cyclase inhibitor 9-(tetrahydro-2-furanyl)-9H-purin-6-amine (SQ 22536, 100 μM); c) the selective small conductance Ca^{2+} activated K^+ ($_{Ca}K^+$) channel inhibitor apamin (1 μM).

In some experiments, rats were pre-treated with the glucocorticoid dexamethasone (5 mg/kg, s.c.) or with vehicle (1 ml/kg, s.c.) 16 and 4 h before administration of endotoxin (40 μg/kg, i.p.) or saline (1 ml/kg, i.p.).

In all cases drugs were selected taking into account their specific mechanisms of action and at concentrations widely reported to be effective. Drugs were added to the organ bath in volumes of less than 1% of the bathing solution. At the end of experiments the tissues were desiccated and weighed to confirm that there were no differences between strips from different treatment groups. Results are analyzed (Power Lab.) as the maximal contraction obtained after each addition of carbachol and expressed as g of contraction.

In vitro incubation with endotoxin

Cumulative concentration-response curves for carbachol (10^{-8} to 10^{-3} M) were performed in strips from non-treated animals. Thereafter, a bathing solution (changed every 30 min) containing endotoxin (100 ng/ml or 1 μg/ml) or its vehicle was added to the organ bath medium, and a second concentration-response curve for carbachol was performed 30 min, 3 h, or 8 h later. Data are expressed as a percentage of the Emax contraction from the first carbachol response curve.

Intracellular mechanisms involved in fundus relaxation induced by activation of the nitrergic pathway

The role of sGC and small conductance $_{Ca}K^+$ channels on relaxation of gastric fundus induced by activation of the nitrergic pathway was analyzed in four parallel strips from non-treated rats.

a) The effect of nicotine (10 μM) on contraction induced by 5-HT (3 μM) was analyzed under NANC conditions (atropine 1 μM and guanethidine 5 μM) in strips incubated (15 min) with vehicle, L-NOARG (100 μM), ODQ (10 μM), or apamin (1 μM). Results are expressed as the percentage of relaxation.

b) The effect of the exogenous NO was analyzed by comparing the contraction induced by a submaximal dose of carbachol (0.1 μM) before and after incubation (10 min) with the NO donor (Z)-1-[2-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DETA-NONOate, 100 μM). Thereafter a third contraction with carbachol was performed, this time pre-incubated (10 min) with ODQ (10 μM) or apamin (1 μM). Results are expressed as a percentage of the contraction from the first curve.

c) In the last group, experiments were performed with the NO-independent sGC activator, BAY 41-2272 (3 μM). Contraction induced by 0.1 μM carbachol was compared before and after incubation (5 min) with BAY 41-2272 (3 μM) in the presence of vehicle, ODQ (10 μM), or apamin (1 μM). Results are expressed as a percentage of the contraction from the first curve.

Effect of endotoxin on NOS expression

Rats were killed 1 h after treatment with endotoxin (40 μg/kg, i.p.) or saline (1 ml/kg, i.p.). Fundal tissue was then frozen in liquid nitrogen and stored at –80°C. Expression of NOS was analyzed by the following techniques:

Quantification of nNOS and inducible NOS (iNOS) mRNA by real-time quantitative RT-PCR

Total RNA was isolated with TriPure Isolation Reagent (Roche Diagnostics) and treated with DNA-free (Ambion) in order to avoid contamination with genomic DNA. The resulting RNA was quantified by UV spectrophotometry, and its integrity was assessed by agarose gel electrophoresis. cDNA was synthesized from 2 μg of total RNA with SuperScript RT RNase H^- (Life Technologies), using 0.8 μg oligo dT_{16} (TIB Molbiol, Roche Diagnostics) and 40U RNase inhibitor (Roche Diagnostics) in a reaction volume of 20 μl. For each sample, controls were performed without reverse transcriptase (RT controls), and a negative control with water in place of the RNA was included.

Quantitative PCR was performed in a LightCycler instrument (Roche Diagnostics) with the use of a LightCycler-FastStart DNA Master SYBR Green I kit. Samples of cDNA (1 μl) were amplified with specific primers (14) for each of the cyclophilin A (CyPA, 0.5 μM), nNOS (0.5 μM), and iNOS (1 μM) genes (Table 1), in a solution containing 2 mM $MgCl_2$ and 5% DMSO (final volume 10 μl). Reactions were performed in duplicate, and a negative control with water instead of cDNA was included in each run. The reaction protocol includes an initial 10 min at 95°C to activate the polymerase. Each PCR cycle involved denaturation (95°C, 30 s), annealing (T_{ann} shown in the table, 30 s), and extension (72°C, 30 s). Fluorescence was measured after each cycle. Reaction specificity was tested by melting curve analysis (T_m, shown in Table 1) and agarose gel electrophoresis. To quantify input amounts of templates, a standard curve for each gene analyzed was obtained with serial dilutions of total RNA from a positive control (Table 1).

To normalize the results, interpolated values for each sample were divided by values for the housekeeping gene CyPA, and results are expressed as NOS/CyPA ratio.

Analysis of nNOS and iNOS protein expression by Western blot

Frozen tissues were homogenized (Ultra-Turrax®, IKA) in an ice-cold buffer (66 mM Tris-HCl pH 7.4; 1 mM EGTA; 1 mM sodium orthovanadate; and 1 mM sodium fluoride). Subsequently, NP-40 was added to a final concentration of 1%, and homogenates were sonicated and centrifuged (10.000 × g for 20 min at 4°C). The supernatant was taken and total protein concentration was determined by the bicinchonic acid method (BCA, Protein Assay Reagent, Pierce). Samples (100 µg of total protein) were resolved on a 7.5% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. Membranes were blocked with 5% non-fat dry milk in TBS-T (20 mM Tris-HCl, pH 7.2; 150 mM NaCl; 0.1% Tween 20) followed by incubation overnight (4°C) with a monoclonal anti-nNOS antibody (1:500, BD Transduction labs.) or a polyclonal anti-iNOS antibody (1:2000, BD Transduction labs). Protein bands were detected by incubation with horseradish peroxidase-conjugated goat anti-mouse IgG (1:2500, Santa Cruz Biotechnologies) or goat anti-rabbit IgG (1:5000, Vector Labs) as required, followed by enhanced chemiluminiscence (ECL Western blotting detection reagent, Amersham).

Determination of NOS activity

NOS activity was measured as the rate of conversion of L-[U-^{14}C]-arginine to L-[U-^{14}C]-citrulline, as described previously (4). Frozen samples were homogenized in an ice-cold buffer (60 mg tissue/ml; pH 7.2) containing 320 mM sucrose, 20 mM HEPES, 1 mM EDTA, 1 mM DL-dithiothreitol, 10 µg/ml leupeptin, 10 µg/ml soybean trypsin inhibitor, and 2 µg/ml aprotinin, followed by centrifuging at 10,000 × g for 20 min at 4°C. Afterwards, 40 µl of supernatant was incubated at 37°C for 10 min in assay buffer (pH 7.4) containing 50 mM KH_2PO_4, 1 mM $MgCl_2$, 0.2 mM $CaCl_2$, 50 mM L-valine, 1 mM L-citrulline, 1 mM DL-dithiothreitol, 100 µM NADPH, 3 µM FAD, 3 µM FMN, 3 µM BH_4, and 2.5 µM L-[U-^{14}C]-arginine (348 mCi mmol^{-1}). The reaction was stopped by addition of 1 ml of 1:1 (v/v) H_2O/Dowex-50W. After allowing the resin to settle for 15 min and centrifugation (2000 × g for 5 min at 4°C), the supernatant was removed (300 µl) for the estimation of the radiolabeled product by scintillation counting (3 ml Pico-Fluor). The specificity of L-arginine conversion to L-citrulline, by NOS, was further confirmed using 1 mM L-NOARG. EGTA (1 mM) was used to differentiate between the Ca^{2+}-dependent (nNOS and eNOS) and Ca^{2+}-independent (iNOS) activity. TRIM (25 mM) was used to differentiate nNOS from endothelial NOS (eNOS). To select the dose of TRIM, an inhibitory dose-response curve (1–30 mM) was performed in homogenates of rat cerebellum; 25 mM TRIM inhibited 88.8% of Ca^{+2}-dependent NOS activity. Protein content was determined by the bicinchonic acid method (BCA, Protein Assay Reagent, Pierce). All activities are expressed as picomol of product generated per minute per mg of protein.

Imaging of NO synthesis by confocal microscopy

NO synthesis was visualized with the cell permeable fluorescent precursor, DAF-FM (4-amino-5-methylamino-2',7'-difluorofluorescein) diacetate. Inside cells, this is hydrolyzed by cytosolic esterases to the non-permeable DAF-FM. In the presence of nitric oxide and oxygen, the relatively non-fluorescent DAF-FM is converted into the highly fluorescent and photo-stable

triazole form, DAF-FM T, whose fluorescence intensity is directly proportional to the NO concentration. The spectra of the adduct of DAF-FM is independent of pH, and the detection limit of NO by this fluorochrome is 3 nM (15, 16).

Tissue staining and confocal microscopy visualization

Five fundal strips were obtained according to the same protocol as described above for functional studies. Four strips were pre-incubated (25 min, 37°C) with carbogenated-Krebs containing vehicle, the NO synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME, 1 mM), TTX (1 μM), or hexamethonium (100 μM). Afterwards, both DAF-FM (1 μM) and the vital nuclear staining fluorochrome, Hoechst 33342 (1 μg/ml) were added and further incubated for 60 min at 37°C. To determine green basal autofluorescence and to establish confocal settings to obtain green fluorescence to minimal levels, Hoechst 33342 only was added to a vehicle-incubated strip. After rinsing, whole-mount preparations were mounted in a tissue slice chamber in carbogenated-Krebs solution. The slice chamber was set on a heated stage (Warner Instrument Corporation) at 37°C. Images were acquired with a Radiance 2100 confocal microscope (Bio-Rad, Hempel Hempstead, UK). A blue diode laser (405 nm) was used to excite Hoechst 33342, and a 488 nm laser line was used to excite DAF-FM. Fluorescence was acquired through 442/45 BP (Hoechst 33342) and 515/30 BP filters (DAF-FM). Confocal microscope settings were adjusted to produce the optimum signal-to-noise ratio. DETA-NONOate (100 μM) was added at the end of each experiment to test the responsiveness of the system. To compare fluorescence signals between different preparations, settings were fixed for all samples from the same animal. To analyze the spatial distribution of DAF-FM fluorescence in each preparation, a complete analysis along the whole-mount Z axis, from serosa to mucosa, was performed (0.6 μm optical section width, 5 μm step width). We used the morphology of cell nuclei to localize the different tissue layers in the gastric wall. Fluorescence analysis was performed by using LaserPix software (Bio-Rad).

Statistical analysis

Data are expressed as mean values ± SEM, and statistical comparison was performed by ANOVA followed by a Newman-Keuls test. Data were considered statistically significant when $P<0.05$. Cumulative concentration curves to carbachol were normalized by sigmoidal non-linear regression to obtain the pD_2 and E_{max} values (Graphpad Prism 3.0).

Drugs used

Escherichia coli endotoxin (serotype 026:B6), carbamylcholine chloride, hexamethonium chloride, L-NOARG, L-NAME, TRIM, VIP 10-28, suramin, PPADS, ODQ, apamin, atropine, guanethidine, nicotine, Hoechst 33342, and all reagents used for determination of NOS activity were purchased from Sigma Chemical Co. (St. Louis, MO). SQ 22536, TTX, and DETA-NONOate were obtained from Calbiochem® (Darmstad, Germany), Tocris Coockson (Bristol, UK), and Alexis corporation (San Diego, CA), respectively. Dexamethasone (Fortecortin®) was used as a clinically available preparation. L-[U-^{14}C]-arginine was obtained from Amersham Life Sciences (London, UK). The fluorochrome DAF-FM diacetate was purchased from Molecular Probes (Eugene, OR). BAY 41-2272 was the kind gift of Salvador Moncada at the Wolfson Institute for Biomedical Research (London, UK). Endotoxin was dissolved in saline, and ODQ,

TRIM, BAY 41-2272 and DAF-FM diacetate were dissolved in DMSO up to 10^{-2} M, 10^{-1} M, 10^{-3}M, and 5 mM, respectively. Further dilutions of these compounds and solutions of all other drugs were in distilled water.

RESULTS

Effect of endotoxin on the mechanical activity of isolated gastric fundus

In vivo pre-treatment with endotoxin decreases fundus contractility

A concentration-response curve to carbachol was performed in fundal strips from saline-treated animals (pD_2 = 6.2 ± 0.2, E_{max} = 6.3 ± 0.4 g, n = 5). Strips from rats treated with endotoxin (40 µg/kg, i.p.) exhibited a decrease in the E_{max} for carbachol (2.6 ± 0.6 g) with no change in the pD_2 (6.2 ± 0.1, n = 5) (Fig. 1).

Involvement of post-ganglionic myenteric neurons in the effect of endotoxin

Inhibition of neural transmission with the neurotoxin TTX or blockade of gangliar nicotinic receptor with hexamethonium both significantly prevented the inhibitory effect of endotoxin on fundus contractility. Neither TTX nor hexamethonium modified the contractile effect of carbachol in control animals (Fig. 2*A*).

Pre-treatment with dexamethasone did not significantly modify the response to carbachol in strips from saline- or endotoxin-treated animals (Fig. 3)

NO synthesis by nNOS mediates the effect of endotoxin

Blockade of NO synthesis by incubation with L-NOARG or the selective nNOS inhibitor TRIM prevented hypo-contractility induced by endotoxin. Neither L-NOARG nor TRIM modified the response to carbachol in strips from control animals (Fig. 2*B*).

Contractility in strips from endotoxin- and saline-treated animals was not influenced by incubation with the VIP antagonist, VIP 10-28 (Fig. 4*A*), nor the P_2-purinoceptor antagonists, suramin plus PPADS (Fig. 4*B*).

sGC and small conductance $_{Ca}K^+$ channels mediate the effect of endotoxin

Inhibition of guanylyl cyclase activity with ODQ prevented the endotoxin-induced hypo-contractility, while having no effect in control rats (Fig. 2*C*). In addition, selective inhibition of the small conductance $_{Ca}K^+$ channels by incubation with apamin blocked the inhibitory effect of endotoxin while leaving the control response unchanged (Fig. 2*C*).

Endotoxin-induced hypo-contractility (E_{max} 2.8±0.2 g) was not significantly modified (P>0.05) by inhibition of adenylyl cyclase activity with SQ 22536 (3.0±0.4 g). In fundus from saline-treated rats SQ 22536 did not significantly alter (5.5±1.1 g) the response to carbachol (5.9±1.2 g) n≥4.

None of the treatments above described modified the pD_2 for carbachol. In addition, at the concentrations used, none of the inhibitors or vehicles significantly affected the basal tone of the fundus strips.

In vitro incubation with endotoxin fails to modify fundus contractility

Incubation with endotoxin (100 ng/ml) for 30 min, 3 h, or 8 h failed to significantly modify the contractile response to carbachol compared with vehicle-incubated strips (89.1 ± 6.8%, 130.8 ± 6.4%, or 134.2 ± 10.0%, respectively vs. 77.4 ± 6.3%, 140.2 ± 8.9%, and 131.4 ± 13.4%, respectively). In a similar manner, incubation with a higher dose of endotoxin (1 µg/ml) for 3 h had no effect (121.0 ± 8.3%) compared with control strips (131.4 ± 13.4%). When incubated for 8 h, this higher dose induced a non-significant (P>0.05) decrease in the contractile response to carbachol compared with vehicle-incubated strips (107.9 ± 16.7% vs. 131.4 ± 13.4%). In no case were changes observed in the pD_2 (6.0 ± 0.2). In all cases n=5.

sGC and small conductance $_{Ca}K^+$ channels are involved in the relaxation of gastric fundus induced by the nitrergic pathway

Nicotine-induced relaxation of strips pre-contracted with 5-HT was significantly prevented by pre-incubation with L-NOARG, ODQ, or apamin (Fig. 5*A*).

Hypo-contractility to carbachol induced by pre-incubation with DETA-NONOate or BAY 41-2272 was significantly reversed in the presence of ODQ or apamin. These drugs did not modify the contractile effect of carbachol in the respective control strips (Fig. 5*B*, *C*).

Effect of endotoxin on NOS expression

In vivo pre-treatment with endotoxin does not modify the expressed levels of nNOS or iNOS mRNA

nNOS and iNOS mRNA were both detected in the gastric fundus of saline-treated rats, as analyzed by real-time quantitative RT-PCR. In fundus obtained from endotoxin-treated rats no significant changes in the amount of nNOS or iNOS mRNA were observed compared with control rats (Table 2).

In vivo pre-treatment with endotoxin does not modify nNOS or iNOS protein content

In the gastric fundus of animals treated with endotoxin, the amount of nNOS protein was similar to that detected in the fundus of saline-treated rats (Fig. 6). iNOS protein was not detected in fundus homogenates of saline- or endotoxin-treated rats. As a positive control, iNOS protein was detected in lung and fundus extracted from a rat treated for 8 h with a higher dose of endotoxin (10 mg/kg, i.p.; Fig. 6).

In vivo pre-treatment with endotoxin does not modify NOS enzyme activity

Pre-treatment with endotoxin for 1 h did not modify total NOS activity in the gastric wall when compared with control rats (5.7±0.5 vs. 6.2±0.3 pmol/min mg protein). In both cases, this activity was due to Ca^{+2}-dependent NOS isoforms, with Ca^{+2}-independent NOS activity (iNOS)

barely detectable. The use of TRIM showed that the Ca^{+2}-dependent NOS activity was mainly due to nNOS (Fig. 7).

In vivo pre-treatment with endotoxin increases NO synthesis in the myenteric plexus of the gastric fundus

Under our experimental conditions, control staining did not show significant green autofluorescence (Fig. 8*A*). After DAF-FM staining, strips from saline-treated rats exhibited a generalized weak green fluorescence signal (Fig. 8*B*, *J*). However, this fluorescence was not indicative of NO synthesis because addition of L-NAME did not reduce it (Fig. 8*C*, *J*). Furthermore, pre-incubation with TTX or hexamethonium also failed to modify the DAF-FM fluorescence signal (Fig. 8*J*).

Fundal strips from endotoxin-treated rats exhibited an intense DAF-FM fluorescent signal in the myenteric plexus (Fig. 8*J*). Moving along the fundus whole-mount Z axis, DAF-FM fluorescence was localized in enteric nerve cell bodies and nerve fibers within myenteric ganglia (Fig. 8*D*), as well as in nerve fibers running parallel to smooth muscle cells (Fig. 8*E*). There was only an extremely weak DAF-FM fluorescent signal in the deep circular smooth muscle layer (Fig. 8*F*).

Pre-incubation with L-NAME prevented the appearance of green fluorescence in strips from endotoxin-treated rats, confirming the NO-specificity (Fig. 8G, 8J). In a similar manner pre-incubation with TTX or hexamethonium impeded the green fluorescence induced by endotoxin-treatment, further confirming the neural cellular origin and suggesting the location of NO synthesis in post-ganglionic myenteric neurons (Fig. 8*H–J*, respectively).

DISCUSSION

In this study we describe the first visual detection of NO synthesis in rat gastric post-ganglionic myenteric neurons, in the fundus of rats treated with endotoxin. We further demonstrate that nitrergic signaling by these neurons mediates the gastric hypo-contractility in response to endotoxin. The effect of NO on gastric smooth muscle is mediated via activation of sGC and small conductance Ca^{2+}-activated K^{+}channels.

Enteric nervous system modulates gastric motor function during early endotoxemia

Endotoxemia caused by infection with gram-negative bacteria is associated with changes in gastric motor function such as a delayed gastric emptying (1, 2, 14). The present study shows that systemic administration of endotoxin to rats causes hypo-contractility to carbachol in isolated fundus strips. This effect is rapid in onset and independent of endotoxin-induced protein synthesis since in vivo pre-treatment with dexamethasone (14, 17) did not prevent its effect, thus suggesting a neural mechanism. This is reinforced by the fact that addition of tetrodotoxin to the organ bath prevented the hypo-contractility. Furthermore, this neural mechanism involves cholinergic synaptic transmission, since hexamethonium also prevented the effect of endotoxin. These observations, and the failure of endotoxin to modify gastric contractility when added to the organ bath, suggest that hypo-contractility is consequence of an extrinsic regulation of motor neurons rather than a direct action of endotoxin on enteric neurons or smooth muscle cells. It is important to note that the effect of endotoxin treatment is maintained after extrinsic denervation

of the gut, suggesting that once the challenge disappears, regulatory mechanisms at the synaptic transmission could be involved (18). Previous studies have reported a role for capsaicin-sensitive afferent vagal neurons in the delayed gastric emptying induced by systemic administration of low doses of endotoxin (2). In addition, these doses have been shown to activate the early-appearing gene product c-fos in the caudal part of the dorsal motor nucleus of the vagus nerve (4, 19) where pre-ganglionic neurons of the inhibitory motor pathway have been reported (20). Taken together the present results suggest that systemic administration of low doses of endotoxin activates a vago-vagal reflex in which the fundus of the stomach dilates. A similar mechanism has been observed in the receptive relaxation of the gastric fundus in response to food (11) or during early stages of vomiting, and might be involved in the delayed gastric emptying associated with endotoxemia.

nNOS-derived NO is involved in early endotoxemia

Inhibitory neurotransmission in the myenteric plexus is mainly mediated by the release of non-adrenergic non-cholinergic neurotransmitters. NO plays an essential role, both in intrinsic inhibitory reflexes that regulate adaptive relaxation of the fundus in response to small increases in intragastric pressure (12) and in extrinsic reflexes involved in the receptive relaxation of the fundus in response to food (11). In the present study, inhibition of NO synthesis in gastric tissue from control animals failed to alter the basal tone and the contractile response to carbachol. It seems that NO, in contrast to what happens in the intestine (21), does not modulate the resting tone of the rat gastric fundus. However, synthesis of NO takes place in fundus of endotoxin-treated rats, since addition of L-NOARG prevented hypo-contractility. This synthesis appears to be mediated by the nNOS isoform, as a selective nNOS inhibitor (22) also blocked the response to carbachol. In addition, nNOS-derived NO synthesis is not regulated at the transcriptional level, because endotoxin treatment did not increase nNOS mRNA or nNOS protein content. These results suggest that in vivo pre-treatment with endotoxin activates a neural mechanism that results in increased nNOS-mediated NO synthesis in the gastric wall. nNOS activity in the enteric nervous system is predominantly regulated by activation of nicotinic receptors and stimulation of a Ca^{2+}-dependent protein kinase C pathway (8, 23). In the present study, analysis of the catalytic activity of NOS in control animals was significantly reduced by incubation with Ca^{2+}-free medium, L-NAME, or TRIM, together suggesting that NO synthesis in the fundus is mainly due to the nNOS isoform. However, no differences in nNOS activity were observed between fundus homogenates from saline- or endotoxin-treated rats. As previously shown (24), regulatory mechanisms operating on constitutive NOS activity in intact tissue, but lost in tissue homogenates, can account for the differences between the results of functional and biochemical experiments.

In light of these discrepancies, we tried to visualize NO synthesis in the gastric fundus by a specific and sensitive technique which exploits the NO-reactive fluorescence indicator DAF-FM in conjunction with confocal laser scanning microscopy (15, 25–28). Previous immunohistochemical studies have demonstrated the presence of NOS in different cell types of the gastric wall (10, 29, 30). Furthermore, a chemiluminescence method allowing the indirect detection of NO in the myenteric plexus has been described (31). However, this is the first study to report the real-time visualization of intracellular NO production in gastric tissue by a direct and non-invasive method. This approach allows functional analysis of NO and offers valuable information about the cellular source of the NO. This is of special interest for this study, given

the wide expression of nNOS in gastric tissue (32, 33). Thus, in whole-mount fundus from control animals, only a slight non-specific background fluorescence signal was observed, suggesting that the basal release of NO was below the detection limit of the fluorochrome (3 nM), that implies a very low basal synthesis of NO in gastric fundus. This observation is consistent with the failure of NOS inhibitors to modulate resting tone and the low spontaneous neuronal activity described in circular muscle strips of the rat gastric fundus (34–36). By contrast, microscopic analysis of fundal strips from endotoxin-treated rats exhibited an intense DAF-FM fluorescence, which was indicative of NO synthesis because pre-incubation with L-NAME significantly prevented it. The fluorescent signal observed was significantly higher than that present in fundal strips from saline-treated rats, suggesting that NO synthesis was induced by endotoxin-treatment. This synthesis was specifically localized in neuronal soma and nerve endings that innervate the circular muscle, and was absent in smooth muscle cells. These observations, coupled with the fact that nNOS is primarily expressed in neurons (32), led us to conclude that neuronal cells are the source of NO synthesis involved in fundus relaxation. Furthermore, by combining microscopic techniques with pharmacological tools such as TTX and hexamethonium, our results locate NO synthesis induced by endotoxin to post-ganglionic myenteric neurons.

iNOS-derived NO is not involved in early endotoxemia

Induction of iNOS has been observed in a variety of tissues, such as liver and lung after in vivo administration of high doses of endotoxin (37). In addition, a role for iNOS-derived NO in the hypo-contractility induced by endotoxin has been shown in both ileal and vascular smooth muscle (30, 38). We, however, found no significant changes in iNOS mRNA expression, iNOS protein content, or Ca^{2+}-independent NOS activity in strips from endotoxin-treated rats compared with controls. Furthermore, our results do not support a role for iNOS-derived NO synthesis in the decrease in gastric tone induced by endotoxin since in vivo pre-treatment with dexamethasone failed to modify the effects of endotoxin. The low doses of endotoxin used and the short time over which its effects are analyzed could explain the lack of iNOS expression, which normally is seen after longer exposure, even to higher doses of endotoxin (39, 40).

sGC and small conductance $_{Ca}K^+$ channels mediate fundus hypo-contractility

Taken together, our results show that nNOS-mediated NO synthesis in gastric post-ganglionic myenteric neurons is involved in the hypo-contractility to carbachol induced by endotoxin. Most of the effects induced by the constitutive synthesis of NO, including relaxation of the circular muscle of the rat fundus, involve activation of sGC (41, 42). In the present study, inhibition of sGC activity by incubation with ODQ prevented the hypo-reactivity induced by endotoxin, suggesting that activation of the sGC is involved, and reinforcing the role of endogenous NO in the effect of endotoxin. In addition, activation of small conductance $_{Ca}K^+$ channels has been reported in the relaxation of gastrointestinal tissue induced by endogenous and exogenous NO (10, 43). Results show that local blockade of small conductance $_{Ca}K^+$ channels by apamin partially prevented the hypo-reactivity induced by endotoxin. Since a direct effect of endotoxin on these channels has been ruled out, these results suggest that it is NO released in response to endotoxin that acts on these channels. This observation is reinforced by the fact that relaxation of gastric fundus induced by exogenous NO (DETA-NONOate) or endogenous release of NO (nicotine) is also reversed by apamin. However, discrepancies still exist in the literature

regarding whether NO acts directly on $_{Ca}K^+$ channels (44) or if it is a cGMP-dependent mechanism (45). The present study shows a role for small conductance $_{Ca}K^+$ channels in the relaxation of the gastric fundus induced by an NO-independent sGC activator (46), suggesting that activation of these channels is due to cGMP rather than a direct action of NO. Taken together, these data suggest an intracellular mechanism of action of NO to relax the circular muscle of the rat gastric fundus based in a sequence of events: increase in cGMP and activation of small conductance $_{Ca}K^+$ channels in the smooth muscle cell membrane.

In addition to NO, other transmitters such as VIP or ATP are implicated in NANC relaxations of the rat gastric fundus (7, 47, 48). The present results do not support a role for VIP or ATP in the hypo-contractility induced by endotoxin. Blockade of the VIP receptor of gastric fundus (49) did not significantly modify the effects of endotoxin. In addition, activation of intracellular adenylyl cyclase, which is involved in VIP-induced relaxation of the gastric fundus (50), does not mediate the effects of endotoxin. In a similar manner blockade of purinoceptors (51–54) also failed to alter the hypo-contractility induced by endotoxin, ruling out a role for ATP.

In conclusion, the present study shows that NO synthesis in gastric post-ganglionic myenteric neurons is involved in the hypo-contractility induced by in vivo pre-treatment with endotoxin. These results and previous studies suggest the existence of a vago-vagal reflex activated in early endotoxemia, which serves to maximize the activity of the constitutive resources before the immune response is fully functioning.

ACKNOWLEDGMENTS

This study has been supported by grant FIS 01/1187 from the Ministerio de Sanidad y Consumo, grant SAF 2001-0763 from the Comisión Interministerial de Ciencia y Tecnología, and grant C 03/02 from Instituto de Salud Carlos III. E. Q. is the recipient of a fellowship from the Ministerio de Sanidad y Consumo.

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Received August 4, 2003; accepted November 21, 2003.

Table 1

Primer sequences, reaction data, and characteristics of specific PCR products for each gene analyzed

Target gene	Primer sequences (5'–3')	T_{ann} (ºC)	PCR cycles	T_m (ºC)	Size (bp)	Positive control
CyPA	CGTCTGCTTCGAGCTGTTTG (s) GTAAAATGCCCGCAAGTCAA (as)	60	30	81.7	464	Cerebellum
nNOS	ATCTCAGACCTGATTCGAGGAG G (s) ACTGTTGAGGATGCTCAGCACA G (as)	55	35	85.1	513	Cerebellum
iNOS	GCTACACTTCCAACGCAACA (s) ACAATCCACAACTCGCTCCA (as)	60	40	84.6	293	Lung (endotoxin 1 mg/kg, 12 h)

T_{ann}, annealing temperature; T_m, melting temperature; s, sense; as, antisense.

Table 2

nNOS and iNOS mRNA expression

Treatment	nNOS/CyPA ratio	iNOS/CyPA ratio
Saline (1 ml/kg, i.p.; 1h)	0.0186 ± 0.0020	0.2207 ± 0.0395
Endotoxin (40 µg/kg, i.p.; 1h)	0.0218 ± 0.0019	0.2097 ± 0.0509

Real-time quantitative RT-PCR of nNOS and iNOS mRNA expressed in gastric fundus of saline- and endotoxin-treated rats. Relative amounts of nNOS, iNOS or CyPA mRNA were read from their respective standard curves. Results express means ± SEM ($n \geq 3$).

Fig. 1

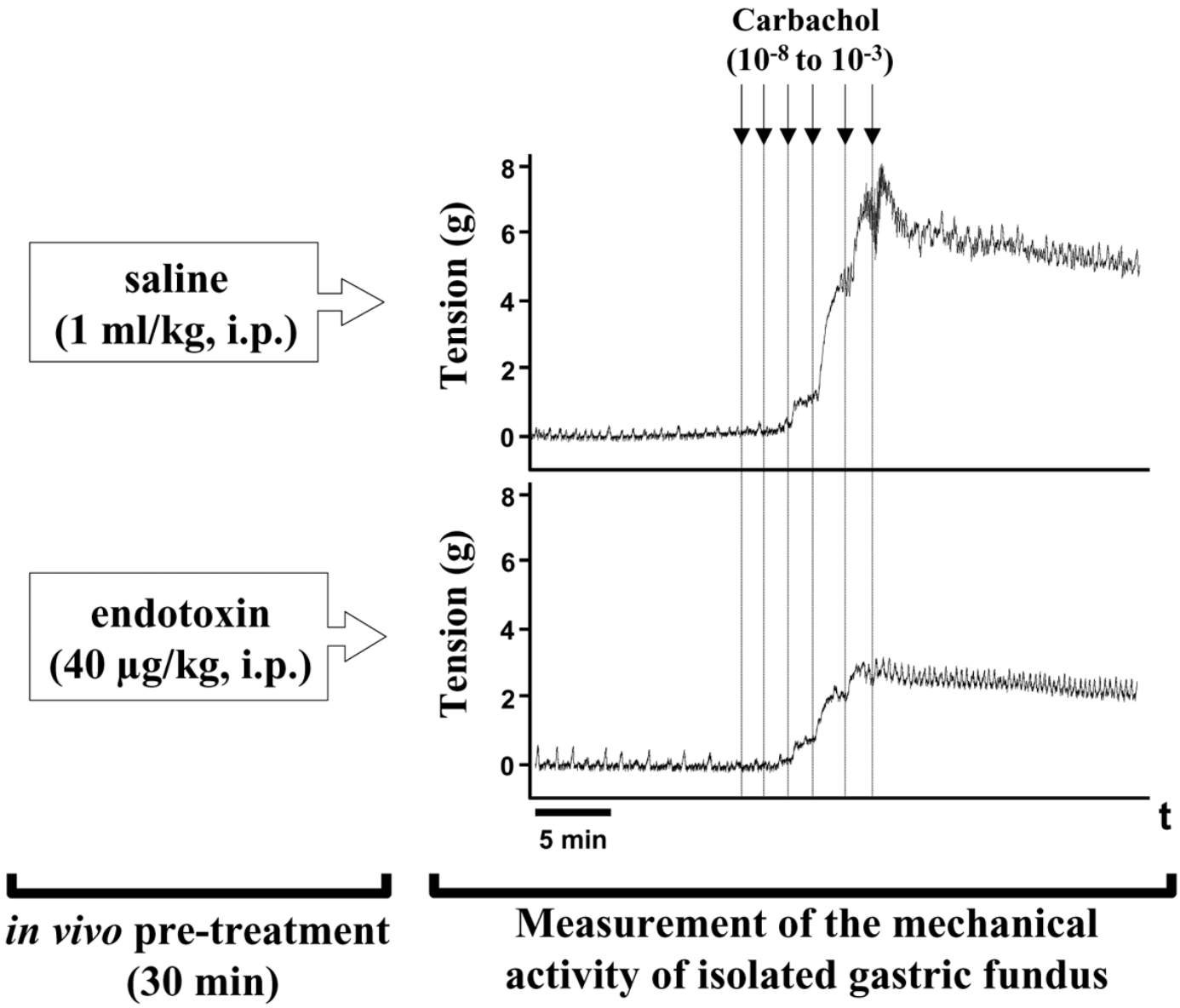

Figure 1. Original recording showing a representative concentration–response curve to carbachol on mechanical activity of isolated gastric fundus from rats treated with saline or endotoxin.

Fig. 2

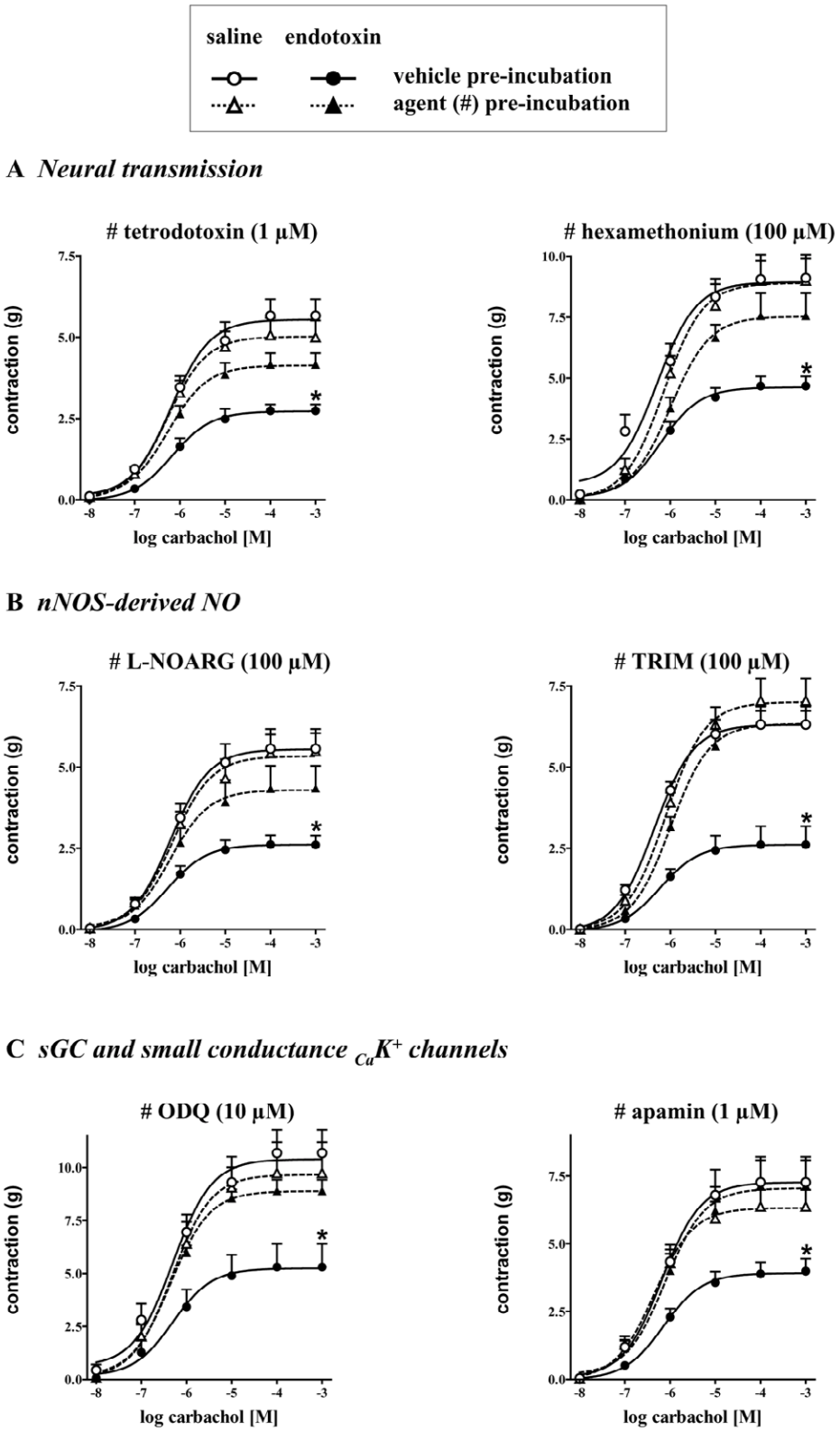

Figure 2. Roles for (***A***) post-ganglionic myenteric neurons, (***B***) nNOS-derived NO, and (***C***) activation of sGC and small conductance $_{Ca}K^+$ channels in fundus hypo-contractility to carbachol induced by in vivo pre-treatment with endotoxin. Results show concentration–response curves to carbachol in fundal strips from rats treated with saline (1 ml/kg, i.p.; open symbols) or endotoxin (40 µg/kg, i.p.; solid symbols). Data are expressed as means ± SEM ($n \geq 5$). *$P < 0.05$ for Emax vs. all experimental groups in each graph.

Fig. 3

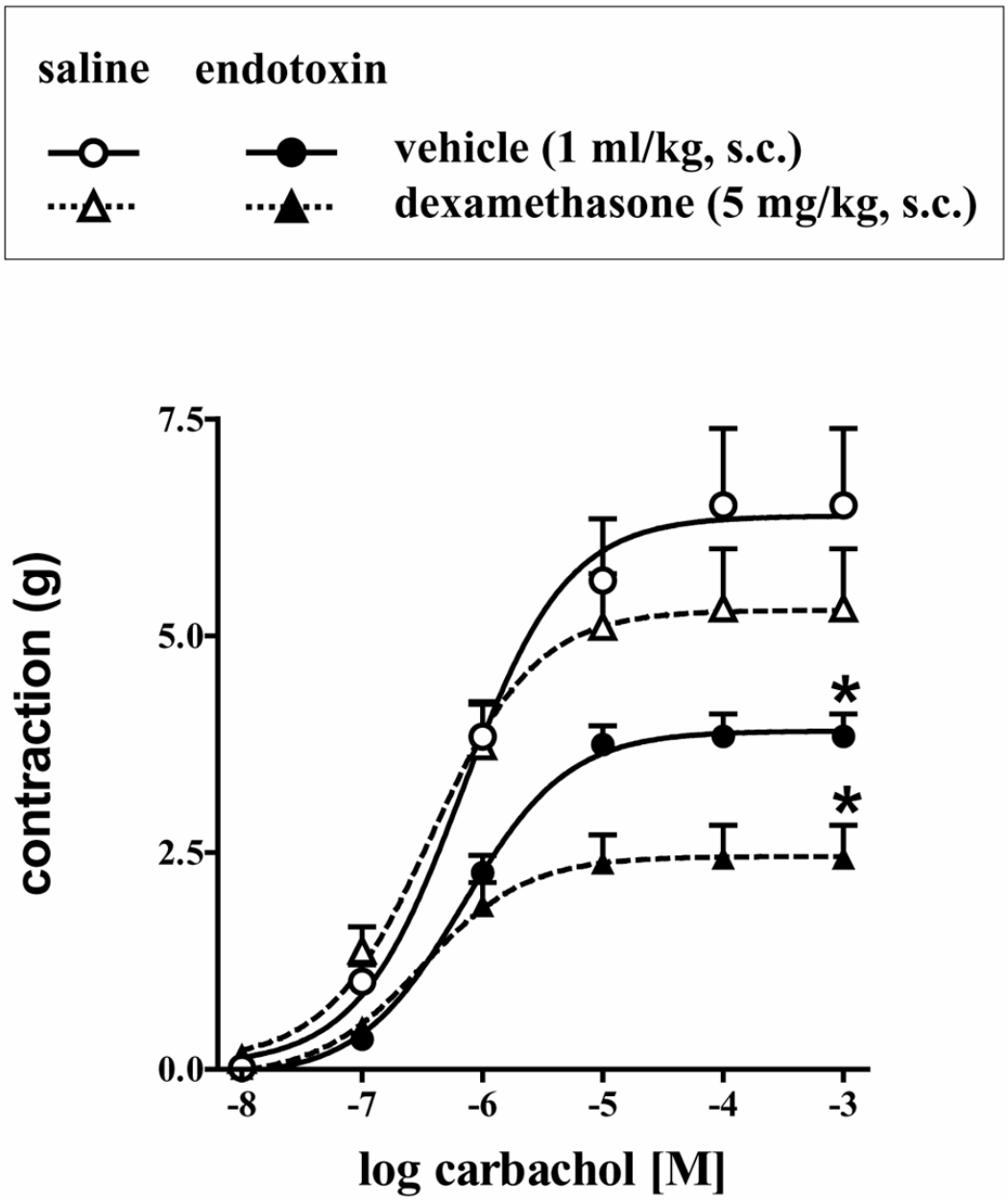

Figure 3. Effect of in vivo pre-treatment with dexamethasone on carbachol-induced fundus contraction in strips from rats treated with saline (1 ml/kg, i.p.; open symbols) or endotoxin (40 μg/kg, i.p.; solid symbols). Data are expressed as means ± SEM ($n \geq 4$). *$P < 0.05$ for E_{max} vs. the corresponding saline-treated group.

Fig. 4

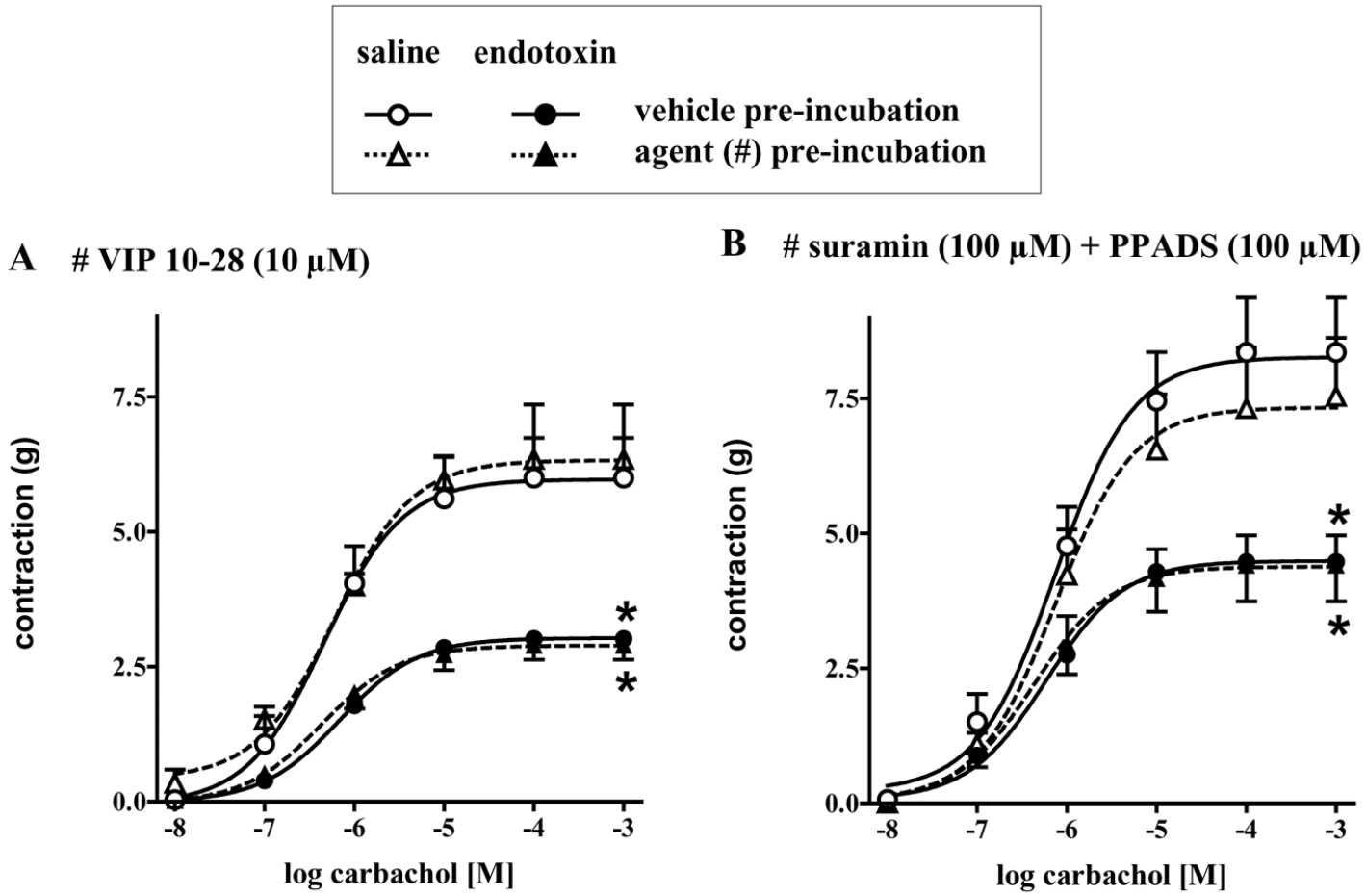

Figure 4. VIP and ATP are not involved in fundus hypo-contractility to carbachol induced by in vivo pre-treatment with endotoxin. Results show concentration–response curves of carbachol in fundal strips from rats treated with saline (1 ml/kg, i.p.; open symbols) or endotoxin (40 µg/kg, i.p.; solid symbols). Data are expressed as means ± SEM ($n \geq 3$). *$P <$ 0.05 for Emax vs. saline-treated groups in each graph.

Fig. 5

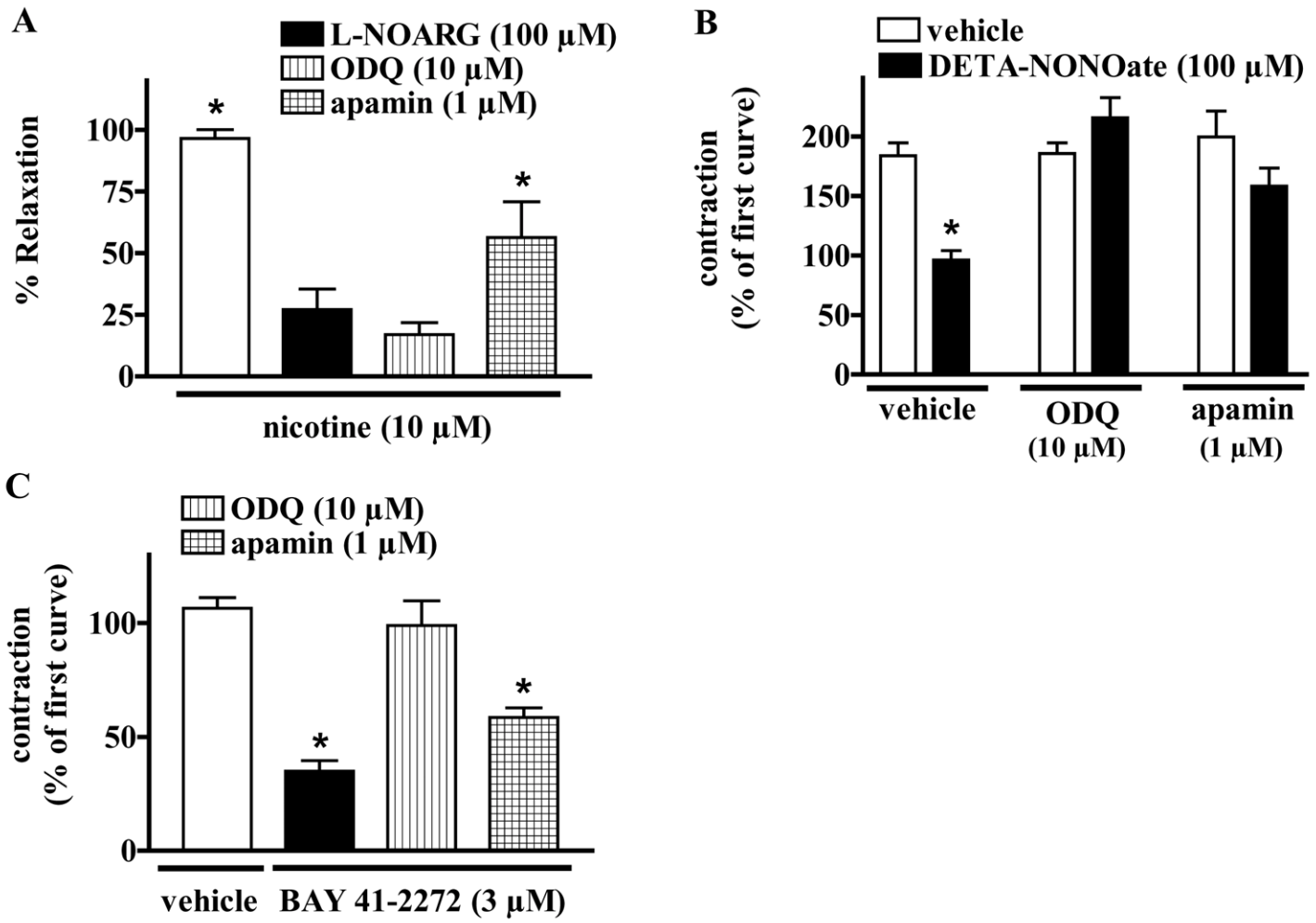

Figure 5. Role of small conductance $_{Ca}K^+$ channels and sGC in fundus relaxation induced by activation of the nitrergic pathway. ***A***) Relaxation induced by nicotine in 5-HT-(3 µM) pre-contracted strips under NANC conditions was evaluated in the presence of L-NOARG, ODQ, or apamin. Hypo-contractility induced by pre-incubation with (***B***) DETA-NONOate or (***C***) BAY 41-2272 on contraction induced by carbachol (0.1 µM) was analyzed in the presence of ODQ or apamin. Bars represent means ± SEM ($n \geq 3$) of the percentage of relaxation induced by nicotine (***A***), or the percentage of the contraction produced by the first addition of carbachol (***B***, ***C***). *$P < 0.05$ vs. all experimental groups in each graph.

Fig. 6

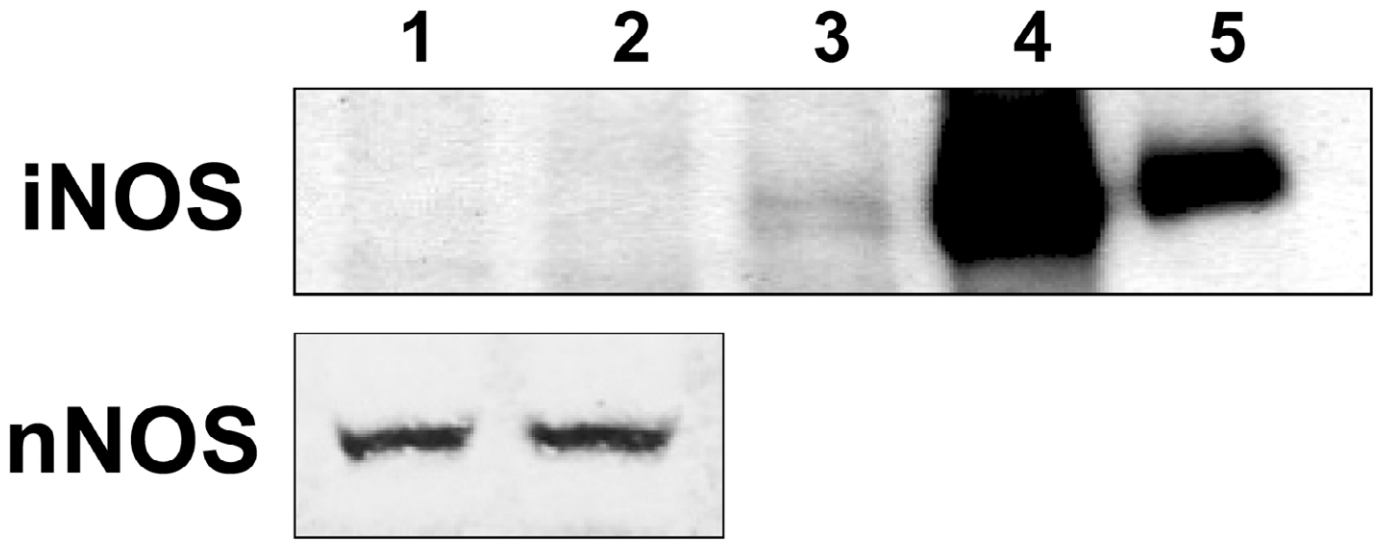

Figure 6. Western blot analysis of nNOS and iNOS in the gastric fundus. Lanes 1 and 2 correspond to fundus from saline-(1 mg/kg, i.p., 1 h) and endotoxin-treated (40µg/kg, i.p., 1 h) rats, respectively. Results are representative of three experiments. Positive controls for iNOS protein expression were obtained from the fundus (Lane 3) and lung (Lane 4) of endotoxin-treated rats (10 mg/kg, i.p., 8 h). Human recombinant iNOS is shown in Lane 5.

Fig. 7

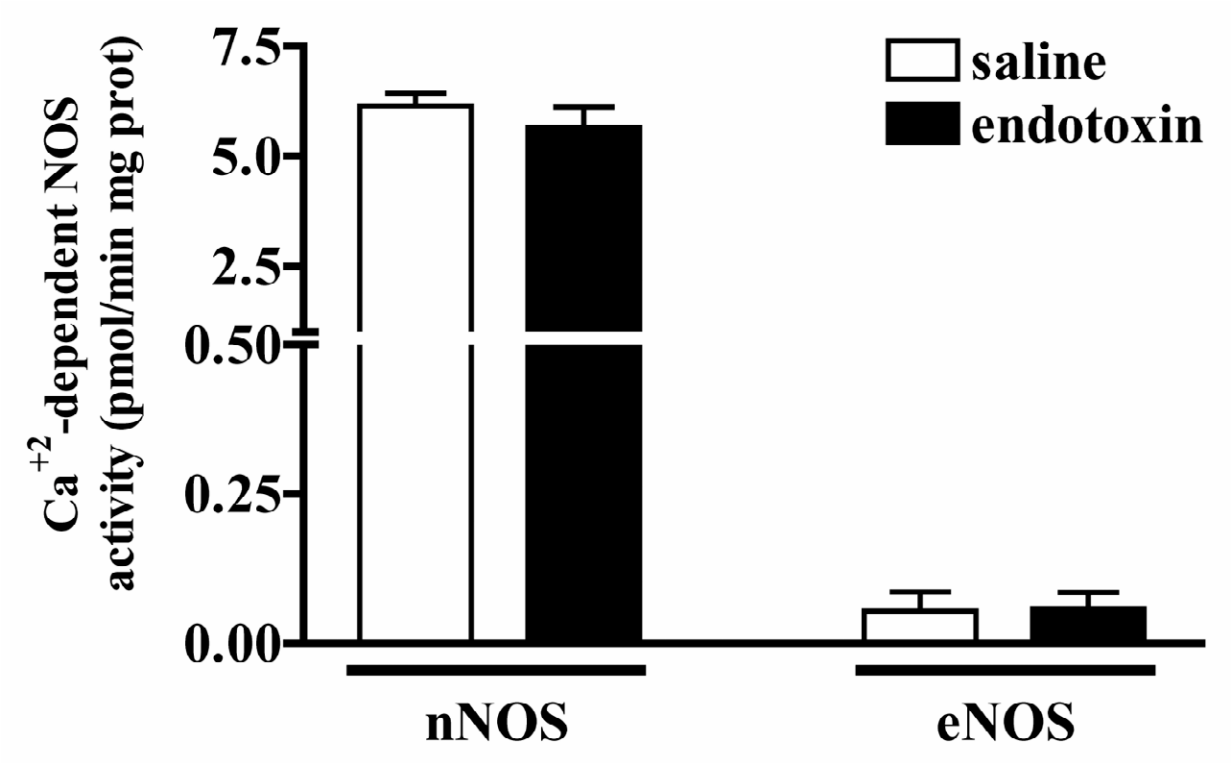

Figure 7. Calcium-dependent NOS activity in tissue extracts of gastric fundus from saline (1 ml/kg, i.p.)- and endotoxin (40 μg/kg, i.p.)-treated rats. Bars represent means ± SEM (*n*=5).

Fig. 8

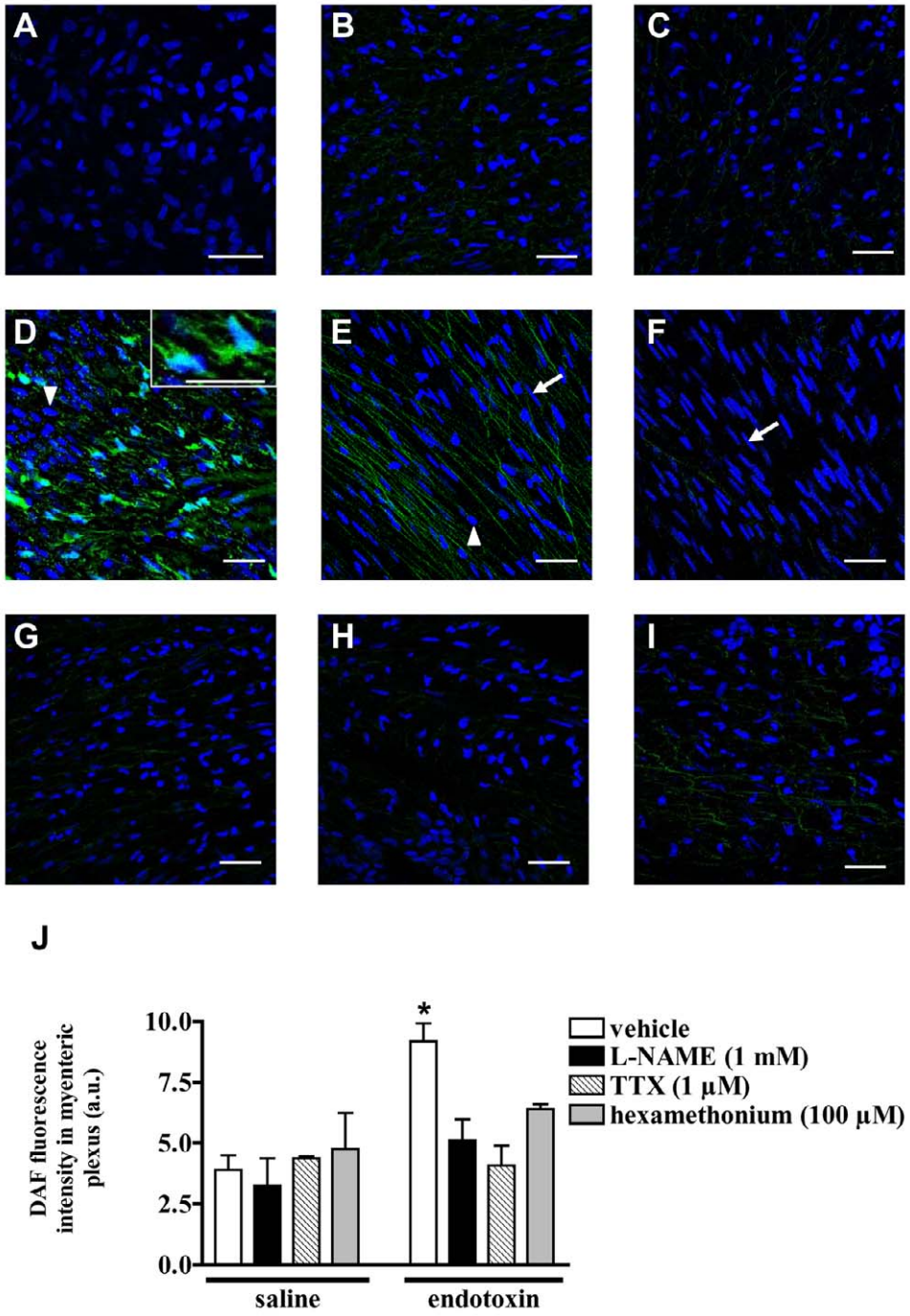

Figure 8. Confocal microscopy images of whole-mount preparations of gastric fundus from saline (1 ml/kg, i.p.)- (***B–C***) or endotoxin (40 µg/kg, i.p.)-treated rats (***D–I***). ***A)*** Only Hoechst 33342 staining (nuclear fluorochrome, blue) showing, almost undetectable, green auto fluorescence; (***B–I***) DAF-FM staining (indicative of NO synthesis; green) and Hoechst 33342. Nuclei from smooth muscle cells (arrows) and nerve cells (arrowheads) have different shape. ***B–C)*** DAF-FM fluorescence present in the myenteric plexus of a saline-treated rat (***B***); L-NAME pre-incubation failed to modify DAF-FM fluorescence (***C***). ***D–F)*** Three confocal images of whole-mount Z projection from the myenteric plexus (***D***) to the circular muscle layer (***F***). DAF-FM fluorescence is present in soma of the myenteric neurons (***D***, a higher magnification of a positive DAF-FM soma is shown in the insert), and nerve endings running parallel to the circular smooth muscle (***E***), while it is absent in the distal circular muscle layer (***F***). ***G–I)*** Pre-incubation with L-NAME (***G***), TTX (***H***), or hexamethonium (***I***) significantly reduced DAF-FM fluorescence in the myenteric plexus of endotoxin-treated rats. ***J)*** Graphic representation showing the quantification of DAF-FM fluorescence present in the myenteric plexus. Results express means ± SEM ($n \geq 3$). *$P < 0.05$ vs. all experimental groups. Scale bar = 25 µm.

Artículo 4

"*Endotoxin inhibits gastric emptying in rats via a capsaicin-sensitive afferent pathway*"

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Naunyn-Schmiedeberg's Archives of Pharmacology (2001) 363:276-280.

Naunyn-Schmiedeberg's Arch Pharmacol (2001) 363 : 276–280
DOI 10.1007/s002100000359

ORIGINAL ARTICLE

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Eugenia García-Zaragozá · Elsa Quintana
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Endotoxin inhibits gastric emptying in rats via a capsaicin-sensitive afferent pathway

Received: 3 December 1999 / Accepted: 28 September 2000 / Published online: 1 December 2000

Abstract The effects of endotoxin on gastric emptying of a solid nutrient meal and the neural mechanisms involved in such a response were investigated in conscious rats.

The intraperitoneal (i.p.) administration of *E. coli* endotoxin (40 μg/kg) significantly reduced the 4-h rate of gastric emptying of a standard solid nutrient meal. Ablation of primary afferent neurons by systemic administration of high doses of capsaicin (20+30+50 mg/kg s.c.) to adult rats did not modify the rate of gastric emptying in control animals but prevented the delay in gastric transit induced by endotoxin. Local application of capsaicin to the vagus nerve rather than application of capsaicin to the celiac ganglion significantly repressed endotoxin-induced delay in gastric emptying. Neither treatment modified the rate of gastric emptying in vehicle-treated animals. Blockade of CGRP receptors (CGRP 8–37, 100 μg/kg i.v.) did not alter gastric emptying in control animals but significantly prevented endotoxin-induced inhibition of gastric emptying. In contrast, a tachykinin receptor antagonist ([D-Pro^2, D-Trp^{7,9}]-substance P, 2 mg/kg i.p.) significantly reduced the rate of gastric emptying in control animals and did not modify the inhibitory effects of endotoxin. Adrenergic blockade with phentolamine (3 mg/kg i.p.) + propranolol (5 mg/kg i.p.) or muscarinic antagonism with atropine (0.1 mg/kg i.p.) failed to reverse the delay in gastric emptying induced by endotoxin.

These observations indicate that endotoxin-induced delay in gastric emptying of a solid nutrient meal is mediated by capsaicin-sensitive afferent neurons.

Keywords Gastric emptying · Transit · Endotoxin · Capsaicin · CGRP · Tachykinins

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Introduction

Administration of low doses of endotoxin to experimental animals induces acute changes in gastric function. Doses of endotoxin that do not modify systemic arterial blood pressure or rectal temperature selectively decrease gastric acid secretion (Barrachina et al. 1995a; Esplugues et al. 1996; Martínez-Cuesta et al. 1994), increase the mucosal resistance to damage (Barrachina et al. 1995b) and decrease gastric emptying of non-nutrient, usually liquid, meals (Collares 1997; Cullen et al. 1995; Takakura et al. 1997; van Miert and de la Parra 1970). These effects of endotoxin do not appear to be the consequence of a local mechanism and seem to be mediated by reflex pathways involving extrinsic innervation of the gut.

A delayed gastric emptying is commonly observed in many pathological circumstances associated with endotoxemia such as cirrhosis (Acalovschi et al. 1997; Isobe et al. 1994), diabetes, chronic renal failure (Kao et al. 1996), head injury (Kao et al. 1998), critical illness (Heyland et al. 1996) or the post-operative period (Ratych et al. 1991; Resnick et al. 1997; van Berge Henegouwen et al. 1997). In these conditions, a delay in the emptying of nutrients from the stomach is the principal cause of dyspepsia, a significant observation considering the lack of prior evidence about the gastric emptying of nutrient solid meals in endotoxemia.

In the present study, we evaluated the effect of small doses of lipopolysaccharide of *Escherichia coli* on the rate of gastric emptying of a standard solid meal in conscious rats. In addition, we analyzed the afferent and the efferent neural pathways involved in endotoxin-induced changes in gastric emptying.

Materials and methods

General. Gastric emptying of solids was measured as previously described (Barrachina et al. 1997). Male Sprague-Dawley rats (250–300 g) which had fasted for 16 h were given access to pre-weighed food for 3 h. The food was then removed and the animals were administered with endotoxin (*E. coli* lipopolysaccha-

ride, 40 μg/kg i.p.; this dose was selected from preliminary studies) or its vehicle (saline, 1 ml/kg). Four hours later, the rats were killed by cervical dislocation. The stomach was exposed through a laparotomy, quickly ligated at both the pylorus and cardia, then removed and its wet content weighed. Gastric emptying (GE) was calculated according to the following formula: GE=(1-wet weight of food recovered from the stomach/weight of food intake)×100.

Treatments. Under brief halothane anaesthesia, capsaicin (20+30+50 mg/kg s.c.) or its vehicle (10% ethanol + 10% Tween-80 + 80% saline) were subcutaneously administered on 3 consecutive days and the experiment was carried out 12–14 days later. The ablation of type C afferent fibers by this treatment was confirmed the day before the experiment by applying the eye wiping test. Animals treated with capsaicin which showed any wiping movements were excluded from the study.

Perineural capsaicin treatments were performed in rats anaesthetized with pentobarbital (50 mg/kg i.p.) and pretreated with atropine sulfate (1 mg/kg i.p.) in order to reduce the acute respiratory and cardiovascular effects of capsaicin. Through a midline neck incision, the cervical vagus was exposed and separated from the carotid artery by a distance of 2–3 mm: Parafilm was placed around the nerve, and a pledget of cotton wool soaked in capsaicin solution (1% w/v) or its vehicle (10% Tween-80 in olive oil) was wrapped around each vagus nerve for 30 min. The area was then thoroughly rinsed with sterile saline and the neck incision closed. Periceliac capsaicin application was performed through a midline abdominal laparotomy. The stomach and spleen were deflected to the right of the animal to facilitate the identification of the vascular references. Then, the celiac-superior mesenteric ganglion complex was exposed through a small incision in the surrounding connective tissue, and a pledget of cotton wool soaked in capsaicin solution (1% w/v) or its vehicle (10% Tween-80 in olive oil) was applied to the ganglion complex for 30 min. Afterwards, the area was rinsed thoroughly with sterile saline and dried with sterile swabs, and the laparotomy was closed. Experiments were performed 12–14 days after perineural capsaicin treatments.

In other experiments, 10 min before administration of endotoxin, rats received one of the following treatments: CGRP 8–37 (CGRP receptor antagonist, 100 μg/kg i.v.), D-Pro^2, D-Trp^{7,9}-substance P (tachykinin receptor antagonist, 2 mg/kg i.p.), propranolol (3 mg/kg i.p.) plus phentolamine (5 mg/kg i.p.), atropine sulfate (0.1 mg/kg or 1 mg/kg i.p.) or saline (1 ml/kg).

Statistical analysis. All data are expressed as means ± SEM. Comparisons between two groups were performed using Student's *t*-test and between three or more groups by one-way analysis of variance followed by Tukey test. *P*-values <0.05 were considered to be significant.

Drugs. Endotoxin (lipopolysaccharide from *E. coli*, serotype 0111:B4), CGRP antagonist (CGRP fragment 8–37), tachykinin receptor antagonist (D-Pro^2, D-Trp^{7,9}-substance P) and atropine sulphate were purchased from Sigma Chemical (USA). Capsaicin was purchased from Fluka Chemie. Propranolol (Sumial, Zeneca) and phentolamine (Regitina, Ciba) were used as clinically available preparations.

Results

Effects of endotoxin on gastric emptying of a solid nutrient meal

The 4-h rate of gastric emptying of a solid nutrient meal in conscious animals (63.6±5.2%, *n*=11) was significantly (*P*<0.05) diminished by a single intraperitoneal administration of endotoxin (28.4±5.1%, *n*=11; 40 μg/kg).

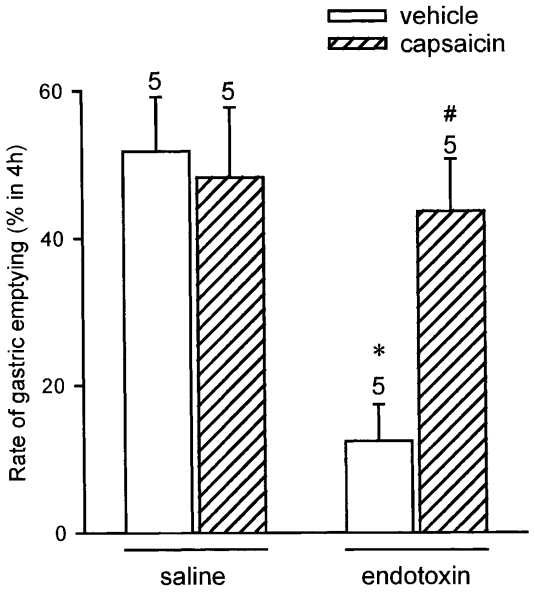

Fig. 1 Gastric emptying of a solid nutrient meal in rats receiving endotoxin (40 μg/kg i.p.) or saline (1 ml/kg i.p.) and pretreated with capsaicin (125 mg/kg s.c.) or its vehicle (ethanol, Tween 80 and saline, 10:10:80) 12–14 days before the experiments. Each *bar* represents mean ± SEM of the number of experiments shown above *column*. *P<0.05 vs. vehicle + saline-treated group, #P<0.05 vs. vehicle + endotoxin-treated group (ANOVA + Tukey test)

Effects of capsaicin on endotoxin-induced changes in gastric emptying

Blockade of afferent neurons with systemic administration of high doses of capsaicin reversed endotoxin-induced delay of gastric emptying by 90.4%. However, these doses of capsaicin did not significantly modify the 4-h rate of gastric transit in control animals (Fig. 1). Selective blockade of vagal capsaicin-sensitive neurons reversed the delay in gastric emptying induced by endotoxin by 39% (Fig. 2a). However, local administration of capsaicin to the celiac ganglion did not significantly modify the 4-h rate of gastric emptying in endotoxin-treated rats (Fig. 2b). In control animals the rate of gastric transit was not altered by local application of capsaicin, either in the vagus nerve or the celiac ganglion (Fig. 2).

Effects of CGRP and neurokinin receptor antagonist on endotoxin-induced changes in gastric emptying

Endogenous blockade of CGRP receptors by pretreatment with CGRP 8–37 (100 μg/kg i.v.) failed to modify gastric emptying in vehicle-treated animals, but significantly prevented endotoxin-induced delay in gastric emptying (Fig. 3).

In control animals, administration of [D-Pro^2, D-Trp^{7,9}]-substance P (2 mg/kg i.p.) significantly reduced (66.9±1.9%, *n*=3, *P*<0.05) the rate of GE compared with that in vehicle-treated rats (87.4±3.4%, *n*=5). Endotoxin-induced delay in gastric emptying (77.5±3.1%, *n*=4) was not sig-

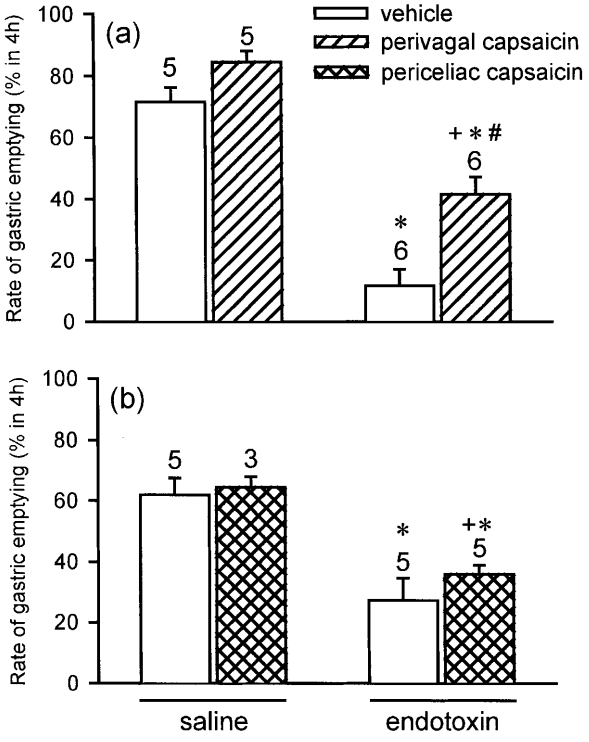

Fig. 2 Gastric emptying of a solid nutrient meal in rats receiving endotoxin (40 μg/kg i.p.) or saline (1 ml/kg i.p.) and pretreated with capsaicin (10 mg/ml) or its vehicle (Tween 80 and olive oil, 10:90) perineurally applied on **a** the cervical vagal trunks or **b** the superior mesenteric-celiac ganglia 12–14 days before the experiments. Each *bar* represents mean ± SEM of the number of experiments shown above *column*. **P*<0.05 vs. vehicle + saline-treated group, ^{+}P<0.05 vs. capsaicin + saline-treated group, $^{\#}P$<0.05 vs. vehicle + endotoxin-treated group (ANOVA + Tukey test)

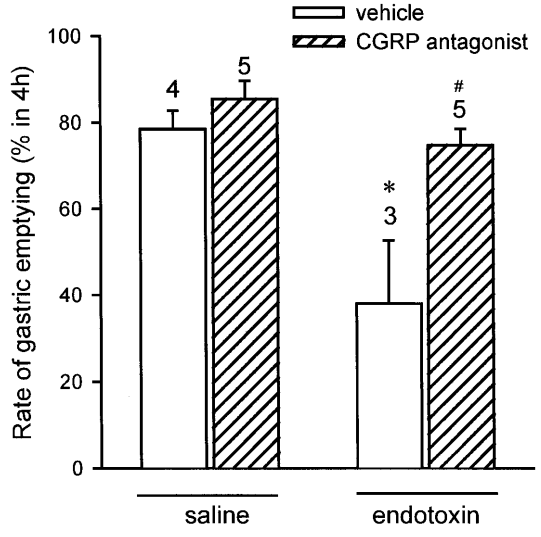

Fig. 3 Gastric emptying of a solid nutrient meal in rats receiving endotoxin (40 μg/kg i.p.) or saline (1 ml/kg i.p.) and pretreated with vehicle (saline, 1 ml/kg i.v.) or a CGRP receptor antagonist (CGRP 8–37, 100 μg/kg i.v.). Each *bar* represents mean ± SEM of the number of experiments shown above *column*. **P*<0.05 vs. vehicle + saline-treated group, $^{\#}P$<0.05 vs. vehicle + endotoxin-treated group (ANOVA + Tukey test)

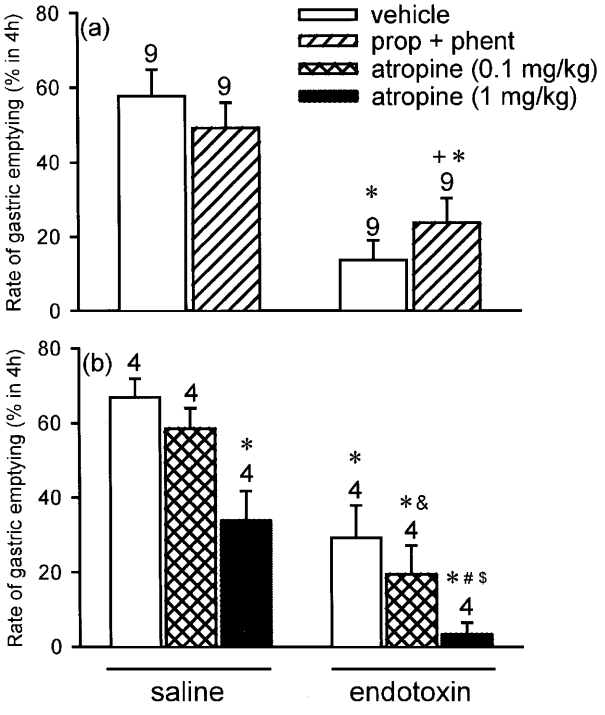

Fig. 4 Gastric emptying of a solid nutrient meal in rats receiving endotoxin (40 μg/kg i.p.) or saline (1 ml/kg i.p.) and pretreated with **a** propranolol plus phentolamine (3 mg/kg and 5 mg/kg i.p., respectively), **b** atropine (0.1 mg/kg and 1 mg/kg i.p.) or the common vehicle (saline, 1 ml/kg i.p.). Each *bar* represents mean ± SEM of the number of experiments shown above *column*. **P*<0.05 vs. vehicle + saline-treated group, ^{+}P<0.05 vs. propranolol + phentolamine + saline-treated group, $^{\&}P$<0.05 vs. atropine (0.1 mg/kg) + saline-treated group, $^{\#}P$<0.05 vs. atropine (1 mg/kg) + saline-treated group, $^{\$}P$<0.05 vs. vehicle + endotoxin-treated group (ANOVA + Tukey test)

nificantly modified by pre-treatment with [D-Pro^2, D-Trp^{7,9}]-substance P (75.1±3.0%, *n*=5; 2 mg/kg i.p.).

Effects of adrenergic or cholinergic blockade on endotoxin-induced changes in gastric emptying

A single i.p. administration of atropine (0.1 mg/kg) did not prevent the delay in gastric emptying induced by endotoxin and failed to modify the rate of gastric transit in vehicle-treated animals (Fig. 4b). Higher doses of atropine (1 mg/kg i.p.) induced a notable delay in gastric emptying in vehicle-treated rats and significantly potentiated the inhibition of gastric emptying induced by endotoxin (Fig. 4b). Adrenergic blockade after administration of propranolol (3 mg/kg i.p.) plus phentolamine (5 mg/kg i.p.) was also ineffective in preventing the inhibitory effect of endotoxin on gastric emptying and it did not significantly modify gastric transit in vehicle-treated animals (Fig. 4a).

Discussion

The present study demonstrates a delayed gastric emptying of a solid nutrient meal under endotoxemia. In con-

scious rats, a single intraperitoneal administration of low doses of endotoxin reduces the 4-h rate of gastric emptying of a solid nutrient meal. The inhibitory effect of endotoxin on gastric emptying has previously been reported for non-nutrient liquid meals (Collares 1997; Cullen et al. 1995; Takakura et al. 1997; van Miert and de la Parra 1970). However, gastric emptying of solids and liquids has been reported as two different physiological processes that are regulated in different ways and depend on distinct motor activities in the stomach, pylorus and proximal duodenum (Mayer 1994).

Capsaicin is a selective neurotoxin that, when administered either systemically or locally, produces a functional ablation of primary afferent C fibers and depletes the terminal fields of the neuropeptides of these neurons, such as tachykinins and CGRP (Holzer 1991). In the present study neither systemic pre-treatment with capsaicin nor blockade of CGRP receptors modified the rate of GE in control animals, suggesting that the afferent type C nervous fibers are not involved in the physiological control of gastric emptying of a solid nutrient meal in conscious animals, as stated in previous reports (Holzer 1986a). However, selective blockade of tachykinins receptors with D-Pro^2, D-Trp^{7,9}-substance P did significantly decrease GE, as previously reported (Holzer 1986b), suggesting a role for these neuropeptides in the control of gastric emptying. There are various possible explanations for the apparent discrepancy between capsaicin pre-treatment and blockade of tachykinins receptors: (a) endogenous tachykinins involved in the physiology of gastric emptying may originate from capsaicin-insensitive nerves (Holzer 1988); (b) chronic treatment with capsaicin vs. acute administration of the receptor antagonist could induce compensatory mechanisms that mask the role of tachykinins on GE. However, the possibility that another inhibitory neuropeptide, such as CGRP, is more prevalent after blocking the tachykinins receptors seems the most probable explanation.

In endotoxin-treated animals, both systemic pre-treatment with capsaicin and blockade of CGRP receptors significantly prevented the delay in gastric emptying associated with endotoxin. In contrast, administration of [D-Pro^2, D-Trp^{7,9}]-substance P did not significantly modify the inhibitory effects of endotoxin. In the gastrointestinal tract, capsaicin-sensitive afferent neurons appear to act through two mechanisms. One is related to the local effector function of the sensory nerve endings by which several neuropeptides are released (Holzer 1988, 1991), which modulates gastrointestinal functions, including gastric motility (Barthó and Holzer 1985; Holzer 1998; Zittel et al. 1994). It seems that endotoxin activates the sensory nerve endings of these fibers, leading to the release of CGRP, which in turn inhibit gastric emptying. The other mechanism activated by capsaicin-sensitive neurons involves the sending of somatovisceral sensory information to the neuronal network in the brain and the spinal cord, which initiates the efferent reflex loop. In the present study, local application of capsaicin to the cervical vagus nerve significantly repressed the inhibitory effect of endotoxin on gastric emptying, while direct application of capsaicin to the celiac ganglion did not induce any change. A dissociation of afferent and local effector roles of capsaicin-sensitive sensory neurons has been reported and there is evidence that both functions are mediated by completely different neurons (Holzer 1988). This hypothesis would explain the differences observed in endotoxin-treated animals between the full normalization of GE after systemic administration of capsaicin and the partial prevention after selective perivagal application.

Considered as a whole, these results suggest that capsaicin-sensitive afferent neurons activated by peripheral endotoxin, in addition to having a local effector function, send a signal through the vagus nerve to the superior neuronal network by which gastric emptying is inhibited. This is supported by immunohistochemical studies reporting fos immunoreactivity in primary afferent neurons of the vagus nerve (Gaykema et al. 1998) and in different areas of the brain after peripheral administration of low doses of endotoxin (Tkacs and Strack 1997).

In order to determine the efferent pathway involved, and considering that some of the central nuclei activated by peripheral endotoxin are involved in the autonomic control of gastric function (Mascarucci et al. 1998; Tkacs and Strack 1997), the role of cholinergic and adrenergic receptors in endotoxin-induced changes in gastric emptying has been evaluated. Administration of atropine at doses that did not modify the rate of gastric transit in control animals failed to prevent the delay in gastric transit induced by endotoxin. Higher doses of atropine significantly decreased the rate of gastric emptying in vehicle-treated animals, confirming the well-known role of muscarinic receptors in the physiological control of gastric motility. In addition, these doses of atropine induced a further inhibition of gastric emptying in endotoxin-treated rats. These data allow us to make two observations: first, the cholinergic receptors are not involved in endotoxin-induced delay in gastric emptying; second, withdrawal of vagal cholinergic activation is not the mechanism through which endotoxin inhibits gastric motor function.

Similar results were obtained with α- and β-adrenoceptor antagonists. The lack of effect of phentolamine and propranolol on gastric transit in endotoxin and vehicle-treated animals suggests that α- and β-adrenoceptors are not involved in the pathophysiological control of gastric emptying, as previously reported in different models (Forster et al. 1991).

In conclusion, the present data indicate that the delay in gastric emptying induced by peripheral endotoxin is mediated through a local effector function of capsaicin-sensitive sensory nerve endings and through the release of CGRP. In addition, these neurons send information through a vagal afferent pathway to the superior neuronal network where an inhibitory non-adrenergic, non-cholinergic mechanism is, in turn, activated.

Acknowledgements The present study has been supported by grants SAF 99-0114, SAF 98-0118 and 1FD97-1029 from Ministerio de Educación y Cultura. S.C. is the recipient of a fellowship from Conselleria de Educación y Ciencia (Generalitat Valenciana),

and E.G. and E.Q. are the recipients of a fellowship from Ministerio de Educación y Cultura and Ministerio de Sanidad y Consumo, respectively. The experiments comply with current Spanish law.

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Artículo 5

"*Downregulation of nNOS and synthesis of PGs associated with endotoxin-induced delay in gastric emptying*"

Sara Calatayud, Eugenia García-Zaragozá, Carlos Hernández, Elsa Quintana, Vicente Felipo, Juan V. Esplugues & María D. Barrachina

American Journal of Physiology (2002) 283: G1360-G1367.

Am J Physiol Gastrointest Liver Physiol 283: G1360–G1367, 2002;
10.1152/ajpgi.00168.2002.

Downregulation of nNOS and synthesis of PGs associated with endotoxin-induced delay in gastric emptying

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Received 9 May 2002; accepted in final form 27 August 2002

Calatayud, Sara, Eugenia García-Zaragozá, Carlos Hernández, Elsa Quintana, Vicente Felipo, Juan Vicente Esplugues, and María Dolores Barrachina. Downregulation of nNOS and synthesis of PGs associated with endotoxin-induced delay in gastric emptying. *Am J Physiol Gastrointest Liver Physiol* 283: G1360–G1367, 2002; 10.1152/ajpgi.00168.2002.—A single intraperitoneal injection of endotoxin (40 μg/kg) significantly delayed gastric emptying of a solid nutrient meal. Blockade of nitric oxide synthase (NOS) with 30 mg/kg ip N^G-nitro-L-arginine methyl ester or 20 mg/kg ip 7-nitroindazole [neuronal NOS (nNOS) inhibitor] significantly delayed gastric emptying in control animals but failed to modify gastric emptying in endotoxin-treated rats. Administration of 2.5, 5, and 10 mg/kg ip N^6-iminoethyl-L-lysine [inducible NOS (iNOS) inhibitor] had no effect in either experimental group. Indomethacin (5 mg/kg sc), NS-398 (cyclooxygenase-2 inhibitor; 10 mg/kg ip), and dexamethasone (10 mg/kg sc) but not quinacrine (20 mg/kg ip) significantly prevented delay in gastric emptying induced by endotoxin but failed to modify gastric emptying in vehicle-treated animals. Ca^{2+}-dependent NOS activity in the antrum pylorus of the stomach was diminished by endotoxin, whereas Ca^{2+}-independent NOS activity was not changed. In addition, decreased nNOS mRNA and protein were observed in the antrum pylorus of endotoxin-treated rats. Our results suggest that downregulation of nNOS in the antrum pylorus of the stomach and synthesis of prostaglandins mediate the delay in gastric emptying of a solid nutrient meal induced by endotoxin.

nitric oxide; prostaglandins; antrum pylorus; nutrient meals

ENDOTOXEMIA AFTER INFECTION with gram-negative bacteria is associated with clinical abnormalities of gastric motor function (such as vomiting) broadly characterized by a decrease in gastric emptying. Experimental administration of the lipopolysaccharide of *Escherichia coli* is known to delay gastric emptying (36, 37, 39), and capsaicin-sensitive afferent neurons and nonadrenergic noncholinergic mechanisms have recently been shown to be involved (6).

Nitric oxide (NO) has a leading role as an inhibitor neurotransmitter of peripheral nonadrenergic noncholinergic nerves (1, 8). In the gastrointestinal tract, neuronal NO plays a role in the physiology of gastric motor function. NO is involved in the reflex relaxation of the gastric fundus to accommodate food or fluid (7) and mediates pyloric relaxation and intestinal feedback regulation, thereby facilitating gastric emptying (2). A recent study (21) analyzing gastric emptying in neuronal NO synthase (nNOS) knockout mice points to a prominent role for this isoenzyme in the modulation of gastric motor function. However, little is known about the role of nNOS in changes in gastric emptying associated with pathophysiological circumstances such as endotoxemia. Taking into account that endotoxin has been widely shown to increase the expression of the inducible NOS (iNOS) in different tissues (5), the specific role of nNOS and iNOS in endotoxin-induced delay in gastric emptying has been evaluated.

Prostaglandins synthesized from arachidonic acid act as local regulatory agents that modulate gastric motor function (32). Endogenous prostaglandins have been involved in the inhibition of gastric emptying induced by IL-1β (35), and exogenous administration of these prostanoids delays gastric emptying (33). Synthesis of prostaglandins is carried out by cyclooxygenase (COX), which exists as two isoenzymes, COX-1 and -2, and the role of prostaglandins on gastric emptying has been established mainly through the use of nonselective COX inhibitors. Both isoenzymes synthesize prostanoids that mediate physiological functions (38). However, the formation of proinflammatory prostaglandins is mostly catalyzed by COX-2, and expression of this isoform is induced by a variety of stimuli, including endotoxin (12). This study also aims to determine the role and the enzymatic source of prostaglandins in the delay of gastric emptying induced by endotoxin.

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MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (250–300 g) (Harlan Laboratories, Barcelona, Spain) were maintained ad libitum on standard Purina laboratory chow and tap water and were housed under conditions of controlled temperature (21 ± 1°C), humidity (30–35%), and lighting (0700–1900 h). All experiments began between 0900 h and 1000 h and were performed in animals deprived of food for 16–18 h but with access to water before and during the experiments.

All protocols comply with the European Community guidelines for the use of experimental animals and were approved by the ethics committee of the Faculty of Medicine of Valencia.

Measurement of Gastric Emptying

Gastric emptying of solids was measured as previously described (6). Rats were placed in individual cages and were given access to preweighed food for 3 h. Food was then removed and 40 μg/kg ip endotoxin (*E. coli* lipopolysaccharide) or 1 ml/kg ip saline was administered to the animals. Four hours later, the rats were killed by cervical dislocation. The stomach was exposed by laparotomy, quickly ligated at both the pylorus and cardias and removed, and its wet content was weighed. Gastric emptying (GE) was calculated according to the following formula: GE = (1 − wet wt of food recovered from stomach/weight of food intake) × 100.

Treatments

To analyze the role of NO in the rate of gastric emptying of a solid nutrient meal, animals received a single intraperitoneal injection of 30 mg/kg N^{ω}-nitro-L-arginine methyl ester (L-NAME) (a NOS inhibitor), 20 mg/kg 7-nitroindazole (a selective nNOS inhibitor), or the respective vehicle (1 ml/kg saline or 0.5 ml/kg DMSO) 2 h after the administration of endotoxin or saline. Some rats received 2.5, 5, or 10 mg/kg ip N^6-iminoethyl-L-lysine (L-NIL) (a selective iNOS inhibitor) or its vehicle (1 ml/kg ip saline) 15 min before endotoxin or saline, and gastric emptying was determined as described above.

To determine the role of prostaglandins in the rate of gastric emptying of a solid nutrient meal, rats were pretreated with 5 mg/kg sc indomethacin (a dual inhibitor of COX-1 and -2), a COX-2-selective dose of 10 mg/kg ip NS-398 (20), or their respective vehicles (1 ml/kg sc $NaHCO_3$ 5%, or 0.5 ml/kg ip DMSO) 60 min before the administration of endotoxin or saline. Some animals received a single intraperitoneal injection of 20 mg/kg quinacrine (a phospholipase 2 enzyme blocker) 15 min before endotoxin or saline.

In the last group, rats were treated with dexamethasone (5 mg/kg sc, 16 h and 1 h before endotoxin) or saline (1 ml/kg sc), and the rate of gastric emptying was analyzed as mentioned above.

Determination of NOS Activity

Rats were killed 4 h after the administration of endotoxin (40 μg/kg ip) or saline (1 ml/kg ip). In short, the antrum pylorus tissue was cut into small pieces, frozen in liquid nitrogen, and stored at −80°C. NOS activity was measured as the rate of conversion of L-[U-^{14}C]arginine to L-[U-^{14}C]citrulline (31). Samples were homogenized (Ultra-Turrax) in an ice-cold buffer (330 mg/ml; pH 7.2) containing 320 mM sucrose, 20 mM HEPES, 1 mM EDTA, 1 mM DL-dithiothreitol, 10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, and 2 μg/ml aprotinin followed by centrifugation at 10.000 *g* for 20 min at 4°C. Afterward, 40 μl of supernatant was incubated at 37°C for 20 min in an assay buffer (pH 7.4) containing 50 mM KH_2PO_4, 1 mM $MgCl_2$, 0.2 mM $CaCl_2$, 50 mM L-valine, 1 mM L-citrulline, 0.02 mM L-arginine, 1 mM DL-dithiothreitol, 100 μM NADPH, 3 μM FAD, 3 μM flavin mononucleotide, 3 μM tetrahydrobiopterin, and 950 nM L-[U-^{14}C]arginine (348 mCi/mmol). The specificity of L-arginine conversion by NOS to L-citrulline was further confirmed using 1 mM N^{ω}-nitro-L-arginine (an NOS inhibitor). To differentiate between the Ca^{2+}-dependent and Ca^{2+}-independent NOS (iNOS activity), 1 mM EGTA (a calcium chelating agent) was used. All activities are expressed as picomoles of product generated per minute per gram of tissue.

Determination of nNOS Protein Content by Western Blot

Rats were killed 4 h after the administration of endotoxin (40 μg/kg ip) or saline (1 ml/kg ip). The content of nNOS protein in the stomach was determined by Western blot analysis. In short, the antrum pylorus tissue was cut into small pieces and homogenized in a boiling medium containing (in mM) 66 Tris·HCl (pH 7.4), 1 EGTA, 1 sodium orthovanadate, and 1 sodium fluoride with 10% glycerol and 1% SDS. Homogenates were treated with ultrasound (cooled on ice for 15 min) followed by centrifugation (10,000 *g* at 4°C) for 20 min. The supernatant was brought to 30% saturation with ammonium sulfate and stirred for 30 min in ice. After centrifugation for 5 min at 12,000 *g*, the pellets were resuspended in the above medium. Lysates were boiled for 5 min, and protein was determined by the bicinchonic acid (protein assay reagent; Pierce) method. Samples were subjected to SDS-PAGE, and immunoblotting was carried out as previously described (11) by using an antibody against nNOS (1:500, Transduction). After development using anti-mouse IgG conjugated with alkaline phosphatase (Sigma) and alkaline phosphatase color development (Sigma), the image was captured using Gelprinter Plus System (TDI), and the densities of the spots were measured using the software Intelligent Quantifier version 2.5.0. (BioImage).

Quantification of nNOS mRNA and iNOS mRNA by Real Time Quantitative RT-PCR

Rats were administered endotoxin (40 μg/kg ip) or saline (1 ml/kg ip) and killed by cervical dislocation 4 h later. In short, the antrum pylorus tissue was frozen in liquid nitrogen and stored at −80°C.

RNA extraction and cDNA synthesis. Total RNA from frozen gastric tissues was isolated with TriPure isolation reagent (Roche Diagnostics) following the manufacturer's protocol. RNA was resuspended in diethylpyrocarbonate-treated H_2O and stored at −80°C. Total RNA was treated with DNA-free (Ambion) to eliminate traces of contaminating genomic DNA. Resulting total RNA was quantified by ultraviolet spectrophotometry, and its integrity was evaluated by agarose gel electrophoresis. RT from 2 μg of total RNA was carried out with SuperScript RT RNase H^- (Life Technologies), using 0.8 μg oligo(dT_{16}) (TIB Molbiol; Roche Diagnostics) and 40 units RNase inhibitor (Roche Diagnostics) in a reaction volume of 20 μl. Controls without RT for each sample and a negative control with water in place of RNA were performed. Synthesized cDNA was stored at −20°C.

Quantitative PCR. Quantitative PCR was carried out in a LightCycler instrument (Roche Diagnostics) with the use of LightCycler-FastStart DNA Master SYBR Green I kit. Samples of 1 μl of cDNA were amplified through specific primers for each gene, 0.5 μM cyclophilin A (CyPA), 0.5 μM nNOS, or

1 μM iNOS, in a solution containing 2 mM $MgCl_2$ and 5% DMSO (final volume 10 μl). Reactions were performed in duplicate, and a negative control with water instead of cDNA was included in each run. Protocol reaction includes an initial period of 10 min at 95°C to activate the polymerase. Each PCR cycle involved denaturation at 95°C for 30 s, annealing at T_{ann} (shown in Table 1) for 30 s and extension at 72°C for 30 s. Fluorescence was measured at the end of each cycle. Specificity of reactions was tested by analysis of the melting curve (T_m, shown in Table 1) and agarose gel electrophoresis. To quantify input amounts of templates, a standard curve was obtained with serial dilutions of total RNA of a positive control (Table 1) for each analyzed gene, also after RT-PCR. To normalize the results, interpolated values for each sample were divided by values for the housekeeping gene CyPA, and results are expressed as an NOS/CyPA ratio.

Statistical Analysis

All data are expressed as means ± SE. Comparisons between two groups were performed using the Student's *t*-test and among three or more groups by one-way ANOVA followed by the Newman-Keuls test. P values < 0.05 were considered significant.

Drugs

Endotoxin (LPS from *E. coli*, serotype 0111:B4), L-NAME, L-NIL, 7-nitroindazole, NS-398, indomethacin, quinacrine, and all reagents used for determination of NOS activity were purchased from Sigma (St. Louis, MO). L-[U-^{14}C]arginine was obtained from Amersham Life Science (London, UK). Dexamethasone (Fortecortin) was used as a clinically available preparation. Unless mentioned otherwise, all drugs were dissolved in saline.

RESULTS

Effects of Endotoxin on Gastric Emptying of a Solid Nutrient Meal

The amount of rat chow eaten for 3 h by Sprague Dawley rats after a 20-h fast was 5.1 ± 0.2 g ($n = 10$). The 4-h rate of gastric emptying in animals treated with vehicle was significantly higher than that observed in endotoxin-treated rats (Fig. 1). This dose of endotoxin has previously been shown to lack effects on rectal temperature and systemic blood pressure in anesthetized animals (9).

Role of NO. Nonisoform-selective blockade of NO synthesis by pretreatment with L-NAME (30 mg/kg ip) significantly decreased the rate of gastric emptying in saline-treated rats, whereas it did not significantly change the rate of gastric emptying in endotoxin-treated animals (Fig. 1). In a similar manner, selective blockade of the nNOS isoform by pretreatment with 7-nitroindazole (20 mg/kg ip) induced a pronounced reduction in the rate of gastric emptying in saline-treated animals, whereas it did not modify the rate of gastric emptying in animals receiving endotoxin (Fig. 1). Selective inhibition of the iNOS isoform by the administration of L-NIL (2.5, 5, and 10 mg/kg ip) did not significantly modify the rate of gastric emptying in vehicle- or endotoxin-treated animals (Fig. 2).

Role of prostaglandins. The delay in gastric emptying induced by endotoxin was significantly prevented by pretreatment with the nonselective COX-1 and -2 inhibitor indomethacin (5 mg/kg sc; Fig. 3). In a similar manner, selective inhibition of the COX-2 isoform by pretreatment with NS-398 (10 mg/kg ip) also significantly prevented the inhibitory effects of endotoxin on gastric emptying (Fig. 3). Both indomethacin and NS-398 when administered to saline-treated animals lacked any effect on the rate of gastric emptying (Fig. 3). Blockade of arachidonic acid supply by pretreatment with a phospholipase 2 inhibitor, quinacrine (20 mg/kg ip), did not alter the rate of gastric emptying in endotoxin- or saline-treated rats (Fig. 4).

Effects of dexamethasone. Pretreatment with dexamethasone (10 mg/kg sc) significantly prevented the inhibition of gastric emptying by endotoxin but did not significantly change the rate of gastric emptying in vehicle-treated animals (Fig. 4).

Effects of Endotoxin on NOS Activity

NOS activity analyzed by the rate of conversion of L-arginine to L-citrulline was present in the antrum pylorus of saline-treated animals. With the use of a calcium chelating agent, we observed that NOS activity in these conditions was mainly Ca^{2+}-dependent, whereas Ca^{2+}-independent NOS activity (iNOS) was almost nonapparent (Fig. 5). Pretreatment (4 h) with endotoxin induced a significant reduction of the Ca^{2+}-dependent NOS activity (26.7%) in the antrum pylorus of the rats, whereas it did not significantly change the Ca^{2+}-independent NOS activity (iNOS), which was similar to that observed in saline-treated rats (Fig. 5).

Table 1. *Primer sequences, reaction data, and characteristics of specific PCR products for each analyzed gene*

Target Gene	Primer Sequences (5′-3′)	T_{ann}, °C	PCR, cycles	T_m, °C	Size, bp	Positive Control
CyPA	**CGTCTGCTTCGAGCTGTTTG (s)** GTAAAATGCCCGCAAGTCAA (as)	60	30	81.7	464	Cerebellum
nNOS	**ATCTCAGACCTGATTCGAGGAGG (s)** ACTGTTGAGGATGCTCAGCACAG (as)	55	35	85.1	513	Cerebellum
iNOS	**GCTACACTTCCAACGCAACA (s)** ACAATCCACAACTCGCTCCA (as)	60	40	84.6	293	Lung, endotoxin, 1 mg/kg, 12 h

Primers for neuronal nitric oxide synthase (nNOS) were taken as reference (24). Others were designed according to reported sequences with GenBank accession no. NM_017101 [cyclophilin A (CyPA)] and D12520 [inducible NOS (iNOS)]. T_{ann}, annealing temperature; T_m, melting temperature.

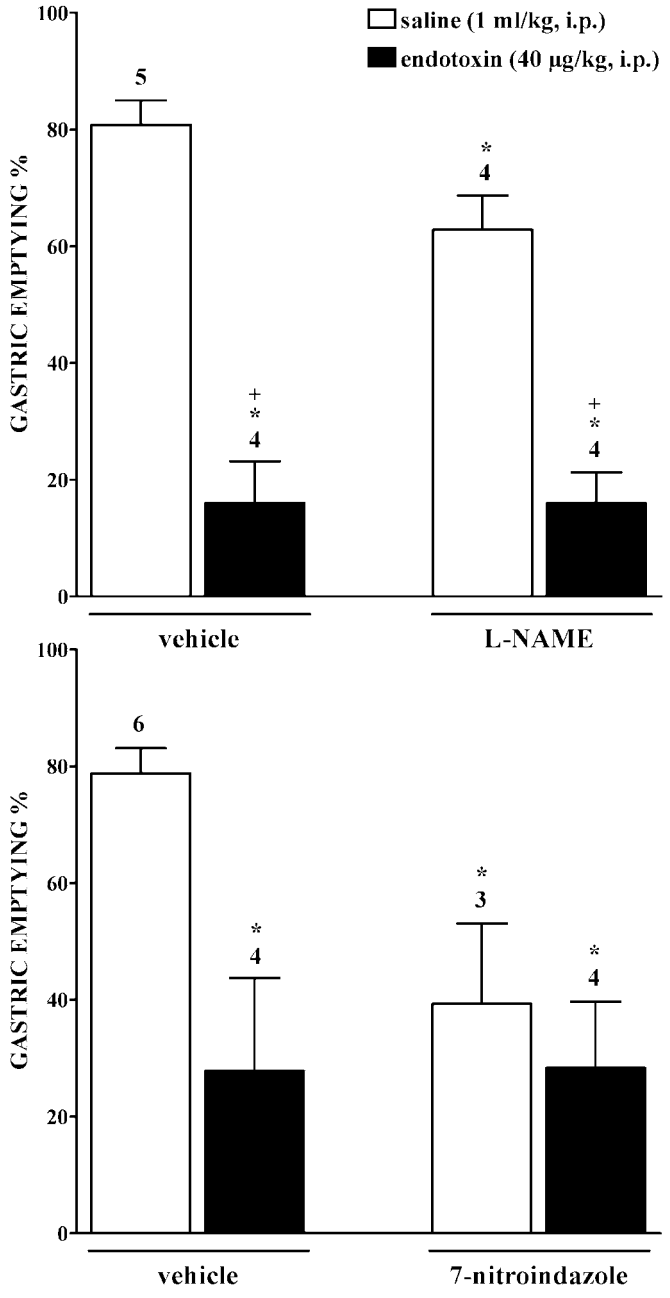

Fig. 1. Gastric emptying of a solid nutrient meal in rats receiving endotoxin or saline and treated with N^{ω}-nitro-L-arginine methyl ester (L-NAME) (30 mg/kg ip), 7-nitroindazole (20 mg/kg ip), or the respective vehicles (1 ml/kg ip saline or 0.5 ml/kg ip DMSO). Bars represent means ± SE of the number of animals shown above columns. *$P < 0.05$ compared with vehicle + saline treated group and $^{+}P < 0.05$ compared with L-NAME + saline-treated group.

Effects of Endotoxin on nNOS Protein Content

Pretreatment (4 h) with endotoxin induced a diminution in the amount of nNOS protein in the antrum pylorus of the stomach analyzed by Western blot (Fig. 6). Densitometry evaluation showed a significant diminution (57.2 ± 6.0% of reduction; $P < 0.05$, $n = 5$) compared with the protein observed in saline-treated animals.

Effects of Endotoxin on nNOS and iNOS mRNA

Both nNOS and iNOS mRNA were present in the antrum pylorus of vehicle-treated animals as analyzed by real-time RT-PCR. A single intraperitoneal injection of endotoxin (40 μg/kg) induced 4 h later a significant diminution in the amount of nNOS mRNA and a significant increase in the amount of iNOS mRNA in the antrum pylorus of the stomach (Table 2).

DISCUSSION

The present study shows a role for NO synthesis in the physiological control of gastric emptying of a solid nutrient meal. Moreover, a downregulation of the nNOS protein in the antrum pylorus of the stomach seems to be involved in the pathophysiological delay of gastric emptying of nutrient meals associated with endotoxemia.

Gastric emptying of nutrient meals is a complex function of the gut mediated by the integrated response of the proximal and distal stomach and duodenum (22), and NO has been shown to play a role in the specific functions carried out by these areas (2, 7, 26). In the present study, blockade of NO synthesis by systemic administration of L-NAME significantly delayed the rate of gastric emptying of a solid nutrient meal, as previously reported for nonnutrient meals (25, 27). This study extends previous observations and shows that selective inhibition of the nNOS isoform by pretreatment with 7-nitroindazole (3) also delays gastric

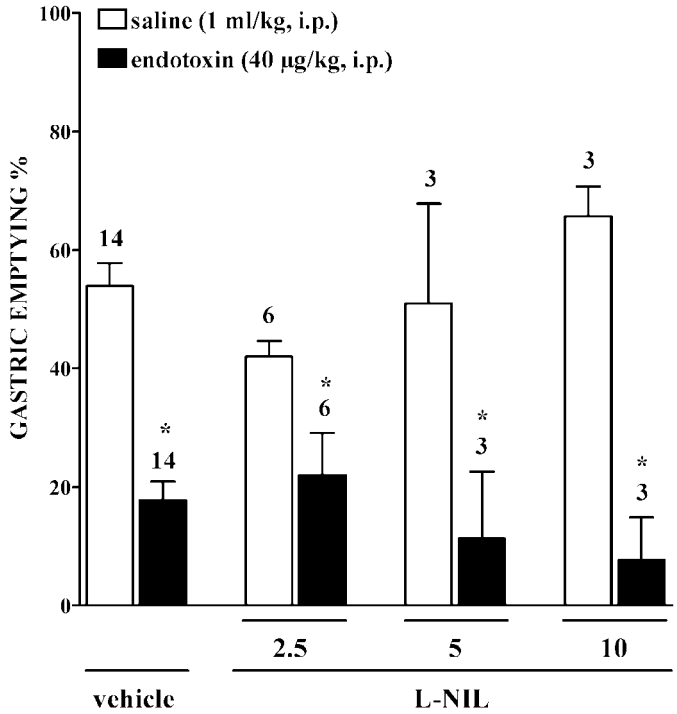

Fig. 2. Gastric emptying of a solid nutrient meal in rats receiving endotoxin or saline and treated with N^{6}-iminoethyl-L-lysine (L-NIL) (2.5, 5, or 10 mg/kg ip) or its vehicle (saline 1 ml/kg ip). Bars represent means ± SE of the number of animals shown above columns. *$P < 0.05$ compared with the respective saline-treated group.

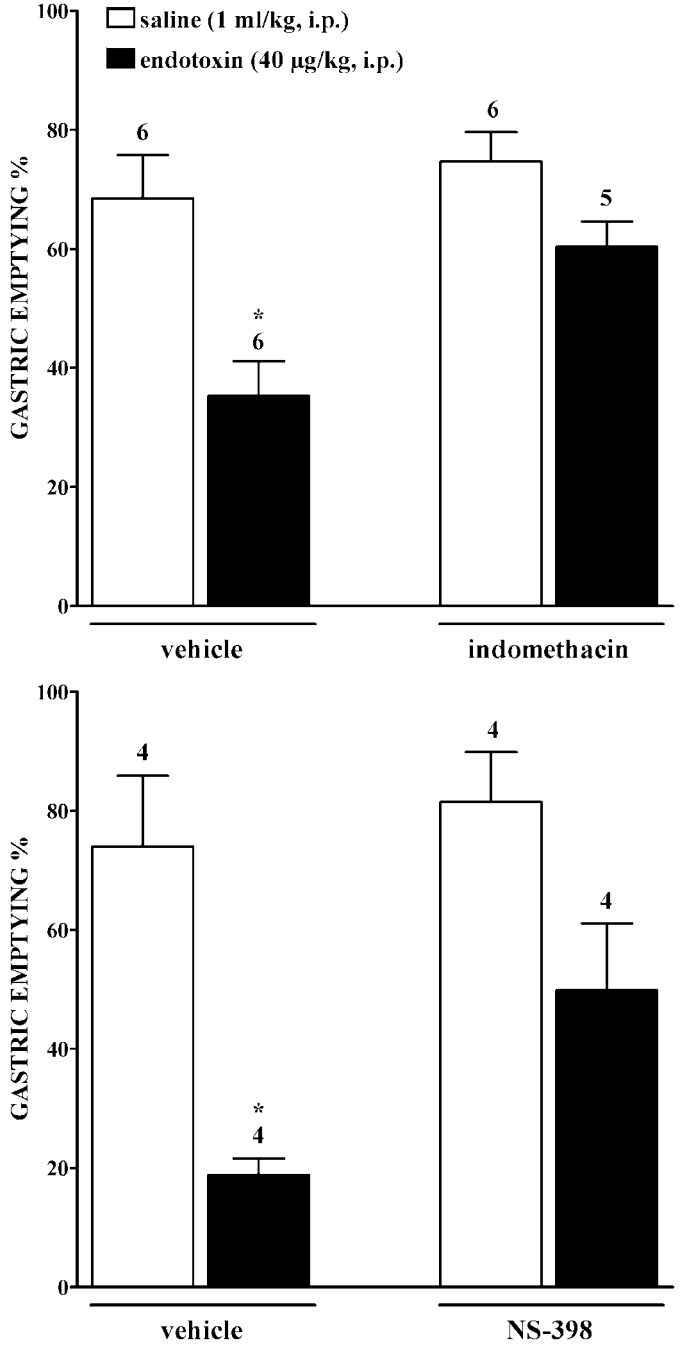

Fig. 3. Gastric emptying of a solid nutrient meal in rats receiving endotoxin or saline and treated with indomethacin (5 mg/kg sc), NS-398 (10 mg/kg ip), or the respective vehicles ($NaHCO_3$ 5% sc or DMSO 0.5 ml/kg ip). Bars represent means ± SE of the number of animals shown above columns. *$P < 0.05$ compared with all experimental groups in the same graph.

emptying, whereas blockade of the iNOS isoform with L-NIL fails to exert the same effect, reinforcing the physiological role of neuronal NO in the modulation of gastric emptying. The specificity of 7-nitroindazole on nNOS has been questioned, and intestinal motor depression through an action unrelated to NOS inhibition has been reported in vitro (17). Taking into account that, in this study, L-NAME also decreased gastric emptying, such a possibility seems unlikely. Additionally, inhibition of nNOS by 7-nitroindazole has recently been reported to increase iNOS expression in the rat small intestine (28). Considering that NO synthesized from iNOS (36) or exogenously administered (13) has been shown to delay gastric emptying, it is possible that increased synthesis of NO by iNOS rather than diminution of nNOS activity is responsible

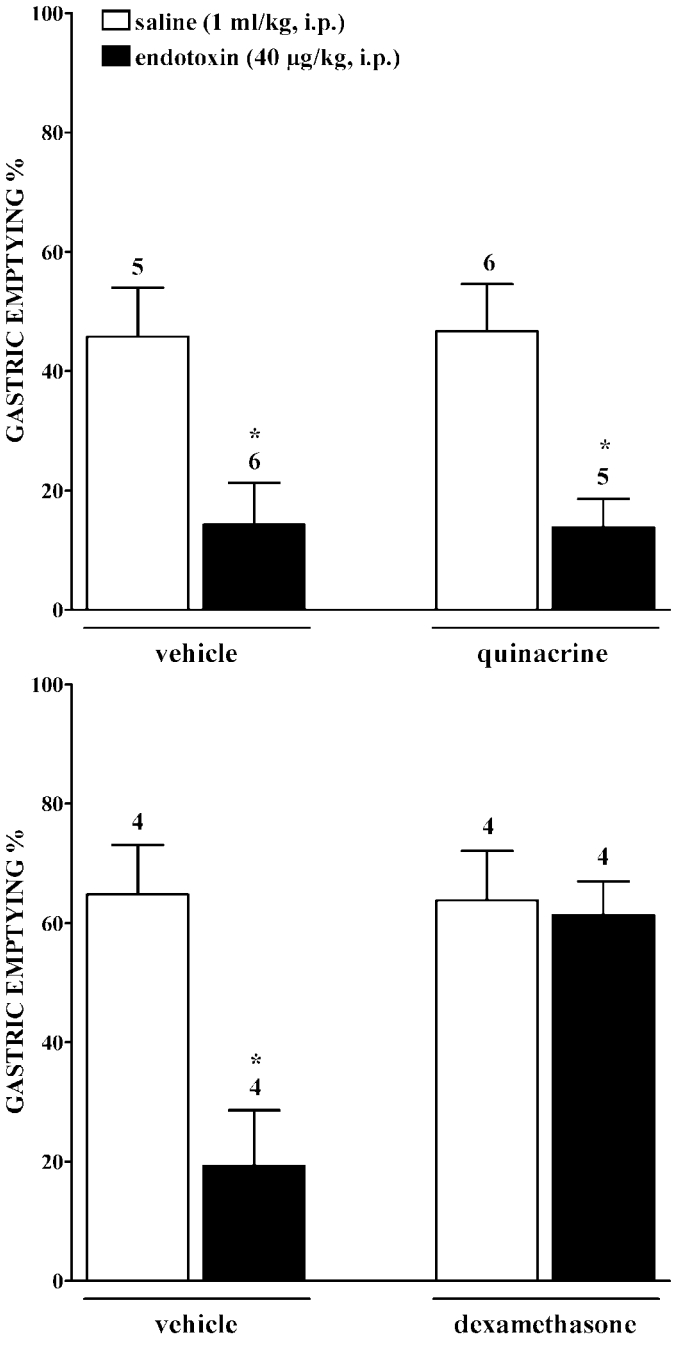

Fig. 4. Gastric emptying of a solid nutrient meal in rats receiving endotoxin or saline and treated with quinacrine (20 mg/kg ip), dexamethasone (5 mg/kg sc), or the respective vehicles (1 ml/kg saline). Bars represent means ± SE of the number of animals shown above columns. *$P < 0.05$ compared with the respective saline-treated group.

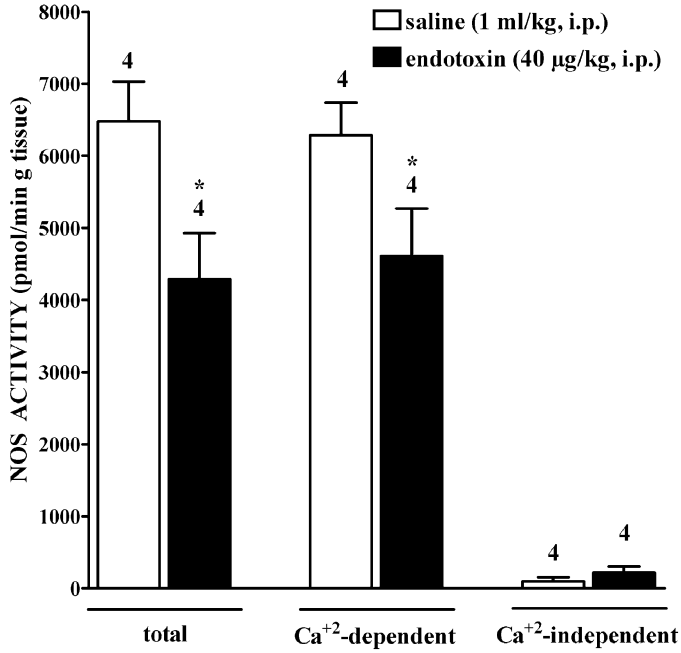

Fig. 5. Nitric oxide synthase (NOS) activity in the antrum pylorus of vehicle- and endotoxin-treated rats. Bars represent means ± SE of the number of animals shown above columns. *$P < 0.05$ compared with the respective saline-treated group.

for the delay in gastric emptying observed with 7-nitroindazole. However, the fact that L-NAME, which inhibits both nNOS and iNOS, also significantly decreased gastric emptying, combined with the following experiments analyzing the relationship between NOS isoforms and gastric emptying, strongly suggests that specific diminution of nNOS activity delays gastric emptying of nutrient meals.

Endotoxemia is associated with delayed gastric emptying, and synthesis of NO has been involved in the modulation of gastric function by endotoxin (9). However, the present study in which both nonselective NOS inhibitors and selective blockade of the nNOS isoform have been used do not support a role for NO synthesis in the delay in gastric emptying of a nutrient meal induced by endotoxin. Gastric emptying is inversely controlled by tones of gastric body and pyloric sphincter, and some evidence supports an inhibitory role of NO on gastric emptying through an action related with relaxation of the gastric fundus (36). However, NO synthesis has been widely associated with an acceleration of gastric emptying mainly due to pyloric sphincter relaxation (2, 25, 27). Lack of effect of NOS inhibitors in the delayed gastric emptying of endotoxin-treated rats, considered in the light of a recent study (10) showing an attenuation of the nonadrenergic noncholinergic relaxation of the pyloric sphincter by endotoxin, led us to think that the antrum pylorus of the stomach is the main target of endotoxin to inhibit gastric emptying.

Analysis of NOS activity in the antrum pylorus of the stomach of endotoxin-treated animals exhibited a significant reduction of Ca^{2+}-dependent NOS activity compared with that observed in vehicle-treated animals. Although Ca^{2+}-dependent NOS activity involves both endothelial NOS and nNOS, the fact that analysis of the NOS activity has been performed in the crude homogenate rather than in the membranous fraction (18) and the predominance of nNOS isoform in the gut (29) led to an important diminution of the nNOS activity associated with endotoxin. More specific analysis of the nNOS isoform showed a marked reduction of the nNOS protein content and nNOS mRNA in the same gastric area 4 h after treatment with endotoxin. Considered as a whole, these results suggest that endotoxin induces a transcriptional downregulation of the nNOS protein that implies diminution of NO synthesis in the antrum pylorus of the stomach, thereby increasing the tone of the pyloric sphincter and impeding gastric emptying.

Downregulation of nNOS protein in the stomach has been described with different proinflammatory stimuli such as platelet activating factor (29) and interferon-γ (4), usually associated with an increased synthesis of NO from iNOS. In addition, iNOS-derived NO has been involved in changes in gastric function over a long period of time, generally related to more severe insults, such as ischemia-reperfusion (15) or higher doses of LPS (36). The present study shows, 4 h after the administration of low doses of endotoxin, an increase in iNOS mRNA in the antrum pylorus of the stomach. However, no changes in iNOS activity were observed in the same area, suggesting that no significant synthesis of NO from the iNOS isoform is taking place at that time. Synthesis of NO from the iNOS, which is an inducible enzyme, requires protein gene expression,

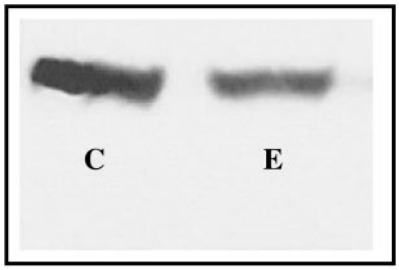

Fig. 6. Western blot analysis shows diminution in the expression of nNOS protein in the antrum pylorus of endotoxin-treated rats (E), compared with control animals (C). Representative experiment of 5 experiments.

Table 2. *nNOS and iNOS gene expression of saline- and endotoxin-treated rats*

Treatment	nNOS/CyPA Ratio	*n*	iNOS/CyPA Ratio	*n*
Saline, 1 ml/kg ip, 4 h	0.0416 ± 0.0045	6	0.2603 ± 0.0312	5
Endotoxin, 40 μg/kg ip, 4 h	0.0272 ± 0.0049*	8	0.9892 ± 0.1714*	8

Results are means ± SE; *n*, no. of animals. Relative amounts of nNOS, iNOS, or CyPA mRNA were obtained from their standard curves (cerebellum, lung of endotoxin-treated animals at 1 mg/kg ip 12 h, or cerebellum, respectively). *$P < 0.05$ compared with the respective saline-treated group.

synthesis of the protein, and functional activity of the protein including dimerization of the iNOS that has been shown to be a slow process (34). It seems that 4 h after the administration of low doses of endotoxin the iNOS gene expression in the antrum pylorus has already been started but synthesis of NO from the iNOS is not significant.

A time lag between iNOS gene expression and synthesis of NO has widely been reported (16, 18, 19). Lack of iNOS-derived NO synthesis 4 h after the administration of endotoxin is reinforced by functional studies showing that pretreatment with both the selective iNOS inhibitor L-NIL and the nonselective NOS inhibitor L-NAME at doses reported to block iNOS-derived NO synthesis (23, 40) did not modify the rate of gastric emptying in endotoxin-treated animals. It is believed that once iNOS is functionally active, it synthesizes high amounts of NO (5, 6), which at the level of the antrum pylorus would cause impaired antral contractions. However, such amounts of NO will necessarily decrease pyloric tone, allowing gastric emptying, an effect not observed in the present study.

The present results support a downregulation of the nNOS protein in the delay of gastric emptying induced by low doses of endotoxin. Mechanisms other than synthesis of NO from the iNOS previously reported (4) seem to be involved. Cross-talk interactions between the NOS and COX systems have been described, and specific modulation of nNOS by prostaglandins has been reported.

A role for endogenous prostaglandins in the inhibitory effects of endotoxin on gastric emptying is shown in the present study. However, these prostanoids do not seem to mediate gastric emptying in vehicle-treated animals. Synthesis of prostaglandins triggered by endotoxin seems to be mediated by the COX-2 isoenzyme, because pretreatment with a COX-2-selective dose of NS-398 (20) significantly prevented the effect of endotoxin in a similar manner to that observed in indomethacin-treated rats. Both induction of COX-2 protein and increase in the supply of arachidonic acid are required to enhance prostanoid production (14). In the present study, the administration of quinacrine, a phospholipase 2-inhibitor, did not significantly modify the rate of gastric emptying in endotoxin-treated animals, suggesting that the increased synthesis of prostanoids induced by endotoxin may be due to an increased expression of COX-2 rather than the release of arachidonic acid from the cellular membrane due to an increased activity of the phospholipase. This is reinforced by the fact that pretreatment with dexamethasone, which inhibits the expression of COX-2 without directly affecting its activity, significantly prevented the inhibition of gastric emptying by endotoxin. Dexamethasone has also been shown to inhibit the expression of iNOS (30). However, taking into account the lack of effect of the iNOS isoform on the delayed gastric emptying induced by endotoxin, the effects of dexamethasone on gastric emptying seem likely due to the inhibition of COX-2 expression.

The present study shows that the delay in gastric emptying of a nutrient meal induced by low doses of endotoxin is mediated by diminution of the nNOS activity in the antrum pylorus of the stomach and synthesis of prostaglandins. Although a possible cross-talk between prostaglandins and NO cannot be ruled out, the present results point to a transcriptional regulation of the nNOS and COX-2 carried out by endotoxin.

The present study has been supported by Comisión Interministerial de Ciencia y Tecnología Grants SAF 98-0118 and SAF 99-0114 and Ministerio de Sanidad y Consumo Grant FIS 01/1187.

E. García-Zaragozá and E. Quintana are the recipients of fellowships from Ministerio de Educación y Cultura and Ministerio de Sanidad y Consumo, respectively.

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II. EFECTOS DE LA ENDOTOXINA SOBRE LA MOTILIDAD COLÓNICA

Por otra parte quisimos estudiar si en estas circunstancias también se altera otro proceso fisiológico como es la excreción fecal. Para ello analizamos esta función a lo largo del tiempo en ratas conscientes pretratadas con la endotoxina.

Estos experimentos han dado lugar a la publicación:

"*Endotoxin stimulates fecal pellet output in rats through a neural mechanism*". Naunyn-Schmiedeberg's Archives of Pharmacology (2003) 367(1):51-5.

En ella demostramos que la endotoxina incrementa la excreción fecal de forma aguda. Este efecto está mediado por la activación de fibras sensoriales sensibles a la capsaicina y por la liberación de neurotransmisores excitadores (Ach y SP) e inhibidores (NO) en la pared colónica, incrementando así la velocidad del reflejo peristáltico.

Artículo 6

"*Endotoxin stimulates fecal pellet output in rats through a neural mechanism*"

Sara Calatayud, María D. Barrachina, Elsa Quintana, Sales Ibiza & Juan V. Esplugues

Naunyn-Schmiedeberg's Archives of Pharmacology (2003) 367(1):51-5.

Naunyn-Schmiedeberg's Arch Pharmacol (2003) 367 : 51–55
DOI 10.1007/s00210-002-0646-7

ORIGINAL ARTICLE

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Endotoxin stimulates fecal pellet output in rats through a neural mechanism

Received: 10 January 2002 / Accepted: 9 September 2002 / Published online: 12 November 2002

Abstract The effects of endotoxin on fecal pellet output and the neural mechanisms involved in this response were investigated in conscious rats.

E. coli endotoxin (40 μg/kg i.p.) significantly increased fecal excretion for 3 h after the injection. Water content in feces was not modified by endotoxin. Ablation of primary afferent neurons by systemic administration of high doses of capsaicin (20+30+50 mg/kg s.c.) to adult rats prevented the stimulatory effect of endotoxin and so did abdominal vagotomy. Adrenoceptor blockade with phentolamine (5 mg/kg i.p.) + propranolol (3 mg/kg i.p.) did not modify pellet output in endotoxin-treated rats while muscarinic receptor blockade with atropine (1 mg/kg i.p.) abolished the stimulatory effect of endotoxin. Finally, the increase in pellet output induced by endotoxin was prevented in animals receiving the substance P receptor antagonist D-Pro^2, D-Trp^{7,9}-substanceP (2 mg/kg i.p.) or the NO-synthase inhibitor L-NAME (10 mg/kg i.p.). None of the above treatments modified pellet output in saline-treated rats.

These observations indicate that endotoxin increases fecal pellet output through a nervous reflex in which capsaicin-sensitive afferent neurons and the release of excitatory (acetylcholine and substance P) and inhibitory (NO) neurotransmitters in the colonic wall are involved.

Keywords Fecal pellet output · Endotoxin · Capsaicin · Substance P · Acetylcholine · Nitric oxide

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Introduction

Endotoxemia is associated with increased susceptibility of the gastrointestinal mucosa to damage (Wallace et al. 1987) and changes in the secretory and motor functions of the gastrointestinal system (Pons et al. 1991, 1994). These alterations may be related to the vomiting and diarrhea that accompany most acute gastrointestinal infections (Mathan et al. 1988). However, low plasma levels of endotoxin, more characteristic of subclinical infections accompanying chronic conditions, induce more subtle, sometimes protective, changes in the gastrointestinal system. This low endotoxemia, which does not modify body temperature or blood pressure, selectively decreases gastric acid secretion (Barrachina et al. 1995b; Esplugues et al. 1996) and increases mucosal resistance to damage (Barrachina et al. 1995a). Changes in gastric motor function such as reduced gastric motility (Quintana et al. 2001) and delayed gastric emptying of solid nutrient meals (Calatayud et al. 2001) are also observed under these circumstances. The effects of endotoxin seem to be neurally-mediated and involve the activation of capsaicin-sensitive afferent neurons and non-adrenergic non-cholinergic nervous fibers (Barrachina et al. 1995a, 1995b; Calatayud et al. 2001; Quintana et al. 2001).

Low endotoxemia increases fecal excretion through the release of inflammatory mediators such as prostaglandins and platelet-activating factor (Pons et al. 1994). Fecal bolus propulsion is caused by the caudad-directed movements of the colonic smooth muscle, which are coordinated by the myenteric plexus. Propulsion of intraluminal contents in the colon depends on a propagated or peristaltic reflex involving the sequential activation of sensory neurons which are coupled, via modulatory interneurons, to excitatory and inhibitory motor neurons. The excitatory neurotransmitters responsible for the ascending contraction are acetylcholine and the tachykinins substance P and neurokinin A, whereas VIP and NO mediate the descending relaxation of circular muscle (Foxx-Orenstein and Grider 1996).

In the present study, we aimed to analyse the effects of low endotoxemia on fecal output in conscious rats and the neural mechanisms involved.

Materials and methods

Experimental protocol. The experiments were carried out in male Sprague-Dawley rats (250–300 g) that had free access to food and water. All experiments were started at 10.00 a.m. to avoid the influence of circadian rhythms. Bowel function was evaluated by counting the number of pellets excreted at different time points after an i.p. injection of saline (1 ml/kg) or endotoxin (40 µg/kg). In some experiments, pellets were collected and weighed immediately (wet weight) and after drying to a constant weight (dry weight). In addition, the amount of food eaten by each rat during the experimental period was measured by weighing the food at the beginning and the food and spill at the end of the experiment.

Some rats received a subcutaneous injection of capsaicin (20+30+50 mg/kg) or its vehicle (ethanol/Tween-80/saline 10:10:80, 1 ml/kg) under brief halothane anaesthesia on 3 consecutive days and, 12 days later, the experiment was carried out. The ablation of type C afferent fibres in capsaicin-treated rats was checked the day before the experiment using the eye-wiping test (Gamse 1982). Animals with abdominal vagotomy or sham-operated rats were obtained from Iffa Credo (France) and the experiments were carried out 10 days after the surgical procedure. In other experiments, rats were pre-treated (10 min) with either atropine sulphate (1 mg/kg i.p.), propranolol hydrochloride (3 mg/kg i.p.) plus phentolamine (5 mg/kg i.p.), the substance P antagonist, D-Pro^2,D-Trp^{7,9}-substanceP (2 mg/kg i.p.), or an NO-synthase inhibitor (L-NAME, 10 mg/kg i.p.). All protocols complied with the European Community guidelines for the use of experimental animals and were approved by the ethics committee of the Faculty of Medicine of Valencia.

Statistics. All data are expressed as means ± SEM. Comparisons between two groups were performed using Student's *t*-test and between three or more groups by one-way analysis of variance followed by Newman-Keuls test. *P*-values <0.05 were considered significant.

Drugs. Endotoxin (lipopolysaccharide from *E. coli*, serotype 0111:B4), substance P antagonist (D-Pro^2,D-Trp^{7,9}-substanceP), L-NAME (N^G-nitro-L-arginine methyl ester) and atropine sulphate were purchased from Sigma (Madrid, Spain), dissolved in sterile saline and administered in a volume of 1 ml/kg. Capsaicin was purchased from Fluka (Madrid, Spain). Salt preparations of (±)-propranolol hydrochloride (Sumial; Zeneca) and phentolamine mesylate (Regitina; Ziba) were used as clinically available preparations and equivalent volumes of sterile water for injection were injected in the respective control group.

Results

Administration of endotoxin (40 µg/kg) induced an acute increase in pellet output. As shown in Fig. 1, the cumulative number of pellets excreted by control and endotoxin-treated rats was significantly higher in the endotoxin group in the first hour after injection and the difference was maintained over 3–4 h. The release of pellets per hour was increased only in the first 2 h (1.4±0.5 vs. 0.2±0.2 pellets in the first hour, $P<0.05$; 1.8±0.7 vs. 0.2±0.2 pellets in the second hour, $P<0.05$). Afterwards, endotoxin-treated animals excreted similar or even lower numbers of pellets per hour than control rats. Water content in the feces was not modified by endotoxin (63±2% vs. 61±5%, w/w). Food intake was also similar in endotoxin- and saline-treated rats (1.6±0.4 g vs. 1.2±0.4 g).

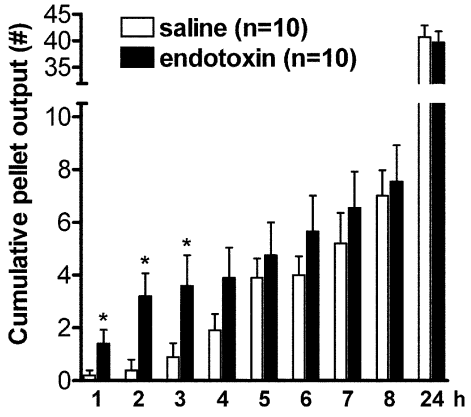

Fig. 1 Fecal pellet output in endotoxin (40 µg/kg i.p.)- or saline (1 ml/kg i.p.)-treated rats evaluated for 24 h after injection and expressed as the cumulative number (#) of pellets excreted at the different time points. Results correspond to means ± SEM of *n* experiments. *$P<0.05$ vs. same time point in saline-treated rats (Student's *t*-test)

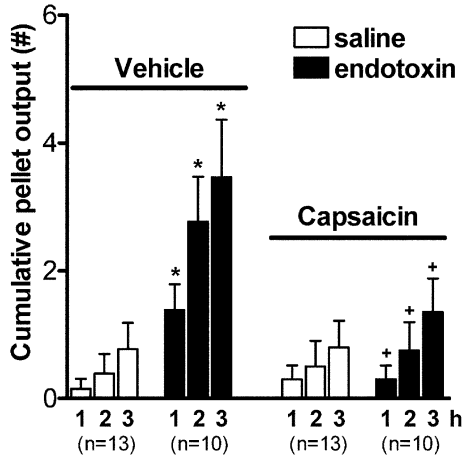

Fig. 2 Effects of capsaicin pre-treatment (20+30+50 mg/kg s.c., 14/12 days) and its vehicle (ethanol/Tween-80/saline 10:10:80 1 ml/kg s.c., 14/12 days) on fecal pellet output in endotoxin (40 µg/kg i.p.)- or saline (1 ml/kg i.p.)-treated rats. Results, expressed as the cumulative number (#) of pellets excreted at the different time points, correspond to means ± SEM of *n* experiments. *$P<0.05$ vs. same time point in vehicle + saline-treated rats, $^+P<0.05$ vs. same time point in vehicle + endotoxin-treated rats (ANOVA + test of Newman-Keuls)

Ablation of afferent C fibres with capsaicin prevented the stimulatory action of endotoxin without inducing noticeable changes in the pellet output of control animals (Fig. 2). A similar effect was observed in rats receiving the substance P antagonist D-Pro^2,D-Trp^{7,9}-substance P (Fig. 3).

The endotoxin effect was also prevented by abdominal vagotomy (Fig. 4). In this experimental group, both control and endotoxin-treated animals exhibited a pellet output higher than in the other groups, probably due to the surgical procedure and the inflammatory process associ-

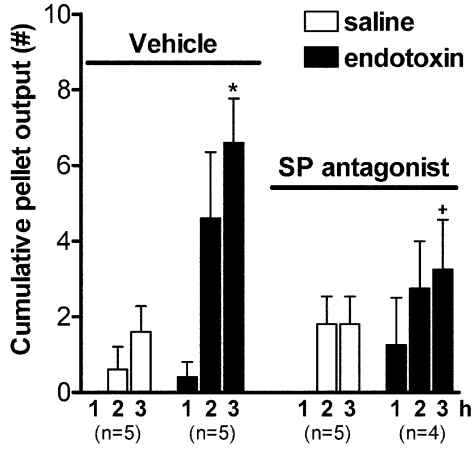

Fig. 3 Effects of pre-treatment with a substance P (*SP*) antagonist (2 mg/kg i.p., 10 min) or its vehicle (saline 1 ml/kg i.p., 10 min) on fecal pellet output in endotoxin (40 µg/kg i.p.)- or saline (1 ml/kg i.p.)-treated rats. Results, expressed as the cumulative number (#) of pellets excreted at the different time points, correspond to means ± SEM of *n* experiments. **P*<0.05 vs. same time point in vehicle + saline-treated rats, ^{+}P<0.05 vs. same time point in vehicle + endotoxin-treated rats (ANOVA + test of Newman-Keuls)

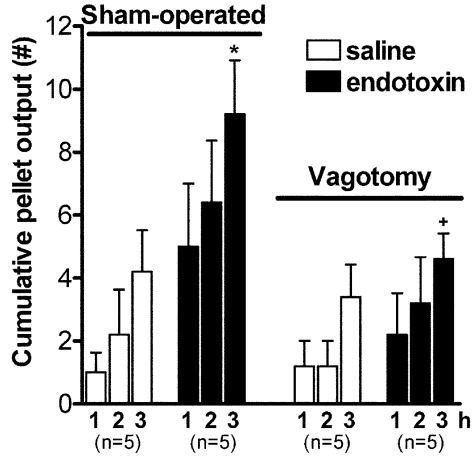

Fig. 4 Effects of abdominal vagotomy or sham operation on fecal pellet output in endotoxin (40 µg/kg i.p.)- or saline (1 ml/kg i.p.)-treated rats. Results, expressed as the cumulative number (#) of pellets excreted at the different time points, correspond to means ± SEM of *n* experiments. **P*<0.05 vs. same time point in sham-operated/saline-treated rats, ^{+}P<0.05 vs. same time point in sham-operated/endotoxin-treated rats (ANOVA + test of Newman-Keuls)

ated with the postoperative period. Muscarinic receptor blockade with atropine abolished the stimulatory action of endotoxin (Fig. 5A) while blockade of α- and β-adrenoceptors with propranolol plus phentolamine had no effect (Fig. 5B). The increase in pellet output induced by endotoxin was also prevented in animals receiving an intraperitoneal injection of the NO-synthase inhibitor L-NAME (Fig. 6). Neither atropine nor L-NAME modified pellet output in saline-treated rats.

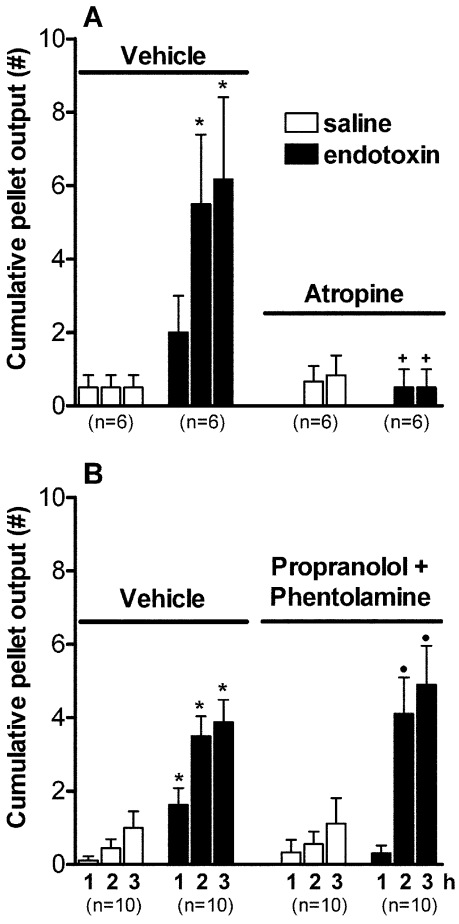

Fig. 5 Effects of **A** atropine (1 mg/kg i.p., 10 min) or its vehicle (saline 1 ml/kg s.c., 10 min), **B** propranolol (3 mg/kg i.p., 10 min) plus phentolamine (5 mg/kg i.p., 10 min) or their vehicle (water for injection 3+1 ml/kg i.p., 10 min), on fecal pellet output in endotoxin (40 µg/kg i.p.)- or saline (1 ml/kg i.p.)-treated rats. Results, expressed as the cumulative number (#) of pellets excreted at the different time points, correspond to means ± SEM of *n* experiments. **P*<0.05 vs. same time point in vehicle + saline-treated rats, ^{+}P<0.05 vs. same time point in vehicle + endotoxin-treated rats, $^{\bullet}P$<0.05 vs. same time point in propranolol + phentolamine + saline-treated rats (ANOVA + test of Newman-Keuls)

Discussion

The present study shows an acute increase in fecal pellet output in rats, induced by doses of *E. coli* endotoxin that do not modify blood pressure or body temperature. Gram-negative bacteria frequently cause diarrheic episodes mainly through the action of bacterial exotoxins. However, it has recently been shown that endotoxins (lipopolysaccharides) released from these bacteria also modify the secretory function of colonocytes (Closs et al. 1998). In the present study, in contrast to that reported with higher doses of endotoxin (Pons et al. 1994), the increased fecal pellet output was not associated with any noticeable change in the consistency of the feces, suggesting that the effect of endotoxin was independent of changes in intestinal secretion. Because the rats were not fasted and rats

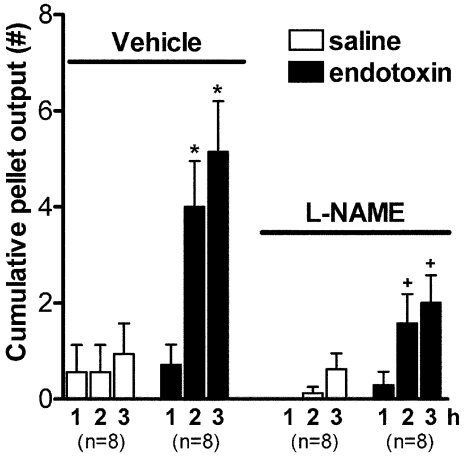

Fig. 6 Effects of pre-treatment with L-NAME (10 mg/kg i.p., 10 min) or its vehicle (saline 1 ml/kg i.p., 10 min) on fecal pellet output in endotoxin (40 μg/kg i.p.)- or saline (1 ml/kg i.p.)-treated rats. Results, expressed as the cumulative number (#) of pellets excreted at the different time points, correspond to means ± SEM of *n* experiments. **P*<0.05 vs. same time point in vehicle + saline-treated rats, ^{+}P<0.05 vs. same time point in vehicle + endotoxin-treated rats (ANOVA + test of Newman-Keuls)

usually follow a nocturnal pattern of feeding, the food intake during the experimental period was very low and not different between control and endotoxin-treated rats. These observations indicate that the effect of endotoxin on fecal excretion was not simply due to a gastro-colonic reflex activated differentially in both groups and suggest that endotoxin stimulates defecation through a direct modulation of colonic-anal motility.

In a previous study we have shown acute modulation of gastric motility with similar doses of endotoxin, an effect mediated by a nervous reflex involving the central nervous system (Quintana et al. 2001). In the present study, the prokinetic effect of endotoxin was prevented by subdiaphragmatic vagotomy, suggesting that afferent and/or efferent transmission between the abdomen and the central nervous system is involved in the effects of endotoxin on pellet output. Previous studies using similar doses of endotoxin reported a role for both afferent and efferent fibers in the inhibitory effects of endotoxin on gastric function (Barrachina et al. 1995a, 1995b; Quintana et al. 2001). We show here that systemic pre-treatment with capsaicin prevented the stimulatory effect of endotoxin on pellet output. In addition to afferent neurons, capsaicin receptors have recently been localized to epithelial cells and enteric neurons in the gut (Anavi-Goffer et al. 2002; Veronesi et al. 1999). However, taking into account that endotoxin has been shown to induce fos immunoreactivity in primary afferent neurons of the vagus (Gaykema et al. 1998), a role for capsaicin-sensitive afferent neurons seems the most likely.

Under physiological conditions, the excitatory motor neurons that regulate ascending contraction of circular muscle release acetylcholine and the tachykinins substance P and neurokinin A (Foxx-Orenstein and Grider 1996). In the present study, the stimulatory effect of endotoxin on fecal pellet output was prevented by blockade of both muscarinic and substance P receptors. Whereas results obtained with atropine imply a role for acetylcholine as an efferent transmitter involved in the control of colonic motility, the role and origin of substance P is less clear. Substance P could be released from afferent neurons and act as a transmitter in the gut or in the spinal cord or brain stem. Taking into account that enteric neurons are the most important source of substance P for smooth muscle contraction (Grider 1989; Holzer et al. 1980), a direct role of this neuropeptide released from enteric neurons on smooth muscle is probably involved.

In addition to excitatory motor neurons, colonic motility is also modulated by inhibitory neurons that regulate the descending relaxation of the circular muscle through the release of VIP and NO (Foxx-Orenstein and Grider 1996). In this study, the increased fecal pellet output observed in endotoxin-treated rats was normalised by inhibition of NO synthesis with L-NAME. Previous studies using higher doses of endotoxin have reported an increased expression of iNOS in enterocytes and a role for iNOS-derived NO in the diarrhea observed in those conditions (Closs et al. 1998). Although we have not analysed the specific NOS isoform involved in the synthesis of NO, the small doses of endotoxin and the short time in which the effects of endotoxin take place do not support a role for iNOS in said effects. Moreover, we have demonstrated in previous studies the absence of iNOS activity in the gastric wall of rats receiving this dose of endotoxin (Quintana et al. 2001). Neurons from the non-adrenergic non-cholinergic system seem to be the most likely source of the NO involved in the endotoxin's action, although further studies are necessary to address this question.

The results reported in the present study and prior evidence show that low endotoxemia modifies bolus transit all along the gastrointestinal system. As previously observed for the delay in gastric emptying (Calatayud et al. 2001), the increased fecal pellet output induced by endotoxin depends on a nervous mechanism. Although additional studies are needed to characterize the specific pathways involved, the present results suggest that endotoxin activates a nervous reflex in which capsaicin-sensitive afferent neurons and the release of excitatory and inhibitory neurotransmitters in the colonic wall are involved. An increased secretion of acetylcholine and substance P would enhance the propulsory effect of the ascending contraction of the circular muscle, while the local release of NO would facilitate the accommodation of the propelled bolus by inducing the descending relaxation of the colonic muscle. This study, put in the light of previous studies with similar doses of endotoxin, led us to hypothesize that different mechanisms activated by endotoxin play a role in the modulation of gastrointestinal function. The initial effects of endotoxin probably depend on a fast, neural mechanism, which would explain the quick onset of the endotoxin's action on fecal excretion, gastric motility, acid secretion and mucosal resistance to damage (first hour post-injection; this study; Barrachina et al. 1995a; Mar-

tínez-Cuesta et al. 1992; Quintana et al. 2001). Reinforcing this neural action, capsaicin-sensitive afferent neurons have been involved in phase 1, but not phase 2 or 3, of the febrile response to endotoxin (Szekely et al. 2000). The later effects of endotoxin would be responsible for the sub-acute effects of endotoxin, and involve expression of cytokines, iNOS and COX-2, among others. According with this, we have recently observed that endotoxin induces COX-2 expression in the stomach and the resultant increase in prostaglandin synthesis mediates the delay in gastric emptying (4 h post-injection; Calatayud et al. 2002). These results are in accordance with the previously reported role of prostaglandins in the stimulatory effect of endotoxin on colonic propulsion (Pons et al. 1994). These mediators, however, do not participate in the acute changes in gastric secretion or mucosal resistance to damage induced by endotoxin (Barrachina et al. 1995a; Martínez-Cuesta et al. 1992). Neural and humoral pathways may even be related since recent reports suggest that the humoral response to endotoxin may partly depend on changes in neural activity, in particular, neuropeptide release from capsaicin-sensitive neurons (Dickerson et al. 1998).

Acknowledgements The present study has been supported by grants 1FD97-1029 and SAF99-0118 from Ministerio de Educación y Cultura, and FISS01/1187 from Ministerio de Sanidad y Consumo. E.Q. is the recipient of a fellowship from Ministerio de Sanidad y Consumo.

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III. ANÁLISIS GLOBAL

Por último, se nos invitó a escribir una revisión que engloba todos los resultados expuestos en la presente tesis junto con otros estudios previos realizados en nuestro laboratorio.

Artículo 7

"*Nitrergic modulation of gastrointestinal function during early endotoxemia*"

Elsa Quintana, María D. Barrachina & Juan V. Esplugues

Current Pharmaceutical Design (2004, en revisión)

NITRERGIC MODULATION OF GASTROINTESTINAL FUNCTION DURING EARLY ENDOTOXEMIA

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ABSTRACT

After bacterial infection, the host reacts by signalling to the central nervous system where a cascade of physiologic, neuroendocrine and behavioural processes is orchestrated, collectively termed the acute phase response. Endotoxemia following Gram-negative bacterial infection induces a wide array of effects, including fever, loss of appetite and changes in gastrointestinal function that attempt to eliminate the challenge and restore homeostasis. Systemic administration of low doses of endotoxin (5-40 μg/kg) to rats is associated with changes in gastrointestinal motor function, inhibition of gastric acid secretion and increase in the gastric mucosal resistance to damage. These changes are rapid in onset (observed within one hour), not related to vascular dysfunction, and appear to be mediated by mechanisms that involve the peripheral and the central nervous system. Nitric oxide (NO) plays a central role in the physiology of the gastrointestinal tract and its response to illness. Accumulated evidence supports an increase of NO synthesis in the brainstem, as well as in the gastric myenteric plexus thirty minutes after endotoxin administration. Such a synthesis is due to constitutive nitric oxide synthase (NOS) and occurs before the induction of NOS takes place. In this review we provide experimental evidence supporting the hypothesis that activation of a physiologic mechanism, mediated by the autonomic and the central nervous systems as well as constitutive NOS isoforms, is involved in acute changes of gastrointestinal function during early endotoxemia.

Key words: neuronal nitric oxide synthase, endotoxemia, central nervous system, vagus nerve, gastric motor function, brain-gut.

INTRODUCTION

The host is continuously exposed to pathogen agents and therefore has developed innate mechanisms that orchestrate a rapid response to eliminate challenge. A common set of physiologic, autonomic, neuroendocrine and behavioural responses are triggered by the organism in what is collectively known as the acute phase response, which is critical for host defence, helping to clear infection [1]. In most instances, the process leads to the elimination of the microorganisms without producing clinically detectable signs of inflammation. However, bacterial components, such as lipopolysaccharide (LPS, or endotoxin) released by Gram-negative bacteria, may activate host cells, such as monocytes/macrophages, neutrophils and endothelial cells, and generate an inflammatory response that will result in phagocytosis and killing of the microorganism [2]. Only under severe endotoxemia, regulatory control mechanisms are overwhelmed and the inflammatory response leads to dysfunction of major organs causing what is termed septic shock, which is characterized by hypotension and vascular collapse, and may culminate in tissue injury, multiple organ failure and death [2].

The acute phase response is clinically characterized by fever or hypothermia, shivering, tachycardia, tachypnea and changes in gastrointestinal function. Experimental work on the neurobiological basis of gastrointestinal function during infection has focused on the examination of physiologic, neural and behavioural changes in rodent models after systemic administration of bacterial endotoxins or cytokines as potential mediators of the acute phase response.

In experimental studies, systemic administration to rats of doses of bacterial endotoxin in the range of mg/kg has been reported to induce profound changes in gastrointestinal function such as diarrhoea, reduction in gastric mucosal blood flow and

gastrointestinal mucosal damage [3,4]. These changes last for several hours after challenge and are related with activation of the immune response and transcriptional synthesis of proteins. In contrast, when low doses of endotoxin (in the range of μg/kg) are administered to rats, gastrointestinal changes such as an increase in the mucosal resistance to damage, diminution of gastric motility and secretion, and increase in intestinal transit [5-9] are observed fast after challenge (30 min). These changes are independent of transcriptional synthesis of proteins and are not related to any clinical sign derived from vascular dysfunction. Strong experimental evidence suggests that activation of physiologic mechanisms, including the autonomic and the central nervous systems as well as constitutive enzymes, mediates acute changes in gastrointestinal function [9-13]. The purpose of this review is to present an overview and new developing hypothesis about the role of nitric oxide and the neural gastrointestinal mechanism triggered by the host to expel the challenge and prevent massive microorganism invasion and to defend itself against further aggression. Unless mentioned otherwise, the review is centred on the gastrointestinal effects observed within the first hour of administration of doses of endotoxin in the range of μg/kg to rats.

INNERVATION OF THE GUT

The gut can be considered as a neurological organ as it contains the largest collection of nerve cells outside the brain [14,15]. It is supplied by extrinsic and intrinsic sensory neurons that, together with endocrine and immune cells form a surveillance network that is essential to gastrointestinal function. While intrinsic primary afferent neurones supply the enteric nervous system with information that is required for its independent control of digestion, afferent fibres of the vagal and spinal

nerves convey information from the gut to the brain participating in autonomic and neuroendocrine reflex circuits. Histochemical and functional evidence suggest complete vagal innervation of the entire gastrointestinal tract. Vagal afferent input to the central nervous system project to the medulla; these fibres have their cell bodies in the nodose ganglion and terminate in the nucleus tractus solitarius (NTS) of the dorsal vagal complex (DVC) at the brainstem, which is the first relay station for sensory signals originating from viscera [16]. From the NTS, projections to other brainstem nuclei and to the midbrain and hypothalamic nuclei are observed [17]. Among them, projections to the dorsal motor nucleus, the nucleus ambiguous and the intermediolateral column of the spinal cord form the basis for vagovagal and vagospinal reflexes involved in the control of gastrointestinal functions such as gastric accommodation induced by distension of the stomach [18-20].

The spinal afferent nerve fibres run with the splanchnic nerves and enter the spinal cord generally through the dorsal roots. Some afferent fibres in visceral nerves project only to prevertebral ganglia and not to the spinal cord and they are likely involved in local autonomic reflexes. Some visceral sensory axons that do project to the spinal cord also give collateral innervation to neurones in prevertebral ganglia and likely also contribute to local control mechanisms as well as to visceral sensations [15]. Noradrenergic nerve endings in the gut wall emerge from the cell bodies located in prevertebral sympathetic ganglia. While many noradrenergic fibres supply the ganglia of the myenteric plexus, few fibres supply the muscle in non-sphincteric regions while these fibres densely supply the pyloric region of the rat.

It has been widely described that vagal afferent neurones are activated by a variety of physiological stimuli such as the appearance of cholecystokinin in the gastric circulation or distension of the gastric wall while spinal afferent neurones are related

with stimuli that potentially are harmful to the tissue and challenge homeostasis [21-23]. However, increasing evidence suggest that vagal afferent neurones signalling information to the central nervous system play a pivotal role in the mechanism triggered by the host to maintain homeostasis after challenge with low doses of endotoxin [5,10,24].

The central nervous system and endotoxemia

The central nervous system mediates multiple aspects of the acute phase response to endotoxemia [1,25]. Immunohistochemical studies have shown the expression of the early gene c-fos or its protein product Fos, markers of neuronal activation, in key autonomic regulatory nuclear groups of the brain after peripheral administration of LPS to rodent animals. Among these nuclei, an intense Fos expression has been observed in the paraventricular nucleus of the hypothalamus, the rostral and caudal levels of the ventrolateral medulla, the NTS and DMN of the DVC and sympathetic preganglionic neurones in the intermediolateral cell column of the spinal cord [10,26-36]. Accumulated evidence suggests that activation of glutamate receptors may represent a final common pathway for the induction of Fos protein in the brain induced by both endotoxin and stress [26]. In this line, several studies have reported a rapid increase in glutamate release in the NTS after a single intraperitoneal injection of LPS [37]. In addition, this effect was completely prevented by local perfusion in the brain (microdialysis studies) of the neurotoxin tetrodotoxin, indicating that glutamatergic nervous terminals in the NTS are activated by endotoxin. Neuronal activation of the brain by peripheral LPS could be triggered by several peripheral signals including immunomodulator transport across the blood brain barrier, humoral or neural pathways [38,39]. Although a role for blood-borne cytokines induced by LPS

cannot be ruled out, the rapidity of the effects observed suggest peripheral sensory nerve stimulation in this process.

Vagal afferent neural signals and endotoxemia

The vagus nerve is the major source of visceral innervation and constitutes the primary neuroanatomic linkage between the brain and gut. For the last years, some studies suggest that vagal sensory neurones play an essential role in signalling information to the brain after intraperitoneal endotoxin administration since: a) c-fos expression was shown in the cell bodies of vagal fibres located in the nodose ganglion, after peripheral administration of endotoxin [29]; b) vagotomy completely blocked c-Fos expression in the brainstem analysed two hours after the intraperitoneal administration of 40 μg/kg of endotoxin to rats [26].

Reinforcing these observations, transection of the abdominal trunks of the vagus nerve has been shown to attenuate or abrogate a number of illness responses to administration of moderate doses of bacterial LPS, including sickness behaviour, activation of the hypothalamic-pituitary axis, hyperalgesia or increase of slow-wave sleep [39-41]. Evidence suggests that LPS activates vagal receptors within the abdominal cavity, which in turn relay immune signal into the central nervous system. In this line, functional studies support that modulation of gastrointestinal function by low doses of LPS (μg/kg) involves a direct neural pathway from periphery to the brain via sensory afferent neurons [5,10]. Indeed blockade of capsaicin-sensitive vagal afferent neurons by local application of this neurotoxin on the vagus nerve, abolished inhibition of gastrointestinal function induced by endotoxin [5,42]. It is important to note that the role of the afferent vagus nerve in signalling information to the CNS has been mainly reported when LPS is administered intraperitoneally to rats. However, some authors

using i.v. LPS have stated that the vagus nerve is not necessary to endotoxin-induced fos expression in the brain [29,35,40], which suggest that alternative pathways may be employed to inform the central nervous system about peripheral challenge.

Vagal efferent neural signals and endotoxemia

The cell bodies of pre-ganglionic motor neurons innervating the gastrointestinal tract are located in the DMN. Fos expression after peripheral administration of LPS has also been observed in the DMN [10], suggesting that LPS may involve this nucleus in acute modulation of gastrointestinal function "Fig. (**1**)". Excitation of DMN neurones has been shown to decrease gastric motor function [43] and a differential distribution of putative neurotransmitters/neuromodulators in the DMN has been reported [44,45]; however the biochemical coding of the DMN neurons involved in gastric inhibition induced by endotoxin remains elusive. It is interesting to note that different hypothesis have been proposed in the literature that could substantiate the inhibition of gastric function by neuronal activation of the DMN: a) Fos expression in the DMN activated by endotoxin would correspond with cholinergic pre-ganglionic neurons. Once modulated by endotoxin, these fibres would synapse in the enteric ganglia and stimulate post-ganglionic inhibitory motor fibres or inhibit excitatory post-ganglionic motor neurons; the final release of inhibitory neurotransmitters from non-adrenergic non-cholinergic (NANC) nerves such as NO or VIP and/or the decrease in the release of excitatory neurotransmitters, such as acetylcholine (Ach) or substance P, from post-ganglionic fibres would be involved in gastrointestinal inhibition. b) Fos expression in the DMN activated by endotoxin would correspond with nitric oxide (NO)-containing neurons, which will be the first in a sequence of nitrergic neurons innervating the gut, as it has been hypothesized [46-48]. Although few nitric oxide synthase (NOS)-containing

neurons have been shown in the DMN, it has been argued that these neurons would be in a "command" position controlling NANC vagally mediated release of NO in the stomach [47]. Several pharmacological studies in rats have demonstrated that doses of endotoxin in the range of μg/kg increase NO synthesis in the brainstem [10,24]. In addition, a recent study has also shown NO synthesis in the stomach of rats treated with the same doses of endotoxin [13]. However the specific relationship between central and peripheral NO needs to be delineated.

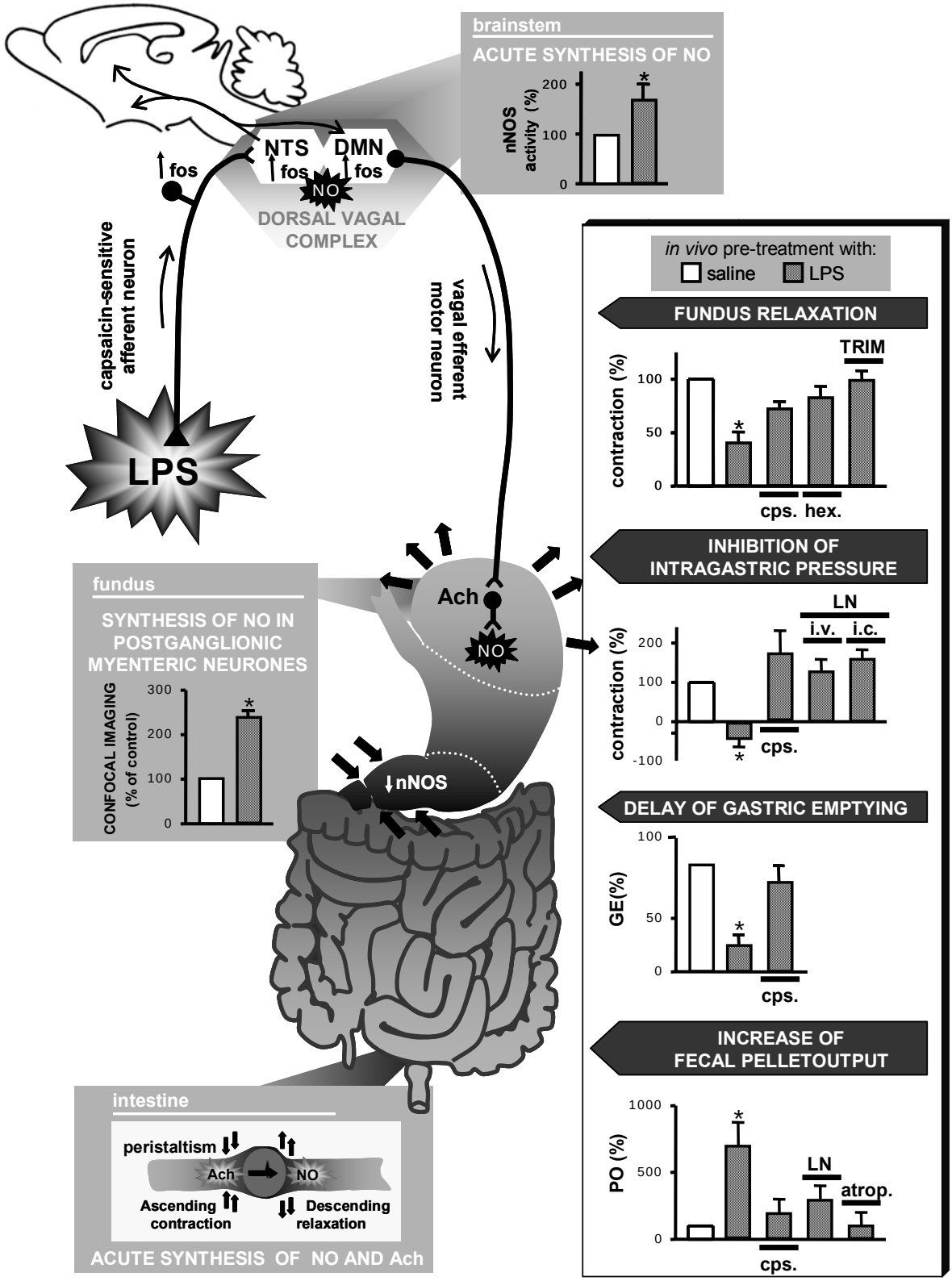

Fig. (1). Schematic diagram showing neural pathways and enzymes involved in the modulation of gastric motor function by endotoxin. Intraperitoneal administration of LPS (40 µg/kg) induces fundus relaxation, diminution in intragastric pressure, delay in

gastric emptying of solid meals and increase in intestinal transit, 30 min, 30 min, 4h and 1h later, respectively. It has been demonstrated that LPS induces the activation of: a) capsaicin-sensitive afferent neurons; b) neurons of the dorsal vagal complex of the brainstem and c) cholinergic pre-ganglionic motor fibres emerging from the DMN, which in turn induce the release of inhibitory neurotransmitters in the myenteric plexus. In addition, NO synthesis from nNOS has been observed by biochemical methods in the brainstem and the myenteric plexus of LPS-treated animals and it has been functionally related with changes in gastrointestinal function. Graphs show original data adapted from [5,7,10,13,42] and represent mean±s.e.m. ($n>3$; $*p<0.05$). Atrop., atropine; cps., capsaicin; DMN, dorsal motor nucleus of the vagus; hex., hexamethonium; LN, L-nitroarginine methyl ester; NTS, nucleus of the solitary tract; TRIM, 1-(2-trifluoromethylphenyl)imidazole, a nNOS inhibitor.

NITRIC OXIDE

NO is synthesized from L-arginine by NOS, an enzyme that has been detected in different cells of the gastrointestinal tract including endothelial, secretor, muscular and nervous cells [49,50]. In addition, immunohistochemical studies have also reported the presence of NOS in vagal afferents and central brainstem nuclei involved in modulation of gastrointestinal function such as intrinsic neurons of the NTS and neurons of the DMN [51,52]. Therefore it is not surprising that NO is an endogenous mediator involved in the pathophysiological regulation of a variety of gastrointestinal functions such as microcirculation and mucosal integrity maintenance, as well as modulation of secretor and motor function. Three different NOS isoforms have been characterized in mammalian cells [53,54]: the neuronal (nNOS) and endothelial (eNOS) NOS isoforms that are constitutively expressed, widely distributed and synthesize NO at low concentrations which regulates numerous physiological functions. The third isoform is named inducible NOS (iNOS) as its expression requires transcriptional activation; it is

induced in many cell types including inflammatory cells, endothelium and neurons after exposure to cytokines and LPS and it is continuously active during inflammation, leading to an overproduction of NO, which presents cytotoxic activity. However, it is now known that the terminology "constitutive" versus "inducible" is an oversimplification as it has been stated that levels of gene expression of "constitutive" isoforms, both eNOS and nNOS, may also be induced under different physiological conditions [55] and, conversely, that iNOS may function as a "constitutive" enzyme in some cells under physiological situations [56].

Nitric oxide and endotoxemia

It was early described that administration of LPS at doses in the range of mg/kg induced a transcriptional increase in the expression of iNOS in a variety of tissues from rodents [57]. Furthermore, elevated levels of circulating nitrite/nitrate, the stable NO bio-reaction products, were reported in septic patients [58,59]. The massive release of iNOS-derived NO during endotoxemia has been associated with vascular dysfunction and severe changes in gastrointestinal function such as an increase in the epithelial barrier function and gastrointestinal mucosal damage [60,61]. In contrast to these effects, a gastrointestinal defensive mechanism is observed rapidly after administration of doses in the range of μg/kg of endotoxin, and NO seems also to be involved. However, a role for the iNOS isoform, as the one responsible for NO synthesis under these circumstances, seems unlikely since iNOS is transcriptionally regulated, a process that requires several hours [62]. Consistent with these observations are biochemical studies showing, one hour after LPS administration (μg/kg), an increase in nNOS enzymatic activity in the brainstem while no changes where observed in iNOS enzymatic activity [10]. Furthermore, parallel experiments on the protein amount

showed no significant changes in nNOS protein in the brainstem at the same time point (unpublished data). It was suggested that endotoxin induced *in vivo* a non-transcriptional regulation of NO synthesis in the brainstem. This mechanism seems extensible to other tissues and NO synthesis from constitutive sources has also been observed in the stomach thirty minutes after administration of low doses of endotoxin [13]. Recent studies using confocal microscopy provided direct evidence of NO synthesis in the gastric fundus of rats treated with endotoxin, "Fig. (**2**)"; such a synthesis was observed in the myenteric plexus and pharmacological tools allowed to specifically localize the cellular source of NO synthesis in post-ganglionic myenteric neurons. In a similar manner to that reported in the brainstem, such a synthesis was not transcriptionally regulated, since no changes in nNOS mRNA nor iNOS mRNA expression were observed in the gastric fundus one hour after LPS administration [13] "Fig. (**1**)".

It seems that nNOS isoform, which plays a main physiological role, may also up-regulate its activity very rapidly after challenge with low doses of endotoxin. [10-13,63]. This mechanism does not seem to be specific of the nNOS isoform and extends to the other constitutive isoenzyme. In this line, NO synthesis has been also involved in the increase in gastric mucosal resistance against deleterious agents induced by doses of endotoxin in the range of μg/kg [11]. Such a synthesis is post-translationally regulated since no changes in eNOS nor iNOS mRNA were observed in the gastric corpus of endotoxin-treated rats at the time were the gastroprotective effect was observed [8]. A recent study demonstrates that, these doses of endotoxin preserve the fall in gastric blood flow induced by non steroidal anti-inflammatory drugs (NSAIDs) [8], a mechanism that is probably involved in the gastroprotective effect of endotoxin. Although no direct evidence of the specific NOS isoform involved was obtained, an

increase in the enzymatic activity of eNOS in vivo by low doses of endotoxin was proposed [8].

Taken together we hypothesized that a rapid and controlled delivery of NO from constitutive sources may be considered as an emergency system by which the host can overcome bacteria infection in a very short time in order to prevent a major insult. This mechanism may be a common mechanism that takes place in situations in which the animal is compromised such as stress situations [24].

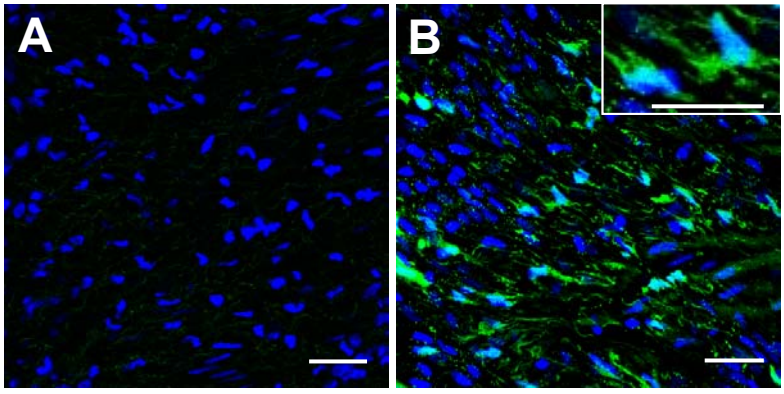

Fig. (2). NO synthesis in the myenteric plexus induced thirty minutes after LPS pre-treatment (40 µg/kg, i.p). Confocal microscopy images of whole-mount preparations of gastric fundus obtained from: saline (1 ml/kg, i.p.; A) or LPS (B) stained with DAF-FM (indicative of NO synthesis; green) and Hoechst 33342 (nuclear fluorochrome, blue). A higher magnification of a positive DAF-FM soma is shown in the insert. Scale bar = 25µm. Microphotographs are adapted from [13]; these results joined to a recent study [98] are the first ones allowing the visualization of NO synthesis in the gastrointestinal tract.

CHANGES IN GASTROINTESTINAL FUNCTION IN ENDOTOXEMIA

Gastrointestinal Motility

Opposite changes between gastric and intestinal motility are observed in the early phase of infection with Gram-negative bacteria. Inhibition of gastric motility and stimulation of intestinal transit occur after administration of doses of endotoxin in the range of μg/kg to rats [5,7,10,64-67].

Inhibition of gastric motor function

In experimental studies, diminution of the gastric tone, relaxation of the gastric fundus and delay in gastric emptying are observed early after systemic administration of bacterial endotoxin to rats. These changes are rapid in onset (30-60 min post-injection of LPS) and mediated by capsaicin-sensitive afferent neurons, since neuronal blockade of these fibres by pre-treatment with the selective neurotoxin capsaicin, completely prevents gastric motor disturbances induced by LPS [5,10]. One of the physiological roles played by these fibres includes a local efferent-like function that allows the release of neuropeptides, a mechanism that seems to be involved in the inhibitory effect of endotoxin, since blockade of CGRP receptors prevented the delay in gastric emptying associated to this circumstance [5]. However, these fibres also trigger a sensory function signalling information from periphery to superior neuronal network [68,69], a mechanism that is also involved in the gastric effect of endotoxin since local application of capsaicin to the cervical vagus nerve prevented such effect “Fig.(**1**)”.

The DVC of the brainstem plays an important role in physiological modulation of gastric motor function. Although presence of different gastric inhibitory neurotransmitters has been reported in this area, increasing evidence for the last fifteen

years support a crucial role for NO synthesis in the DVC on the inhibition of gastric motor function [10,47]. It has been reported that gastric relaxation evoked by the central nervous system is completely abolished by an NOS inhibitor in the DVC [70] while administration of NO producing compounds in the DMN increase the spontaneous firing rate of the neurons [71]; furthermore microinjection of L-arginine (the NO synthesis precursor) into the DVC has been shown to decrease intragastric pressure while L-NAME (a NOS inhibitor) increased it [47]. It is likely that a mechanism based on the activation of the NOS in the DVC and the subsequent synthesis of NO is triggered by peripheral endotoxin and plays a role in the diminution of gastric motor function associated with this circumstance. In this line, NO synthesis in the brainstem has been involved in endotoxin-induced diminution of intragastric pressure stimulated by 2-deoxy-D-glucose [10]. These results, joined to the fact that no changes in the amount of nNOS protein in the brainstem are observed under these circumstances, suggest that a non-transcriptional regulation of NO synthesis from nNOS in this area during early endotoxemia is involved in gastric motor inhibition "Fig. (**1**)".

Previous studies have reported that inhibition of gastric motor function by NO synthesis in the DVC is an effect mediated by the vagus nerve [47], although the nature of the vagal efferent fibres involved has not been elucidated. In a recent study we have reported that fundus relaxation induced by intraperitoneal administration of LPS is mediated by a neural mechanism that involves extrinsic innervation of the gut and NO synthesis in the myenteric plexus of the gastric fundus [13]. Such a synthesis is mediated by the nNOS isoform since both L-NAME and a selective nNOS inhibitor, TRIM, significantly prevented fundus relaxation induced by endotoxin. Different experimental studies have demonstrated that NO synthesis in both the brainstem and myenteric plexus is involved in gastric motor inhibition induced by low doses of

systemic LPS. These results led us to hypothesise that a sequence of nitrergic neurons could be the efferent mechanism involved in the inhibitory effect of endotoxin on gastric function, as previously reported [47]. However, it has also been reported that endotoxin-induced NO synthesis in the myenteric plexus is regulated by nicotinic receptors in the ganglia which support a role for vagal cholinergic pre-ganglionic fibres in the effects of endotoxin [13]. These results ruled out the above hypothesis and make necessary further studies to draw the specific efferent pathway activated by endotoxin [10,72] "Fig. (**1**)".

Most of the effects induced by the constitutive synthesis of NO, including relaxation of the gastric smooth muscle involve activation of the soluble guanylate cyclase (sGC) [53]. It has been reported that relaxation of the gastric fundus by low doses of endotoxin is mediated by activation of the sGC. This intracellular messenger reinforces the synthesis of NO by endotoxin and suggests a constitutive enzymatic source [13]. In the same study it is shown that the inhibitory effect of LPS was also mediated by activation of small conductance calcium sensitive K^+ ($_{ca}K^+$) channels [13], which have been involved in gastrointestinal relaxation induced by endogenous and exogenous NO [13,73-75]. In addition, by using pharmacological tools it is shown that NO, once released from enteric neurons, would increase the levels of cGMP which in turn phosphorylates the small conductance $_{ca}K^+$ channels [76] which leads to relaxation of the smooth muscle cell under early endotoxemia "Fig. (**3**)".

In summary, consistent evidence supports that inhibition of gastric motor function in early endotoxemia is mediated by a complex neural mechanism that involves activation of the vagus nerve, synthesis of NO in the brainstem and the final release of NO from enteric neurons acting on the smooth muscle cell. Such a

mechanism might be involved in the relaxation of gastric fundus associated with the delayed gastric emptying observed in endotoxemia.

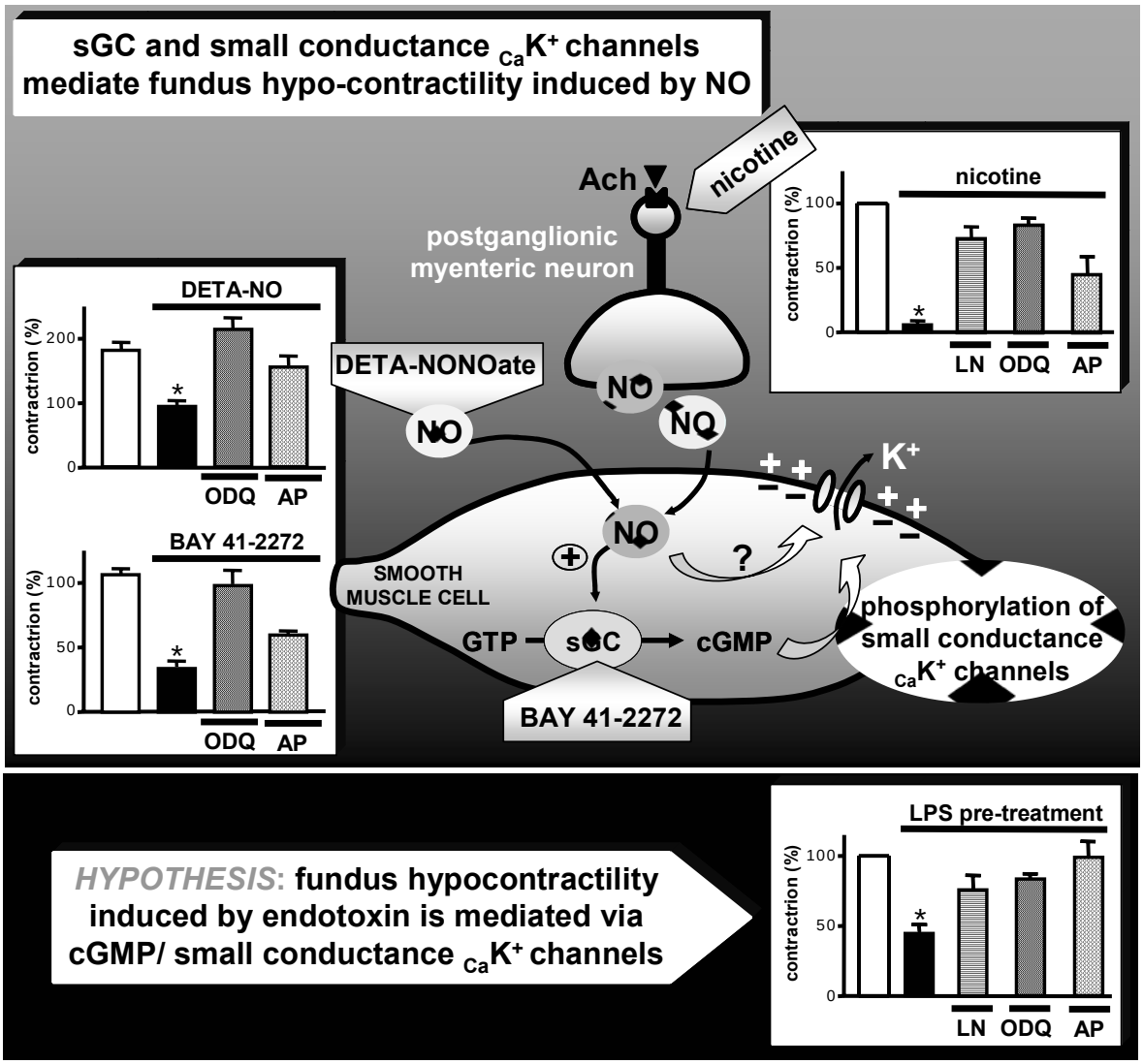

Fig. (3). Schematic diagram showing the intracellular mechanisms involved in NO induced fundus relaxation. NO, endogenously released by nicotine or LPS and exogenously administered with an NO donor (DETA-NO), induces relaxation of the smooth muscle cell mediated by activation of sGC and phosphorylation of small conductance $_{Ca}K^+$ channels. These channels are involved in relaxation of gastric fundus induced by direct activation of the sGC with BAY 41-2272. We propose that fundus relaxation during early endotoxemia is mediated via NO/cGMP/$_{Ca}K^+$ channels. Graphs show original data adapted from [13] and represent mean±s.e.m. (n>3; *p<0.05). AP,

apamine; DETA-NO, (Z)-1-[2-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate); LN, L-nitroarginine methyl ester; ODQ, 1H-(1,2,4,)oxadiazolo(4,3-a)quinoxalin-1-one).

Gastric emptying of meals is a complex function of the gut mediated by the integrated response of the proximal (fundus) and distal (antrum-pylorus) stomach and duodenum [77], and nitric oxide has been shown to play a role in the specific functions carried out by these areas [78-80]. Gastric emptying is inversely controlled by tones of gastric body and pyloric sphincter. In this context, some evidence supports an inhibitory role of NO on gastric emptying through an action related with relaxation of the gastric fundus [81] while NO synthesis has been associated with an acceleration of gastric emptying mainly due to pyloric sphincter relaxation [78,82,83]. Experimental studies have shown a delayed gastric emptying of a solid nutrient meal after administration of doses of endotoxin in the range of μg/kg. Due to the intrinsic kinetic of this physiological process, studies were performed four hours after endotoxin administration. Interestingly, when constitutive NOS activity was analyzed in the antrum-pylorus of the stomach, a significant reduction was observed parallel to delayed gastric emptying [7]. In addition, nNOS mRNA expression and protein was also significantly reduced, which suggest that these doses of endotoxin induce four hours later a transcriptional down-regulation of the nNOS protein expression in the antrum-pylorus; it was hypothesised that diminution of NO synthesis would increase the sphincter tone and, therefore, delay gastric emptying “Fig. (**1**)”. It is interesting to note the area-specific modulation of NO synthesis in the stomach induced by endotoxin: an increase of NO synthesis in the gastric fundus is observed 30 min after challenge while a trancriptional down-regulation of nNOS in the antrum pylorus is observed four hours later. The specific relationship between these two mechanisms needs to be addressed.

Increase of intestinal transit

Besides the abnormal gastric motility, intestinal motility pattern is also altered during early endotoxemia. Administration of doses of μg/kg of *E. coli* endotoxin induce a significant increase of colonic motility and transit in conscious rats, an action that was reported to be partly mediated by PAF and PGs [84]. In addition, a similar dose of endotoxin has been shown to increase intestinal transit in anaesthetized rats and faecal excretion in conscious rats within one hour of administration [42,85]. The effect of endotoxin on fecal excretion was only observed within the first three hours after challenge and was not associated with changes in the consistency of the faeces, suggesting that the effect of endotoxin was independent of changes in intestinal secretion, in contrast to that reported with slightly higher doses of intravenous endotoxin [84]. Such a prokinetic effect of endotoxin on intestinal motility seems to be mediated by a long nervous reflex, similar to what happens in the stomach, as endotoxin effect on faecal excretion is prevented by subdiaphragmatic vagotomy or systemic pre-treatment with capsaicin [42]. Systematic progression of intestinal contents is known to be dependent of the peristaltic reflex, which consists of two sequential components: descending relaxation caudal and ascending contraction oral to the site of stimulus. The excitatory neurotransmitters responsible for ascending contraction are Ach and the tachykinins substance P and neurokinin A, whereas VIP and NO mediate the descending relaxation of circular muscle [86]. Once again the effects of early endotoxemia on faecal excretion involve the activation of a physiological mechanism based on the release of the excitatory neurotransmitters Ach and substance P and the inhibitory neurotransmitter NO. Previous studies using higher doses of endotoxin have reported an increased expression of iNOS in enterocytes and a role for iNOS-derived NO in diarrhoea observed in those conditions [87]. However, the small doses of

endotoxin used and the short time in which its effects on faecal excretion are observed, point towards the regulation of constitutive NOS isoform rather than an induction of iNOS. As a whole, results suggest that the increase in faecal excretion induced by endotoxin is consequence of a nervous mechanism in which capsaicin-sensitive afferent neurons and the release of excitatory and inhibitory neurotransmitters in the colonic wall are involved. An increased secretion of Ach and substance P would enhance the propulsory effect of ascending contraction of the circular muscle, while local release of NO would facilitate the accommodation of the propelled bolus by inducing the descending relaxation of the colonic muscle "Fig. (**1**)".

In conclusion we hypothesised that after bacterial invasion the host responds acutely by maximizing its physiological resources, before the immune system is active, in order to prevent further absorption of challenge. In this process, gastrointestinal motility is wisely altered and opposing effects on motility depending on the region analysed are observed. A hypo-contractility of proximal stomach and a delayed gastric emptying would prevent access of possible toxins to the duodenum and absorption, while increased intestinal propulsion and faecal excretion would help to expel challenge. A perfect interplay between the central and enteric nervous systems is necessary, and nitric oxide seems to be a crucial molecule in this process.

Gastric acid secretion

Gastric acid secretion constitutes one of the most important endogenous aggressive factors for the gastric mucosa. Although hyper-secretion has been observed in severe stress situations, inhibition of gastric acid secretion has been reported during moderate clinical endotoxemia [88], a situation that has been reproduced after endotoxin administration to rats [9,12,24,63,89]. Doses of LPS in the range of μg/kg inhibit gastric

acid secretion in a reversible way, indicative of a biological effect on gastric physiology rather than a toxic or destructive effect on the parietal cell [90]. Early studies demonstrated that inhibition of gastric acid secretion by endotoxin was rapid in onset (observed 15 min post-injection), not related to vascular changes and independent of protein synthesis [9,12,24]. In addition, experimental evidence demonstrated that a neural mechanism involving the central nervous system and activation of non-adrenergic non-cholinergic fibres was involved in endotoxin-induced gastric hyposecretion [9]. Pharmacological studies using administration of NOS inhibitors into the cisterna magna showed a role for central NO synthesis in the hypo-secretory effects of endotoxin [12]. Further immunohistochemical and electrochemical studies localized the enzyme responsible for NO synthesis in the DVC of the brainstem, suggesting that a nervous mechanism involving the activation of the vagus nerve could mediate the inhibitory effects of endotoxin on gastric acid secretion [24]. In line with this observation, inhibition of pentagastrin-stimulated acid secretion by endotoxin was prevented by cervical vagotomy [63]. Furthermore, the acid inhibitory effect of endotoxin was also prevented by intracisternal administration of an NMDA glutamate receptor antagonist, reinforcing the involvement of the afferent vagus nerve in the hypo-secretion [63]. However, still little evidence exists regarding the efferent mechanism that finally reduces gastric acid secretion in endotoxemia. Taking into consideration that the hypo-secretory doses of endotoxin have been shown to increase NO synthesis in the myenteric plexus [13], a direct role for NO acting on the parietal cell and inhibiting gastric acid secretion might be inferred. Consistent with this observation is the fact that NO donors have been shown to inhibit the secretory activity in rat isolated parietal cells [91].

Endotoxin may be considered as a challenge to which the body responds with stress. In this line, endotoxin has been shown to activate neurons immunoreactive to stress peptides such as oxytocin or corticotrophin releasing factor in the brain [27,92] and the endogenous release of oxytocin has been shown to play a key role in the hypo-secretory response associated with endotoxemia [93]. Other experimental models of moderate somatic stress such as mild hyperthermia or hypotension have been associated with an acute and long-lasting inhibition of gastric acid secretion, and a nervous reflex involving NO synthesis in the DVC [24] mediated such a response. It seems that acute hypo-secretion associated with stress is a consequence of a physiological protective response activated under these circumstances.

Gastric blood flow and mucosal integrity

Gastric mucosal lesions are important complications of septic shock. However, administration of doses of endotoxin in the range of μg/kg to rats has been reported to protect gastric mucosa against the damage induced by ulcerogenic stimuli such as stress, NSAIDs and ethanol [6,8,11]. The gastroprotective action of LPS is observed one hour after challenge, mediated by activation of capsaicin-sensitive afferent neurons and independent of transcriptional synthesis of proteins. In addition NO synthesis-induced by endotoxin seems to play an important role since blockade of the NOS significantly prevented the gastroprotective effect. Such a synthesis was not related to the iNOS isoform, since: a) iNOS mRNA expression was not detected in gastric corpus one hour after LPS administration, time at which the gastroprotective effect was observed [8] and b) dexamethasone pre-treatment did not modify LPS effect [11]. Once again, low doses of endotoxin induce NO synthesis from constitutive sources, which is involved in mucosal gastroprotection. Constitutive synthesis of NO by nNOS or eNOS isoforms has

been involved in the maintenance of the gastrointestinal mucosal integrity through modulation of gastric mucosal blood flow, prevention of leukocyte adhesion to the endothelium, epithelial secretion and barrier function [49,94,95]. Among these mechanisms, modulation of gastric mucosal blood flow, [95] seems to play an essential role. Neither, endogenously generated NO by endotoxin, nor exogenous NO liberated by an NO donor, such as nitroglicerin, modifies resting gastrointestinal blood flow in experimental animals. However, both situations have been shown to prevent the reduction in gastric blood flow induced by an ulcerogenic dose of NSAIDs and consequently counteract its detrimental effects [8,96]. The eNOS is presumably the main isoform involved in the maintenance of gastric blood flow; however, synthesis of NO in the myenteric plexus, probably due to nNOS isoform, which has been demonstrated during early endotoxemia [13], could also be contributing to gastric mucosal protection. In this line, evidence exists for a role of endogenous NO in gastric mucosa protection following central vagal stimulation [97]. However, biochemical studies trying to demonstrate an increase in the NOS activity of the constitutive isoforms in the gastric corpus of endotoxin-treated rats showed no significant changes. It is believed that factors increasing the activity of these enzymes that are only present in vivo were responsible for the enhanced NO synthesis after endotoxin administration. In summary, we believe that rapidly after bacterial infection the host tries to maintain gastric mucosal integrity mainly by preserving the fall in microcirculation and, in order to get this, maximizes the activity of its constitutive resources.

CONCLUDING REMARKS

Gastrointestinal function is modulated by endotoxin. The initial effects depend on a fast, neural mechanism involving activation of capsaicin sensitive afferent neurons,

which explains the rapid onset (first hour post-injection) of the endotoxin effect on faecal excretion, gastric motility, acid secretion and mucosal resistance to damage. In such a response, NO synthesis from calcium-dependent NOS isoforms occurs in both, the central (DVC) and enteric nervous systems. The perfect interplay of afferent/efferent neurons and nNOS constitutes an emergency system, which allows the organism to adapt to slight alterations of the physiology, and enhances the body ability to withstand ulterior aggressions. Understanding the functional neuroanatomy by which the nervous system responds to immune challenge would greatly help to control the deleterious and promote the beneficial aspects of the acute phase reaction to infection.

ACNOWLEGDMENTS

Research in the authors' laboratory pertaining to this topic was supported by Grant FIS 01/1187 from the Ministerio de Sanidad y Consumo and grant C 03/02 from Instituto de Salud Carlos III. Elsa Quintana is the recipient of a fellowship from the Ministerio de Sanidad y Consumo.

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RESUMEN DE RESULTADOS Y DISCUSIÓN GENERAL

El grupo de investigación al que pertenezco ha caracterizado durante los últimos años los mecanismos involucrados en las alteraciones agudas de la función gastrointestinal en la endotoxemia. A diferencia de lo que ocurre en estadios más avanzados, en donde prevalecen los mecanismos inmunes, la activación de vías nerviosas parece desempeñar un papel esencial en los cambios gastrointestinales durante la endotoxemia temprana. De este modo, la inhibición de la secreción ácida gástrica y el incremento del flujo sanguíneo y resistencia de la mucosa gástrica, que acontecen de forma rápida tras la administración de endotoxina a un animal de laboratorio, son mediados por un mecanismo nervioso que involucra el sistema nervioso periférico y central (Barrachina *et al.*, 1995a y b; Calatayud *et al.*, 2003; Esplugues *et al.*, 1996; Garcia-Zaragoza *et al.*, 2000).

El presente trabajo ha sido diseñado para estudiar las alteraciones de la motilidad gastrointestinal asociadas a los primeros estadios de la endotoxemia y los mecanismos que la median. A continuación se resumen y discuten los resultados obtenidos agrupándolos en dos partes generales según se refieran a la alteración de la función motora gástrica o colónica.

I. EFECTOS DE LA ENDOTOXINA SOBRE LA MOTILIDAD GÁSTRICA

La administración periférica de endotoxina de *E. coli*, a dosis subsépticas, produjo una inhibición aguda de la función motora gástrica caracterizada por una hipocontractilidad del estómago proximal y un enlentecimiento del vaciamiento gástrico de sólidos nutrientes (**artículos 1-5**).

Estos efectos se observan rápidamente tras la administración de la endotoxina y son independientes de síntesis proteica lo que sugiere la participación de mecanismos nerviosos. Gracias al uso de herramientas farmacológicas y técnicas inmunohistoquímicas hemos demostrado la

participación de un reflejo nervioso que involucra el nervio vago y el SNC en las alteraciones motoras inducidas por la endotoxina.

Análisis de la vía nerviosa aferente

La activación de fibras aferentes sensoriales sensibles a la capsaicina participa en la inhibición de la presión intragástrica (P_{IG}) y en el retraso del vaciamiento gástrico inducido por la endotoxina, ya que el pretratamiento sistémico con la capsaicina previno los efectos inhibidores de la endotoxina (**artículos 1,4**).

Las neuronas aferentes primarias en el tracto gastrointestinal pueden actuar a través de dos mecanismos. En primer lugar, su activación se ha relacionado con un efecto local (reflejo axonal) a través del cual se liberan neuropéptidos, tales como taquicininas y CGRP, que modulan la función gastrointestinal (Holzer, 1988; Holzer, 1991). El bloqueo de los receptores del CGRP previno significativamente el efecto de la endotoxina sobre el vaciamiento gástrico sugiriendo por tanto que la activación de las terminaciones nerviosas sensoriales de estas fibras está mediando dicho efecto (**artículo 4**). Por otra parte, las fibras aferentes también mandan la información somatovisceral sensorial a centros nerviosos superiores (Holzer, 1998). En nuestros experimentos, la aplicación local de capsaicina en el nervio vago cervical previno la inhibición del vaciamiento gástrico inducido por la endotoxina, mientras que la aplicación directa de capsaicina en el ganglio celíaco no alteró esta función en ningún caso (**artículo 4**). Estos resultados sugieren que un reflejo nervioso largo, mediado por el nervio vago, que involucra la participación del SNC también está implicado.

El conjunto de estos resultados proponen que la endotoxina altera la motilidad gástrica mediante la activación de neuronas aferentes sensibles a la

capsaicina. Estas neuronas, además de tener una función efectora local, envían a través del nervio vago la información aferente a centros nerviosos superiores.

Participación del sistema nervioso central

La administración periférica de endotoxina en la rata consciente induce un incremento en la expresión de la proteína Fos, marcador de activación neuronal, en diferentes núcleos del CDV, sugiriendo la participación del SNC en los efectos de la endotoxina. Así, el pretratamiento con la endotoxina, a dosis que modula la función motora gástrica, incrementó significativamente las células inmunoreactivas a Fos en el CDV del tronco del encéfalo extraído de ratas pretratadas con 2-DG (**artículo 1**). El CDV se compone del NTS, el cual recibe las fibras sensoriales aferentes, y el NDV, que contiene los cuerpos de las neuronas preganglionares motoras eferentes que inervan el tracto gastrointestinal (Kalia & Mesulam, 1980). Estos núcleos participan en los reflejos vago-vagales que regulan la función motora gástrica (Rogers *et al.*, 1995). Un análisis detallado de la localización de las células inmunoreactivas a Fos demostró que el incremento se dio específicamente en el NTS y en el NDV, implicando a ambos núcleos en la acción de la endotoxina.

La activación de neuronas del NTS sugiere que neuronas aferentes primarias vagales se activan durante la endotoxemia. La activación de dicho núcleo podría también deberse a un acceso directo de la endotoxina al SNC ya que, aunque está descrito que a estas dosis es incapaz de atravesar la barrera hematoencefálica (BHE), podría estar accediendo a través de pequeñas estructuras centrales carentes de BHE como es el área postrema situada también en el CDV. Sin embargo, la participación de las neuronas aferentes sensibles a la capsaicina refuerza la hipótesis de que es un mecanismo nervioso el responsable de la activación de núcleos centrales. En este sentido, se ha descrito en la literatura que los cuerpos neuronales de fibras vagales

aferentes, localizados en el ganglio nodoso, también expresan Fos tras la administración periférica de endotoxina y dicha expresión está bloqueada con la vagotomía (Gaykema *et al.*, 1998). Además, se ha descrito que la endotoxina induce la liberación de glutamato, principal neurotransmisor de las terminales aferentes vagales, en el NTS (Mascarucci *et al.*, 1998) y se ha demostrado un papel central para este mediador en la alteración de la función gástrica asociada a estas circunstancias (Garcia-Zaragoza *et al.*, 2000).

Por otra parte, la activación de neuronas del NDV durante la endotoxemia sugiere la participación de fibras motoras eferentes vagales en los efectos de la endotoxina. En este sentido, la inhibición de la función motora gástrica tras la excitación de neuronas del NDV por diversos estímulos ha sido previamente descrita (Blackshaw *et al.*, 1987; Miolan & Roman, 1984). Parece que la administración periférica de endotoxina induce la activación de neuronas sensoriales y motoras a nivel del tronco del encéfalo.

El NO se ha descrito como un mensajero intercelular de los circuitos nerviosos del tronco del encéfalo que controlan la función gástrica (Esplugues *et al.*, 1996; Krowicki *et al.*, 1997). En nuestros experimentos, el pretratamiento con L-NAME previno el incremento de la expresión de Fos inducido por la endotoxina periférica en el CDV, sugiriendo que las neuronas que se activan en respuesta a la endotoxina son dependientes de la síntesis de NO (**artículo 1**). Sin embargo, el hecho de que en animales control el pretratamiento con L-NAME indujo la activación de núcleos del CDV hace que estos resultados no sean concluyentes. La participación del NO en la activación de núcleos del CDV se ve reforzada por el hecho de que la administración de la misma dosis de endotoxina incrementó rápidamente (30 min) la actividad enzimática NOSn en el tronco del encéfalo (**artículo 1**). Además, el NO sintetizado media la inhibición de la P_{IG} que tiene lugar durante la endotoxemia ya que el pretratamiento intracisternal (i.c.) con L-NAME previno dicho efecto (**artículo 1**). En línea con

estos resultados, nuestro grupo de investigación ha demostrado previamente que la endotoxina y situaciones de estrés como son una ligera hipertermia o la administración i.c. de oxitocina, inhiben la secreción ácida gástrica mediante un reflejo nervioso que implica síntesis de NO específicamente en el NDV (Esplugues *et al.*, 1996).

El análisis de la expresión génica de la NOSn en una sección del tronco del encéfalo que contiene el CDV demostró un incremento del ARNm de la NOSn 2 horas tras la administración de la endotoxina, mientras que no se observaron cambios a tiempos más tempranos (**artículo 2**). Este incremento también se vio reflejado en un aumento de la cantidad de proteína NOSn, analizado por inmunohistoquímica. El análisis de la distribución de la NOSn a lo largo del eje rostro-caudal del CDV en los animales control refleja una mayor proporción a nivel rostral, sugiriendo un papel para el NO en esta zona en la regulación fisiológica de funciones autonómicas. En este sentido, se ha descrito un papel tónico para la síntesis de NO en el CDV rostral modulando la P_{IG} (Krowicki *et al.*, 1997). En el tronco del encéfalo procedente de animales pretratados con la endotoxina, la distribución de la NOSn fue muy similar, reflejándose un incremento significativo específicamente a nivel rostral. Estos resultados sugieren la participación de las neuronas nitrérgicas del CDV rostral mediando los efectos de la endotoxina.

En conjunto, los diferentes estudios muestran que la activación de núcleos nerviosos del CDV en respuesta a la endotoxemia lleva consigo un incremento agudo (30 min) de la síntesis de NO, regulado a nivel postraduccional, que es responsable de la inhibición de la P_{IG} asociada a esta circunstancia. Además, un poco más tarde (2h), se produce una regulación transcripcional de la isoforma NOSn específicamente en el nivel más rostral del CDV que probablemente esté implicada en la modulación de la función gastrointestinal que tiene lugar en estadios más avanzados.

Análisis de la vía nerviosa eferente

El nervio vago constituye la principal vía de comunicación entre el SNC y el SNE. La activación de neuronas del NDV nos indicó que las fibras eferentes vagales podían estar mediando los efectos de la endotoxina. El nervio vago contiene fibras eferentes colinérgicas preganglionares que inervan el estómago y regulan su motilidad (Chang *et al.*, 2003). En su interacción con el SNE, estas fibras pueden hacer sinapsis con: (a) neuronas postganglionares entéricas colinérgicas excitadoras, provocando un incremento de la actividad motora gástrica (vía colinérgica); o (b) neuronas postganglionares entéricas inhibidoras que relajan la musculatura gástrica (vía no-adrenérgica no-colinérgica o NANC). Además, se ha descrito un tercer tipo de neuronas cuya transmisión es independiente de receptor nicotínico por lo que no hacen sinapsis ganglionar, y que producen una relajación del músculo liso dependiente de NO (Krowicki *et al.*, 1999). Una inhibición de la vía colinérgica y/o una activación de la vía NANC o de las neuronas vagales nitrérgicas independientes de transmisión ganglionar, conduciría a una relajación del tono muscular gástrico. En nuestros experimentos, el pretratamiento con el antagonista muscarínico atropina, a dosis que no alteraron la respuesta control, no modificó el efecto inhibidor de la endotoxina sobre el vaciamiento gástrico. Una dosis superior de atropina inhibió el vaciamiento gástrico en un animal control y produjo una inhibición más acusada aún en los animales tratados con la endotoxina, descartando la participación de estos receptores en los efectos inhibidores de la endotoxina (**artículo 4**). Además, la hipocontractilidad observada en el fundus inducida por la endotoxina fue revertida totalmente por el bloqueante del receptor nicotínico ganglionar hexametonio, indicando que está implicada una sinapsis nerviosa ganglionar (**artículo 3**). Por otra parte, el estómago también recibe inervación extrínseca simpática procedente de nervios postganglionares noradrenérgicos esplácnicos. Aunque la influencia del sistema nervioso simpático sobre la

motilidad gástrica es mucho menos importante que la regulación parasimpática vagal, la excitación de estas fibras también provoca la inhibición de la motilidad gástrica. Sin embargo, el bloqueo de los receptores adrenérgicos α y β no alteró el vaciamiento gástrico en animales control ni tratados con la endotoxina, descartando la participación de estas fibras en nuestro modelo (**artículo 4**). El conjunto de estos resultados descarta un efecto inhibidor de la vía colinérgica y adrenérgica y apunta hacia la hipótesis de que durante la endotoxemia se pone en marcha la activación de una vía inhibidora vagal NANC.

Con el bloque experimental expuesto hasta ahora podemos concluir que, en estadios tempranos de la endotoxemia, la actividad motora gástrica se ve alterada de manera aguda, efecto que está mediado por un reflejo nervioso en el que participan fibras aferentes sensibles a capsaicina, los núcleos centrales NTS y NDV situados en el tronco del encéfalo, la síntesis de NO a este nivel y una vía eferente NANC. Con estos resultados confirmamos que la alteración de la función motora gástrica durante la endotoxemia es debida a un mecanismo nervioso similar al que ya se había descrito previamente para la modulación de otras funciones tales como la inhibición aguda de la secreción ácida gástrica asociada a estas circunstancias (Barrachina *et al.*, 1995b; Esplugues *et al.*, 1996; Garcia-Zaragoza *et al.*, 2000). Sin embargo, hasta ahora no se había analizado la parte final de este reflejo nervioso. Uno de los objetivos de la presente tesis ha sido analizar cuáles son los mediadores periféricos últimos responsables de dicho efecto. A continuación se exponen y discuten los resultados obtenidos.

Mediadores periféricos implicados

La inervación NANC constituye la principal vía nerviosa inhibidora responsable de la relajación de la musculatura lisa gastrointestinal. Diferentes

estudios farmacológicos han demostrado que NO, VIP y ATP son los mediadores finales de esta vía (Curro & Preziosi, 1998). En nuestros experimentos *in vivo*, la administración intravenosa de L-NAME previno de manera significativa la disminución de la P_{IG} asociada a la endotoxemia, sugiriendo por tanto que la síntesis periférica de NO está mediando dicho efecto (**artículo 1**). Sin embargo, estos resultados no son concluyentes dado que el L-NAME es capaz de atravesar la BHE inhibiendo así la síntesis de NO central, que como ya hemos demostrado previamente, está involucrada. Con la finalidad de analizar de manera aislada la parte final del reflejo nervioso, diseñamos un protocolo experimental que combina el tratamiento *in vivo* con la endotoxina durante 30 min, periodo en el cual se desencadena el reflejo nervioso, con el análisis posterior de la capacidad contráctil del tejido aislado *in vitro*, mediante la técnica de baño de órganos (**artículo 3**). De esta manera pudimos comprobar que la hipocontractilidad fúndica derivada del pretratamiento con la endotoxina fue revertida tras la incubación del tejido con la tetrodotoxina (TTX) o el hexametonio. Además, cuando se incubó la endotoxina *in vitro* sobre el fundus aislado de animales sin tratar no se vio afectada su capacidad contráctil, descartando un efecto directo de la endotoxina sobre el territorio gástrico. El conjunto de estos resultados demuestra la participación de una regulación nerviosa extrínseca en nuestro modelo. Por otra parte, la inhibición local de la síntesis de NO mediante la incubación con L-NOARG, o con TRIM (inhibidor selectivo de la NOSn) revirtió por completo la hipotonicidad del fundus inducida por la endotoxina. Así, obtuvimos la primera evidencia directa que demuestra que la síntesis de NO en el fundus media los efectos de la endotoxina. El bloqueo de los receptores del VIP o del ATP no modificó la respuesta contráctil en ningún caso, descartando por tanto la participación de estos dos mediadores en estas circunstancias.

El análisis de la cantidad de ARNm de NOSn en el fundus (1h tras el tratamiento) nos indicó que la endotoxina no modifica la expresión génica de esta isoenzima (**artículo 3**). Este resultado indica que la síntesis de NO inducida por la endotoxina se debe a una regulación postranscripcional, hecho que no sorprende dada la rapidez con la que se observan los cambios en la motilidad.

Con el afán de demostrar la síntesis de NO de una manera directa y de analizar el origen celular empleamos la microscopía confocal, la cual nos permitió visualizar en el tejido íntegro dicha síntesis gracias al empleo del DAF-FM, un fluorocromo "vital" específico del NO (Nagano & Yoshimura, 2002) (**artículo 3**). Este sistema permite observar directamente la síntesis intracelular del NO *in situ* y en tiempo real proporcionando así una valiosa información de la distribución espacial y temporal del NO en el organismo. Así, observamos que la fluorescencia del DAF-FM, principalmente presente en células nerviosas, se vio incrementada en el fundus de los animales pretratados *in vivo* con la endotoxina. Además, dicho incremento fue revertido tras la incubación con L-NAME, TTX y hexametonio. Estos resultados demuestran en su conjunto que la endotoxemia induce la síntesis de NO en neuronas postganglionares del plexo mientérico del fundus, y refuerzan los resultados funcionales que demuestran un papel para dicha síntesis modulando el tono del fundus gástrico.

La principal diana del NO involucrada en la relajación del músculo liso descrita hasta la fecha es la enzima GCs (Lefebvre, 1998; Moncada *et al.*, 1991). Además, los canales de potasio dependientes de calcio (canales $_{Ca}K^{+}$) también han sido relacionados con la relajación muscular inducida por el NO (Kitamura *et al.*, 1993; Shuttleworth & Sanders, 1996). En nuestros experimentos (**artículo 3**) hemos comprobado que la relajación del fundus inducida tanto por el NO exógeno (DETA-NO), como por el NO liberado endógenamente (nicotina) fue revertida al bloquear la actividad enzimática de la GCs con ODQ y al bloquear los canales $_{Ca}K^{+}$ de baja conductancia con

apamina. Además, la hipocontractilidad del fundus inducida por la endotoxina fue revertida también con el ODQ y con la apamina. Estos resultados demuestran que el NO liberado durante la endotoxemia relaja el fundus gástrico a través de la enzima GCs y de la hiperpolarización inducida por la apertura de los canales $_{Ca}K^+$ de baja conductancia. Actualmente en la literatura encontramos discrepancias sobre si el NO activa directamente estos canales (Bolotina *et al.*, 1994; Serio *et al.*, 2003) o si es una acción mediada por el propio GMPc (Robertson *et al.*, 1993). En este sentido, nosotros demostramos que la vía GCs/canales $_{Ca}K^+$ de baja conductancia media la relajación del fundus ya que la relajación inducida por un activador directo de la enzima GCs es revertida por la apamina. En base a estos resultados proponemos que la endotoxina relaja el fundus gástrico mediante la vía NO/GMPc/canales $_{Ca}K^+$; sin embargo, no podemos descartar una acción directa del NO sobre estos canales.

Todos estos resultados sugieren que la administración de dosis bajas de endotoxina activan un reflejo nervioso vagal en el que participa la síntesis neuronal de NO en el tronco del encéfalo y en el plexo mientérico y como consecuencia se induce rápidamente la relajación del fundus gástrico. Es posible que este mecanismo pueda estar involucrado en el retraso del vaciamiento gástrico que se observa en estas circunstancias. En la literatura se ha descrito un papel inhibidor para el NO sobre el vaciamiento gástrico a través de la relajación del fundus (Takakura *et al.*, 1997).

El vaciamiento gástrico de sólidos nutrientes es una función compleja regulada por la actuación integrada de la parte proximal (fundus) y distal (antro-píloro) del estómago y del duodeno (Mayer, 1994). En el estómago, el tono de la parte proximal y distal regulan de manera inversa el vaciamiento siendo el tono del esfínter pilórico el último responsable que permite la salida del contenido gástrico. Mediante técnicas de inmunohistoquímica se ha demostrado una elevada densidad enzimática NOS en este esfínter (Feher *et*

al., 2001; Ward *et al.*, 1994). Además, la síntesis de NO se ha asociado con una aceleración del vaciamiento gástrico principalmente debido a la relajación del píloro (Allescher & Daniel, 1994; Orihata & Sarna, 1994; Plourde *et al.*, 1994). En nuestros experimentos (**artículo 5**), el tratamiento con L-NAME o 7-NI (inhibidor selectivo de la NOSn) inhibió de manera significativa el vaciamiento de sólidos en animales control, reforzando un papel fisiológico para la enzima NOSn en el control de esta función. Sin embargo, dicho tratamiento no produjo una inhibición más acusada del vaciamiento en los animales tratados con la endotoxina. Podríamos decir que la inhibición de la síntesis de NO en condiciones fisiológicas mimetiza el efecto de la endotoxemia sobre el vaciamiento gástrico. El análisis de la expresión génica, cantidad proteica y actividad de la enzima NOSn en la región antro-pilórica demostró que 4h tras el tratamiento con la endotoxina, tiempo en el cual está inhibido el vaciamiento gástrico, se produce una disminución transcripcional de la NOSn con la consecuente disminución de su actividad en esta región. La inervación NANC juega un papel muy importante mediando la relajación del píloro. Nosotros pensamos que dicha disminución de la NOSn en el píloro puede estar causando una relajación NANC atenuada del esfínter con el consiguiente incremento del tono, impidiendo así el vaciamiento del contenido gástrico.

Resulta interesante la reacción del organismo mediante la cual está modulando la síntesis del NO de manera diferencial dependiendo de la región del estómago (incremento en la parte proximal y disminución en la parte distal), retrasando así el vaciamiento gástrico, probablemente con la finalidad de prevenir la absorción de toxinas.

Como se ha demostrado hasta ahora por distintas vías, la isoforma implicada en todos estos efectos es la NOSn. Está ampliamente descrito que la endotoxina incrementa la expresión de la NOSi (Barrachina *et al.*, 2001;

Moncada *et al.*, 1991) y se le ha atribuido un papel modulador de la motilidad a esta isoforma (Eskandari *et al.*, 1999; Takakura *et al.*, 1997). Sin embargo, este hecho está asociado a tiempos más largos y a dosis de endotoxina superiores a la empleada en este estudio, generalmente relacionadas con un *shock* endotóxico (Thiemermann, 1997). En nuestro modelo el análisis de la expresión de la NOSi en el estómago 1h tras la administración de la endotoxina, tiempo en el que la P_{IG} y el tono del fundus están inhibidos, demuestra que esta isoforma no está incrementada (**artículos 1,3**). Además, el pretratamiento con la dexametasona no altera dicho efecto, hecho que nos indica que no existe síntesis proteica (**artículo 3**). Por otra parte, el análisis de la expresión de la NOSi en la región antro-pilórica 4h tras el tratamiento con la endotoxina, tiempo en el cual observamos la inhibición del vaciamiento gástrico, sí revela un incremento de la cantidad de ARNm de esta isoenzima (**artículo 5**). Sin embargo, el análisis de la actividad enzimática NOSi no demuestra síntesis de NO derivada de esta isoforma. Además, el tratamiento con un inhibidor selectivo de la NOSi (L-NIL) no impidió el efecto de la endotoxina sobre el vaciamiento gástrico. Estos resultados demuestran que 4h tras el tratamiento con la endotoxina, la inducción de la NOS comienza a tener lugar, sin embargo la síntesis de NO derivada todavía no existe y por lo tanto no se le puede atribuir ningún papel funcional en la inhibición de la motilidad gástrica observada en este momento.

Además del NO, las prostaglandinas (PGs) también actúan modulando de manera importante la función motora gástrica (Sanders, 1984). La síntesis endógena (Suto *et al.*, 1994) o la administración exógena (Stein *et al.*, 1994) de PGs se ha asociado con la inhibición del vaciamiento gástrico. En nuestros experimentos (**artículo 5**), la inhibición de la ciclooxigenasa (COX) con indometacina no alteró el vaciamiento gástrico en los animales control descartando un papel regulador de las PGs en condiciones fisiológicas. Sin

embargo, este tratamiento impidió la inhibición del vaciamiento gástrico asociado a la endotoxemia indicando por tanto un papel para las PGs en estas circunstancias. Además, dicha síntesis parece consecuencia de una inducción de la isoforma COX-2 ya que el tratamiento con el inhibidor selectivo de esta isoforma (NS-398) o con la dexametasona también previno el efecto de la endotoxina.

II. EFECTOS DE LA ENDOTOXINA SOBRE LA MOTILIDAD COLÓNICA

Además de las alteraciones en la motilidad gástrica, la endotoxemia también se caracteriza por una alteración de la función motora intestinal. Estudios previos en nuestro laboratorio demuestran un incremento del tránsito intestinal en estas circunstancias (Martinez-Cuesta *et al.*, 1997). El presente estudio amplía dichos resultados y demuestra que la endotoxina incrementa de manera aguda (en las tres primeras horas) la excreción fecal (**artículo 6**). Durante este proceso no se observó un cambio en la consistencia de las heces lo que sugiere que el efecto de la endotoxina es independiente de alteraciones en la secreción intestinal, hecho que se observa en los procesos diarreicos característicos de una endotoxemia más severa (Closs *et al.*, 1998; Pons *et al.*, 1994).

Nuestros experimentos demuestran que el efecto procinético inducido por la endotoxina es consecuencia, al igual que en el caso anterior, de un reflejo nervioso vagal en el que las neuronas aferentes sensibles a la capsaicina están implicadas ya que el tratamiento con esta neurotoxina, así como una vagotomía abdominal, revirtieron tal efecto.

El avance del bolo fecal está regulado por el reflejo peristáltico el cual se compone de dos fases secuenciales: la relajación descendente caudal y

contracción ascendente oral. La coordinación de estas dos fases determinará la velocidad del tránsito intestinal. En circunstancias fisiológicas, los neurotransmisores excitadores responsables de la contracción ascendente son principalmente Ach y SP mientras que el NO está implicado en la relajación descendente. Nuestros resultados demuestran que durante la endotoxemia, la liberación de Ach, SP y NO están mediando la aceleración de la excreción fecal.

La mayoría de los estudios realizados en este campo demuestran un papel para la síntesis de NO derivada de la NOSi mediando la diarrea asociada a una endotoxemia generalmente más severa que la que presentamos nosotros. Sin embargo, aunque no hemos analizado en este caso la isoforma de la NOS involucrada, las dosis bajas de endotoxina que usamos y el mecanismo nervioso rápido que hemos demostrado está teniendo lugar, nos hace pensar principalmente en una regulación postraduccional de la isoforma NOSn una vez más.

Estos resultados sugieren que durante la endotoxemia se produce un incremento agudo de la excreción fecal mediado por un reflejo nervioso en el que neuronas aferentes sensibles a capsaicina y liberación de neurotransmisores excitadores (Ach y SP) e inhibidores (NO) están involucradas. Estudios adicionales harían falta para caracterizar la vía nerviosa implicada. Nosotros proponemos que la liberación en la pared colónica de Ach y SP promueve el efecto propulsor de la contracción ascendente, mientras que la síntesis local de NO permite el avance del bolo fecal al mediar la relajación descendente.

Recopilando toda la información reflejada en esta tesis podemos concluir que durante una invasión bacteriana el organismo reacciona rápidamente modulando la función motora gastrointestinal con la finalidad de prevenir la absorción futura de agentes extraños. Así, una hipocontractilidad del estómago proximal asociado a un enlentecimiento del vaciamiento gástrico prevendría el acceso de posibles toxinas al duodeno, retrasando de esta manera su posible absorción; en la misma línea, una aceleración de la excreción fecal facilitaría su pronta expulsión. Esta rápida respuesta es posible gracias a la interacción y perfecta sincronización de los sistemas nerviosos central y periférico. Así, el SNC recibe la señal de "alerta" en la periferia gracias a las neuronas aferentes sensibles a la capsaicina, rápidamente procesa esta información, principalmente en el CDV, y envía el mensaje "ejecutor" a través de las neuronas eferentes vagales que están en íntimo contacto con el SNE. De esta manera modula no sólo la función motora gastrointestinal, sino que también desencadena una inhibición de la secreción ácida gástrica, incrementa el flujo sanguíneo mucoso y protege la mucosa gástrica frente a agresiones externas (**artículo 7**). Todas estas acciones se pueden considerar como una reacción general de defensa que pone en alerta al organismo, intenta mantener la homeostasis y le prepara para una posible agresión posterior.

Además, hemos observado que la regulación postraduccional de la NOSn tiene un papel protagonista en todo este proceso. Un incremento de la síntesis de NO en el tronco del encéfalo, en neuronas postganglionares del plexo mientérico del fundus gástrico y en la pared colónica sucede en los primeros estadios de la endotoxemia. Pasado el efecto agudo de la endotoxina, empezaría a observarse lo que se considera parte de una respuesta inmune generalizada mediada por la expresión de las enzimas NOSi y COX-2. En respuesta a una invasión bacteriana, parece que el organismo maximiza la actividad de sus recursos fisiológicos mientras se alcanza la plena actividad del sistema inmune.

CONCLUSIONES

I. Tras una invasión bacteriana se observan cambios agudos en la función motora gastrointestinal caracterizados por un enlentecimiento del vaciamiento gástrico y una aceleración de la excreción fecal. Este comportamiento constituye un mecanismo de respuesta temprana cuya finalidad podría ser evitar la absorción de toxinas y facilitar su expulsión.

II. Estas alteraciones se producen rápidamente gracias a la actuación del sistema nervioso, tanto central como periférico, mediante la activación secuencial de fibras sensoriales aferentes, núcleos centrales del complejo dorsal vagal, fibras eferentes vagales y neuronas del plexo mientérico.

III. En este proceso, la síntesis aguda de óxido nítrico de origen nervioso juega un papel esencial como neurotransmisor en el tronco del encéfalo, y como molécula efectora en la unión neuro-muscular en el territorio gastrointestinal.

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