UNIVERSIDAD DE ALCALÁ

FACULTAD DE MEDICINA

MECANISMOS DE REGULACIÓN DEL ENZIMA DE CONVERSIÓN DE LA ENDOTELINA-1 (ECE-1). IMPORTANCIA EN ALGUNAS SITUACIONES FISIOPATOLÓGICAS.

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MECANISMOS DE REGULACIÓN DEL ENZIMA DE CONVERSIÓN DE LA ENDOTELINA-1 (ECE-1). IMPORTANCIA EN ALGUNAS SITUACIONES FISIOPATOLÓGICAS.

Tesis presentada por Dña. Ingrid Viviana Raoch Michaels

para optar al grado de Doctor en Medicina

Alcalá de Henares, 2009

Dña. María Luisa Díez Marqués, Catedrático y Director del Departamento de Fisiología de la Universidad de Alcalá.

CERTIFICA:

Que la presente tesis doctoral elaborada por Dña. Ingrid Viviana Raoch Michaels para optar al grado de Doctor en Medicina, con el título "**Mecanismos de regulación del enzima de conversión de la endotelina-1 (ECE-1). Importancia en algunas situaciones fisiopatológicas",** ha sido realizada en la Unidad de Investigación del Hospital Universitario Príncipe de Asturias y en el departamento de Fisiología de la Universidad de Alcalá, bajo la dirección de los Dres. Susana López Ongil y Diego Rodríguez Puyol, actuando como tutor Manuel Rodríguez Puyol.

Y para que así conste, expide y firma el presente certificado en Alcalá de Henares, a 01 de Julio de 2009.

Fdo. María Luisa Díez Marqués

Dña. Susana López Ongil, Investigadora del sistema nacional de salud adscrita al Hospital Universitario Príncipe de Asturias y D. Diego Rodríguez Puyol, Jefe de Nefrología del Hospital Universitario Príncipe de Asturias y Profesor Asociado del Departamento de Medicina de la Universidad de Alcalá, como Directores de Tesis, y D. Manuel Rodríguez Puyol, Catedrático del Departamento de Fisiología de la Universidad de Alcalá, como Tutor.

CERTIFICAN:

Que el presente trabajo, titulado "**Mecanismos de regulación del enzima de conversión de la endotelina-1 (ECE-1). Importancia en algunas situaciones fisiopatológicas"**, presentado por Dña. Ingrid Viviana Raoch Michaels para optar al grado de Doctor en Medicina, ha sido realizado bajo su dirección en la Unidad de Investigación del Hospital Universitario Príncipe de Asturias. Hallándose concluido y reuniendo, a su juicio, los requisitos formales, científicos y de rigor metodológico suficientes, autorizan su presentación a fin de que pueda ser defendido ante el tribunal correspondiente.

Y para que así conste, expedimos y firmamos la presente certificación en Alcalá de Henares, a 01 de Julio de 2009.

Fdo. Susana López Ongil. Fdo. Diego Rodríguez Puyol.

Fdo. Manuel Rodríguez Puyol.

A mis abuelos.

A mis padres.

Y a todos aquellos, que de una u otra

forma, no han dejado que desfallezca.

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ABREVIATURAS

ADN	Ácido desoxirribonucleico
ARN	Ácido ribonucleico
CEAB	Células endoteliales de aorta bovina
dB-GMPc	Dibutiril GMP cíclico: *"N'2-O-Dibutirylguanosine 3',5'-cyclic monophosphate sodium salt hydrate"*
DEA	Complejo óxido nítrico-dietilamina: *"Diethylamine/nitric oxide complex sodium salt; 2-(N,N-Diethyl-amino)-diazenolate 2-oxide"*
DNI	Dinitrato de isosorbide
DRB	Inhibidor de la transcripción DRB: *"5,6-Dichlorobenzoimidazole 1-beta-d-ribofuranoside"*
ECE-1	Enzima de conversión de la endotelina-1
eNOS	Óxido nítrico sintetasa endotelial
ET-1	Endotelina-1
GAPDH	Gliceraldehído 3-fosfato deshidrogenasa
GCs	Guanilato ciclasa soluble
GMPc	Guanosina 3',5'-monofosfato
H	Hora
HTA	Hipertensión arterial
LDL	Lipoproteína de baja densidad
Min.	Minuto
NO	Óxido nítrico
O_2	Oxígeno
ODQ	Inhibidor de GCs, ODQ: *"1H-(1,2,4) Oxadiazolo-(4,3-α)quinoxalin-1-one"*
PBS	Solución salina tamponada con fosfato
PKG	Proteína quinasa G
SNP	Nitroprusiato sódico
SOD	Superóxido dismutasa
SSC	Solución salina con citrato
SSPE	Solución salina con fosfato
TTBS	Solución salina tamponado con Tris-HCl, conteniendo Tween
UTR	Región no traducida: *"untraslated region"*
VASP	Vasodilator-stimulated phosphoprotein

1. ESTRUCTURA Y FUNCIÓN DEL ENDOTELIO

Las paredes de los vasos sanguíneos están dispuestos formando tres capas concéntricas, íntima, media y adventicia. La íntima consta de una monocapa de células endoteliales llamada endotelio con poco tejido conjuntivo subendotelial y en contacto directo con la luz del vaso, separada de la capa media por una densa membrana elástica denominada *lámina elástica interna.* La capa media está formada por varias capas de células musculares lisas inmersas en matriz extracelular y se separa de la adventicia por la *lámina elástica externa.* La adventicia, última capa del vaso, está constituida principalmente por fibroblastos formando un tejido conjuntivo de revestimiento junto a fibras nerviosas y vasos sanguíneos.

La integridad estructural y funcional del endotelio es esencial para el mantenimiento de la homeostasia de la pared vascular y la función circulatoria. El endotelio vascular es un tejido multifactorial con numerosas propiedades metabólicas y de síntesis. Entre sus funciones se pueden citar el control del tono vascular, la regulación del paso de macromoléculas desde el torrente circulatorio hacia el espacio extravascular, la participación en el proceso de remodelación vascular, la modulación de la coagulación sanguínea, el metabolismo de las lipoproteínas, el control de las respuestas inflamatorias e inmunes y la regulación de la neovascularización.

La disfunción endotelial hace referencia a un estado de desequilibrio homeostático, en donde la respuesta del endotelio frente a un estímulo ambiental no es la adecuada. Aunque inicialmente se describió como una respuesta vasodilatadora inadecuada ante la infusión de acetil colina y se relacionó con un déficit en la biosíntesis y/o actividad de óxido nítrico (NO), en la actualidad se considera un fenómeno más generalizado, donde la pérdida de las distintas propiedades reguladoras del endotelio puede construir uno de los sustratos fisiopatológicos responsable de las distintas alteraciones que caracterizan las enfermedades cardiovasculares. Los desencadenantes de la disfunción endotelial han sido y son todavía objeto de estudio en nuestros días, habiéndose propuesto, entre otros, la presencia de niveles anormales de citoquinas, productos bacterianos, compuestos lipídicos, proteínas anormales, radicales libres y fármacos, así como cambios en las propias fuerzas mecánicas intravasculares. Como consecuencia de la presencia de estos estímulos, se produciría un desequilibrio entre la síntesis o biodisponibilidad de factores vasodilatadores como el NO (1, 2) y vasoconstrictores como la endotelina-1 (ET-1) (3, 4), o incluso por una mayor producción de radicales libres (5), con el subsiguiente desarrollo de cambios en la función endotelial. En concreto, el endotelio sintetizaría moléculas de adhesión, moléculas protrombóticas, factores de crecimiento

y otras sustancias que favorecen el desarrollo y mantenimiento de las complicaciones asociadas a las enfermedades cardiovasculares, como la hipertensión o la aterosclerosis (5).

2. FACTORES VASOACTIVOS ENDOTELIALES

Para mantener la homeostasis del organismo, el endotelio sintetiza una serie de factores bioactivos. Entre estos destacan: el óxido nítrico (NO) y la prostaciclina como sustancias vasodilatadoras, y la endotelina-1 (ET-1), la angiotensina II (AII) y el tromboxano A_2 (TxA_2) como sustancias vasoconstrictoras. A continuación se describirán en detalle sólo las que son más relevantes en este trabajo.

2.1. Óxido nítrico (NO)

- **Estructura molecular**

En 1980, Furchgott y Zawadzki (6) descubrieron un factor derivado del endotelio que tenía una acción relajante sobre el músculo liso. Posteriormente, Palmer et al., en 1987 (7), demostraron que el componente mayoritario de dicho factor era el NO. El NO es una molécula pequeña, de 30 Dalton de peso molecular, de naturaleza gaseosa, incolora e inodora. Es altamente lipófila y atraviesa fácilmente la membrana celular. Químicamente resulta de la combinación de un átomo de oxígeno y un átomo de nitrógeno, posee un electrón desapareado, y por tanto, es un radical libre altamente reactivo. Tiene una semivida muy corta, de 3 a 5 segundos en medio oxigenado. Se inactiva con oxígeno dando nitritos y nitratos, y con el anión superóxido dando peroxinitritos, que se acumulan en el medio. La superóxido dismutasa aumenta la semivida del NO porque secuestra al anión superóxido. El NO tiene alta afinidad por el grupo hemo de la hemoglobina, mioglobina y guanilato ciclasa soluble, siendo esta última su ligando biológico natural más específico. Además, puede reaccionar con grupos tiol, nitrosilando proteínas.

- **Biosíntesis del óxido nítrico**

El NO es generado enzimáticamente por un grupo de enzimas denominadas óxido nítrico sintetasas (NOS). Estas enzimas catalizan la reacción estequiométrica de oxidación del aminoácido L-Arginina a L-Citrulina, liberando una molécula de NO (Figura 1). Las NOS pertenecen a la familia de las flavoproteínas, y se conocen al menos 3 isoformas distintas (8, 9), que son productos de 3 genes diferentes. Todas las isoformas son proteínas con pesos moleculares que oscilan entre 125-160 KDa y se encuentran formando homodímeros en su estado nativo. Poseen dos dominios, un dominio reductor en el extremo carboxilo terminal, que contiene secuencias consenso

de unión a distintos cofactores como FAD, FMN, NADPH, y otro dominio oxidativo con actividad catalítica en el extremo amino terminal, donde se encuentra la secuencia de unión al grupo hemo, al cofactor BH_4 y a la L-Arginina. Los dos dominios se unen a través de un lugar de reconocimiento para la calmodulina.

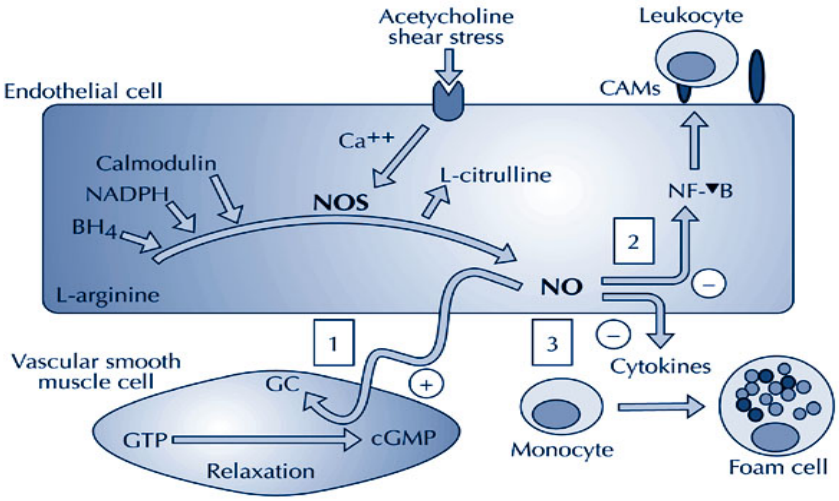

Figura 1. Biosíntesis del óxido nítrico.

Las tres isoformas descritas se clasifican atendiendo a dos tipos de nomenclatura: a) la **NOS1 o nNOS** es la primera que se descubrió en el tejido neuronal, es una isoforma constitutiva y el NO producido aquí actúa como neurotransmisor, regulando entre otros procesos de aprendizaje y memoria, b) la **NOS2 o iNOS** es la isoforma inducible expresada en macrófagos, músculo liso, hepatocitos, células mesangiales, neutrófilos, e incluso en el propio endotelio, y actúa fundamentalmente en la respuesta inmune e inflamatoria, y c) la **NOS3 o eNOS** es una isoforma constitutiva sintetizada fundamentalmente en el endotelio pero también en menor cantidad en plaquetas, epitelio renal o neutrófilos, que está implicada en mantener la homeostasia vascular y el tono vascular. La eNOS y nNOS, isoformas constitutivas, dependen de calcio y calmodulina para su activación, que ocurre en segundos, produciendo cantidades pequeñas, pero constantes de NO. Por su parte, la iNOS es independiente de calcio, necesita horas para activarse, pero cuando lo hace produce grandes cantidades de NO, que pueden llegar a ser tóxicas; de ahí su efecto citotóxico en la respuesta inmune e inflamatoria.

Los mecanismos reguladores de las tres isoformas son muy diferentes. En general, la nNOS y eNOS se activan por estímulos solubles, fundamentalmente autacoides, de forma rápida y por un mecanismo que requiere la activación del

sistema calcio-calmodulina (Ca-CaM). Por el contrario, la iNOS requiere para su activación una modulación transcripcional, dependiente de la acción de ciertas citoquinas. A su vez, nNOS y eNOS pueden ser reguladas post-trasduccionalmente por fosforilación mediante quinasas dependientes de AMP cíclico, GMP cíclico, PKC y Ca-CaM. En general, la fosforilación disminuye la actividad catalítica del enzima y en el caso de la eNOS, no sólo su actividad enzimática sino su distribución subcelular, ya que al fosforilarse se transloca de la membrana al citosol, siendo la eNOS citosólica catalíticamente inactiva. También son reguladas mediante un proceso de retroalimentación negativo por el propio NO, que inhibe la actividad del enzima que lo sintetiza (10).

- **Efectos del óxido nítrico**

De todas las funciones del NO la más ampliamente descrita es su capacidad de regular el tono vascular, pero destacan otras como su papel neurotransmisor en el sistema nervioso central y periférico, su acción bactericida y citotóxica en la respuesta inmune e inflamatoria por parte de macrófagos y neutrófilos, su capacidad para regular el crecimiento y la muerte celular, así como la síntesis de matriz extracelular y sus efectos anticoagulantes.

El NO sintetizado por el endotelio difunde libremente a través de la membrana celular y llega a la capa de células musculares lisas del vaso, donde se une a su ligando natural, una metaloproteína citoplasmática similar a la hemoglobina conocida como guanilato ciclasa soluble (GCs). El NO se une a la GCs a través del hierro del grupo hemo de este enzima, dando lugar a la síntesis de GMP cíclico (GMPc). Este, por un lado, disminuye los niveles de calcio de la célula y por otro activa a una proteína dependiente de GMPc (PKG), que activa a la fosfatasa de la cadena ligera de la miosina, defosforilando esta proteína e induciendo así la relajación de la célula muscular lisa. La relajación del músculo liso permite que se dilate el vaso y disminuya la presión sanguínea. Experimentos con ratones knockout para eNOS (11-13) revelan la importancia del NO en la regulación del tono vascular, puesto que estos ratones, que carecen de eNOS, desarrollan hipertensión arterial.

En las plaquetas, el NO también genera GMPc, disminuyendo su afinidad por la superficie endotelial y por otras plaquetas, actuando así como antiagregante plaquetario y anticoagulante. Evita, además, la adhesión leucocitaria a las paredes vasculares, inhibiendo la activación de estas células y el proceso de la inflamación.

En lo referente a la capacidad de defensa del organismo, el NO es un potente agente bactericida y citotóxico, formando parte de las reacciones de inmunidad no específica de células endoteliales, hepatocitos o músculo liso. Estas acciones las

realiza porque es capaz de inhibir el crecimiento y la replicación de los agentes patógenos (bacterias, virus y tumores), interfiriendo con los grupos hemo de enzimas claves de la cadena respiratoria y de la síntesis de ADN de las células diana, interaccionando con radicales libres derivados del oxígeno, generando así moléculas que podrían aumentar su citotoxicidad.

En el sistema neuronal es un agente neurotransmisor que participa en procesos de aprendizaje, memoria y procesamiento de impulsos nerviosos, erección del pene, y también en la diferenciación y regeneración de neuronas.

Algunos autores han demostrado la capacidad del NO para inhibir la síntesis de componentes de matriz extracelular (14-17) o la síntesis de ET-1 a través de prepro-ET-1 (18), influyendo en mayor o menor medida en las funciones comentadas que ejerce el NO.

- **Importancia del óxido nítrico en situaciones fisiopatológicas**

El NO puede comportarse como un arma de doble filo. Por un lado es beneficioso como mensajero o modulador y frente a la defensa de microorganismos, pero por otro, en concentraciones elevadas, es potencialmente tóxico, y esta toxicidad es debida fundamentalmente al hecho de que el NO se une con gran afinidad al anión superóxido formando peroxinitritos, que son aún más citotóxicos. Además, el NO puede unirse directamente a las tirosinas de algunas proteínas, nitrosilándolas e inactivándolas irreversiblemente. Niveles anormalmente elevados de NO están involucrados en la hipotensión asociada al shock séptico, en la reacción inflamatoria en respuesta al daño tisular, en la artritis o en enfermedades neurodegenerativas. Por el contrario, el NO está disminuido en enfermedades cardiovasculares como la aterosclerosis, hipertensión o diabetes.

Como terapias farmacológicas, en relación con las posibles alteraciones del NO, se han considerado tres: los inhibidores de la NOS, los donadores de NO y los inhibidores de la fosfodiesterasa 5. Los inhibidores de las NOS podrían ser efectivos cuando los niveles de NO están incrementados, en trastornos patológicos como el shock séptico, inflamación y enfermedades neurodegenerativas. Estos inhibidores son los análogos de la L-arginina y compiten con éste aminoácido por el lugar de unión a la enzima. Los donadores de NO podrían ser útiles en aquellas patologías donde el NO está disminuido, como la hipertensión, la hiperreactividad bronquial o la disfunción eréctil. Los donadores de NO difieren en sus características bioquímicas a la hora de liberar NO, en la duración de su acción y en la selectividad tisular. En general, los inhibidores de la fosfodiesterasa reducen la degradación del AMPc generando una acción inotrópica positiva en el corazón y vasodilatación periférica. Es un grupo de

medicamentos con un amplio campo de acción determinado por el bloqueo selectivo de las 5 isoenzimas que lo conforman. En la actualidad, son usados en el tratamiento de la insuficiencia cardiaca congestiva, en asma y la enfermedad pulmonar obstructiva crónica (IV) y en la disfunción eréctil (V).

2.2. Endotelina-1 (ET-1)

- **Estructura molecular**

La ET-1, descubierta en 1988 (19), es el péptido vasoconstrictor más potente identificado hasta la fecha. Formado por 21 aminoácidos con un peso molecular de 2492 Da, contiene 2 puentes disulfuro en su molécula, lo que le confiere una estructura inusual como péptido endógeno de mamíferos, asemejándose a algunas neurotoxinas o venenos del reino animal. Posee una alta homología entre especies, y pertenece a una familia de péptidos relacionados estructuralmente (ET-1, ET-2 y ET-3), difiriendo de las dos últimas en 2 y 6 aminoácidos respectivamente.

- **Biosíntesis de la endotelina-1**

La ET-1 se produce fundamentalmente en células endoteliales como un péptido largo, prepro-endotelina (prepro-ET-1), de 201 aminoácidos en el hombre, que requiere un proceso proteolítico inusual de rotura por una endopeptidasa que reconoce pares dibásicos (Lys-Arg o Arg-Arg), dando un péptido intermedio de 38 aminoácidos en humanos, denominado Big-ET-1 y que es inactivo. La Big-ET-1 a su vez sufre una proteolisis por acción del enzima de conversión de la endotelina-1 (ECE-1), que ataca grupos Trp-Val, dando lugar a la ET-1 madura y activa. La ET-1 sintetizada se libera, no existiendo depósitos intracelulares para almacenarla. La síntesis de la ET-1 consta, pues, de dos pasos, la formación del precursor prepro-ET-1 y la acción acoplada de dos enzimas, la endopeptidasa y ECE-1, dependiendo su regulación de los niveles de precursor y de la última de estas enzimas (Figura 2).

El gen de la prepro-ET-1 comprende 5 exones y se encuentra en el cromosoma 6. Se trata de un gen con un promotor típico con región TATA, que posee diferentes elementos de regulación tipo cis y tipo trans, entre las que podemos destacar secuencias de unión a factores de transcripción como AP-1, NF-1 o GATA. El gen de la prepro-ET-1 ha sido bastante estudiado y se sabe que se regula positiva o negativamente por distintas sustancias y factores de transcripción conocidos, entre los que se incluyen la trombina, la angiotensina II, la vasopresina, el TGF-β, ionósforo de calcio, ésteres de forbol, fuerzas de rozamiento vascular (“*shear stress”*), e incluso la propia ET-1 (20-23).

- **Efectos de la endotelina-1**

La ET-1 es un péptido vasoconstrictor muy potente, que causa una respuesta intensa y sostenida en la mayoría de las arterias y venas de los mamíferos, aumentando así la presión arterial. Dada la amplia distribución de sus receptores, se ha descrito un amplio rango de funciones, además de la vasoconstricción, en diversos órganos y tejidos, como son la modulación de la síntesis de péptidos natriuréticos en miocitos, de eicosanoides y de NO en vasos, de noradrenalina en terminaciones simpáticas y de renina en riñón. Además, la ET-1 es capaz de inducir proliferación de células musculares lisas, sugiriendo su papel en procesos de hipertrofia celular, aterosclerosis y en enfermedades renales. La ET-1 puede inducir liberación y expresión de factores quimiotácticos, factores de crecimiento y moléculas de adhesión, jugando probablemente un papel relevante en la inflamación.

Los efectos de la ET-1 son la consecuencia de la unión a receptores específicos en las células diana. Se conocen dos subtipos de receptores, ET_A y ET_B. El receptor ET_A se encuentra en músculo liso y reconoce selectivamente ET-1 y ET-2, siendo responsable de la contracción y proliferación del músculo liso. Por el contrario, el receptor ET_B se encuentra en el endotelio y no es selectivo, siendo su efecto predominante la liberación de NO, con la subsiguiente relajación del músculo liso. Ambos receptores tienen la estructura típica de siete regiones transmembrana y están acoplados a proteínas G, de forma que al unirse la ET-1 determinan un aumento de calcio intracelular mediado por la activación de la fosfolipasa C y el inositol trifosfato.

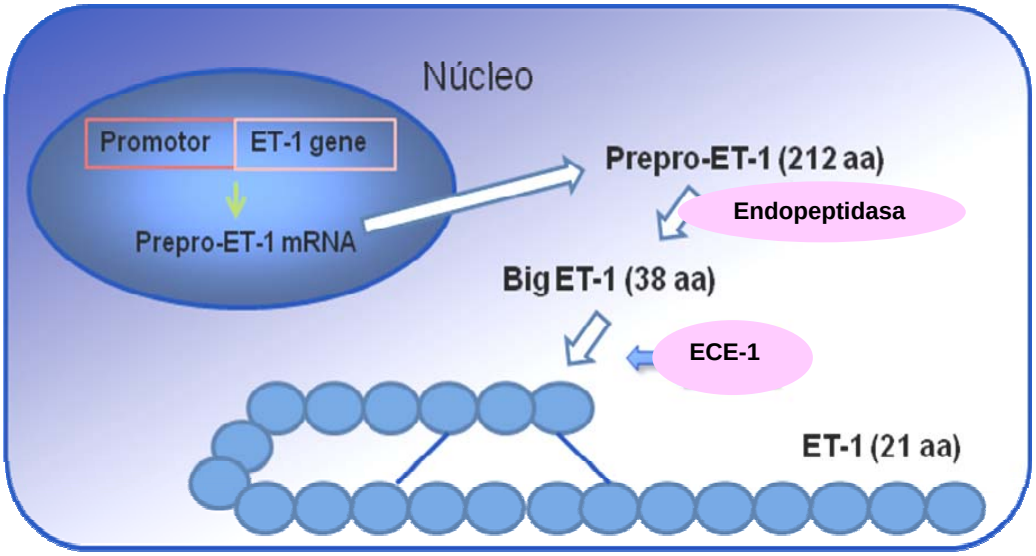

Figura 2.- Síntesis de la endotelina-1.

- **Importancia de la endotelina en situaciones fisiopatológicas**

Los niveles de ET-1 en voluntarios sanos son muy bajos, por lo que puede considerarse que su acción preferencial es local, no actuando como hormona circulante. Se han detectado niveles elevados de ET-1 en el plasma de sujetos hipertensos, en pacientes en hemodiálisis, en el infarto agudo de miocardio y en la angina vasoespástica, en la insuficiencia renal aguda, en la sepsis, en el síndrome hepatorrenal, en episodios agudos de asma y en algunos tipos de cáncer. Estos hallazgos, junto a evidencias experimentales obtenidas en cultivos celulares o en animales de experimentación, han sugerido un papel de la ET-1 en la génesis de estas patologías (24-28).

No obstante, la única enfermedad en la que se ha demostrado un papel relevante de la ET-1 en seres humanos es la hipertensión pulmonar. De hecho, la administración de bosentan, un antagonista de ambos receptores de la ET-1, a pacientes con determinadas formas de hipertensión pulmonar, mejora el curso de la enfermedad.

3. ENZIMA DE CONVERSIÓN DE LA ENDOTELINA-1

3.1. Estructura molecular

Descrita por primera vez por Sawamura et al. (29) en medula adrenal bovina, y más tarde caracterizada como metaloproteinasa sensible a fosforamidón (30), se creyó inicialmente que era específica para la ET-1; sin embargo, se sabe que interviene también en el metabolismo de la bradiquinina y del péptido beta amieloide. Existen tres tipos de enzima de conversión de la endotelina (ECE): ECE-1, ECE-2 y ECE-3. La ECE-1 es la enzima mayoritaria y objetivo de este trabajo, es una metaloproteinasa de tipo II unida a membrana que procesa tanto la Big ET-1 endógena como la exógena. Por su parte, la ECE-2 es una metaloproteinasa intracelular que procesa sólo la Big ET-1 endógena (31). Respecto a la ECE-3 es una metaloproteinasa distinta a ECE-1 y ECE-3, que se encontró en el iris bovino (32) y que parece que procesa específicamente Big-ET-3.

- **Estructura genómica**

Hasta el momento se han identificado cuatro isoformas distintas del ARNm de ECE-(1a,1b,1c,1d) (Figura 3), generadas por “splicing” alternativo de distintos promotores dentro del mismo gen (33-37), las cuales comparten el mismo dominio carboxilo-terminal (extracelular) y poseen las mismas propiedades bioquímicas, sin embargo, difieren en el dominio corto amino-terminal (intracelular), el cual ha sido relacionado con la distinta localización de cada isoforma dentro de la célula (38,39). El

gen está compuesto de 19 exones que se extienden en más de 68 Kb y se localiza en el cromosoma 1, banda p36 (36, 40).

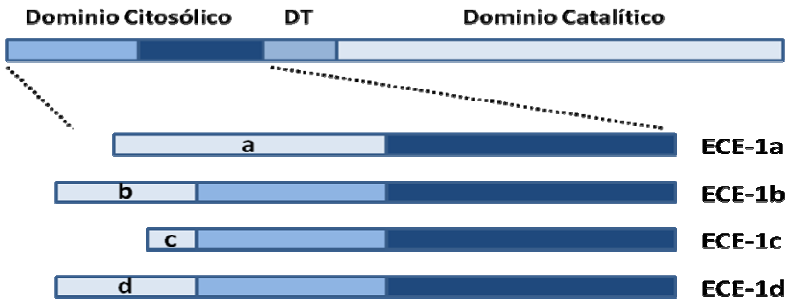

Figura 3. Dominio amino terminal de cada isoforma de la ECE-1.

Inicialmente, fueron descritas dos isoformas (41, 42), la isoforma ECE-1a de 758 aminoácidos codificada en el exón 3 y la isoforma ECE-1b de 770 aminoácidos codificada en los exones 1b y 2. Más tarde, se identificó la isoforma ECE-1c, de 754 aminoácidos (33, 43), codificada en el exón 1c, y por último, la isoforma ECE-1d, de 767 aminoácidos (36), codificada en el exón 2 (Figura 4). Cada una de las regiones anteriores a los exones, además de contener los promotores, presentan múltiples sitios de unión a factores de transcripción entre los que se incluyen NFκB, AP1, AP2 y c-ets1, entre otros, siendo así potencialmente regulables.

Recientemente, se ha descubierto una variante adicional que carece del dominio transmembrana o péptido señal, que se origina por transformaciones del ARN mensajero de ECE-1b, 1c o 1d, conocida como ECE-1sv que al no tener el dominio transmembrana se cree que permanece en el citosol. Tiene un peso molecular aparente de 75 KDa y está altamente expresada en células endoteliales derivadas de los lechos vasculares (44).

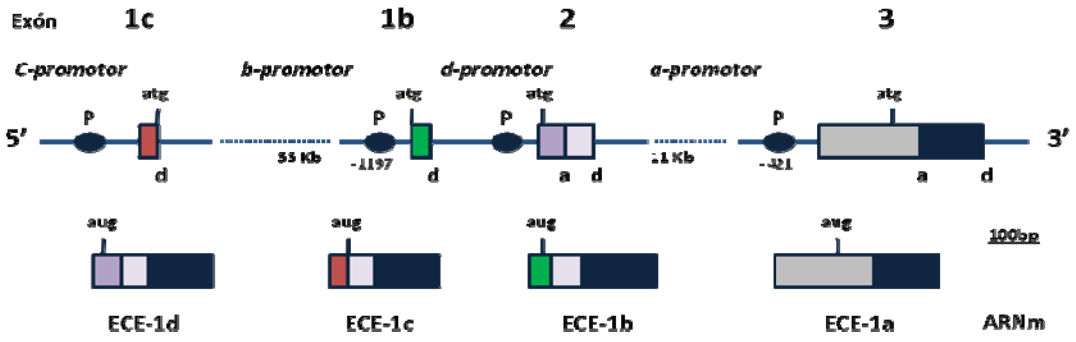

Figura 4. Organización de la región 5' del gen humano de la ECE-1. Delante de cada exón se encuentra cada uno de los cuatro promotores (elipses negras) que van a dirigir la transcripción de cada isoforma. (Extraído de *Eur. J. Biochem*. **264**, pag.343).

- **Estructura proteica**

ECE-1 es una metaloproteasa transmembrana tipo II dependiente de zinc, que pertenece a la familia de la endopeptidasa neutra como la neprilisina (NEP), el antígeno del grupo sanguíneo Kell, el gen regulador de la excreción de fosfato (PEX), la enzima conversora X (XCE) y la endopeptidasa secretora (SEP). Todas ellas mantienen una estructura proteica parecida, con un dominio carboxilo-terminal extracelular responsable de la actividad catalítica y un dominio amino-terminal intracelular corto. Su secuencia de aminoácidos presenta un 37 % de homología con la NEP (45), compartiendo además 10 de los 14 residuos cisteína (37, 41), por lo que tiene una estructura terciaria similar a la NEP.

La secuencia predicha tiene 85.000 Da mientras la ECE purificada tiene 130.000 Da. Esta diferencia puede deberse a la alta glicosilación que sufre dicha enzima en su dominio extracelular (41). Es una proteína monomérica de unos 120 a 130 KDa de peso molecular, aunque en estado nativo puede encontrarse formando dímeros de 250 KDa, bien homodímeros (αα, ββ) o bien heterodímeros (αβ) unidos por medio de 2 puentes disulfuro (33). La dimerización ocurre rápidamente después de la síntesis, probablemente en el retículo endoplasmático, ya que se observan transitoriamente dímeros intermedios compatibles con una glicosilación incompleta o ECE-1 no plegada (38). El motivo de unión al zinc, HEXXH, se encuentra en el dominio carboxilo-terminal (Figura 5), en donde E corresponde al ácido glutámico, el cual es crucial para la actividad catalítica porque es el ligando para el zinc. Los residuos de histidina en los extremos actúan como aminoácidos coordinadores del zinc y estabilizan la configuración tetraédrica intermedia durante el estado de transición (33, 41, 46).

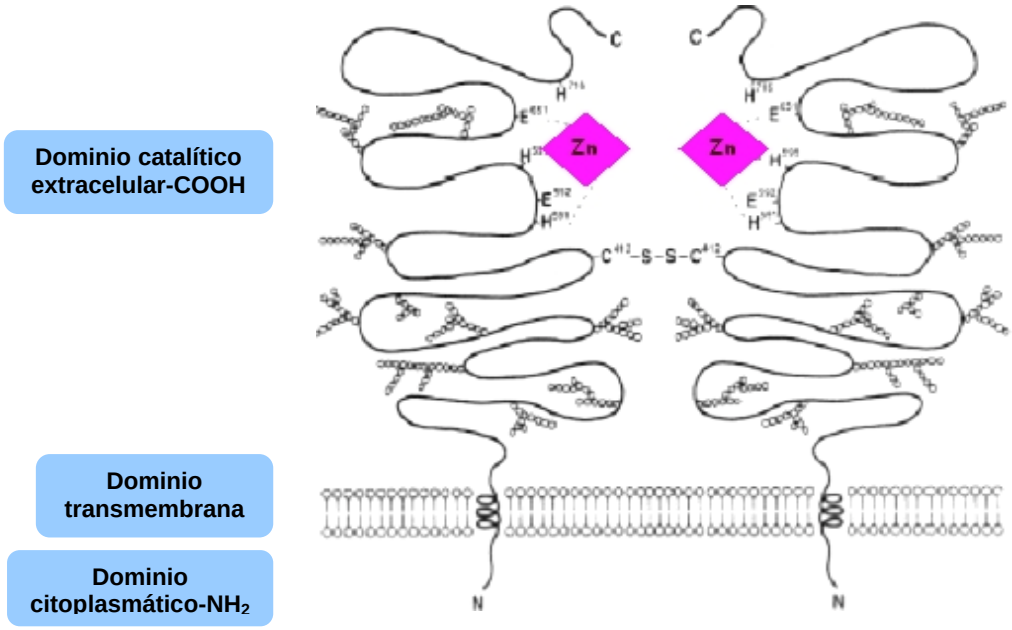

Figura 5. Estructura de la ECE-1

Es una proteína altamente distribuida en distintos tejidos y especies, al igual que la ET-1. Es una proteína expresada en varios tejidos coincidiendo con el hecho de que la prepro-ET-1 esta también ampliamente distribuida. La secuencia de aminoácidos se encuentra altamente conservada entre especies. La proteína ECE en la rata tiene una secuencia de 754 aminoácidos, y se expresa abundantemente en pulmón, glándula adrenal y en menor proporción en riñón, corazón y cerebro. La proteína bovina tiene una secuencia de 758 residuos y presenta un 91 % de homología con la de rata, expresándose mayoritariamente en pulmón. La proteína humana contiene 754 aminoácidos con un alto grado de conservación con la proteína bovina (94 %) y de rata (91 %) (46, 47). Se expresa en pulmón, páncreas y placenta, órganos que también son ricos en ET, y muy poco en cerebro y riñón.

- **Regulación**

La expresión de ECE-1 puede estar regulada en paralelo con la prepro-ET-1, aunque no necesariamente en el mismo sentido. Puesto que el promotor de ECE-1 contiene secuencias de reconocimiento para distintos factores de transcripción, es susceptible de ser regulado por cualquiera de ellos. Se sabe que la expresión de ECE-1 se estimula por el factor de crecimiento vascular endotelial (VEGF) en células endoteliales (48), por los esteres de forbol activando el factor Ets-1 en células endoteliales humanas (EA.hy926) (49), por el agua oxigenada activando el factor STAT3 en células endoteliales de aorta bovina (1) y por la glucosa en HuVEC (50).

Por el contrario, la expresión disminuye por sustancias como la propia ET-1 en células endoteliales (51), por el “shear stress” en HuVEC (52, 53) y por la Angiotensina II liberada en cocultivos de células musculares lisas con HuVEC (54). El anión superóxido es capaz de inhibir la actividad de ECE-1 al eliminar el Zn del centro activo del enzima (55). No obstante, a pesar de todos los estudios que existen en la literatura, poco se sabe sobre el verdadero papel fisiopatológico de este enzima, clave para la síntesis de ET-1.

3.2. Mecanismo de acción

- **Acción del enzima de conversión de la endotelina**

ECE-1 tiene la capacidad de cortar específicamente en la posición Trp^{21}-Val^{22} de la Big ET-1. El sitio de corte es fundamental a la hora de determinar los niveles de actividad de la enzima, ya que se ha demostrado que los péptidos ET-1 (1-20) y ET-1 (1-22) muestran una actividad tres veces menor que la forma auténtica de ET-1 (1-21) (56).

No es capaz de catalizar un sustrato con el dominio C-terminal truncado, Big ET-1 (1-31), lo que indica que es un requisito indispensable para su reconocimiento. La incubación de Big ET-1 con ECE-1 durante 24 horas resulta en una tasa de conversión mayor al 90% generando dos productos peptídicos: ET-1 (1-21) y Big ET-1 (22, 47); siendo mucho más efectiva la conversión de la Big ET-1 endógena (50-90%) que la exógena (5-10%) (57).

ECE es activa entre pH 6.8-7.4, con un máximo a pH 7 (57, 58). Como la NEP, el dominio carboxilo-terminal es grande y extracelular, encontrándose allí el centro catalítico del enzima, mientras que el dominio amino-terminal es corto y citoplasmático. Así, ECE convertiría Big ET en ET-1 en la superficie de la membrana después de que las células secreten Big ET al exterior de la célula. ECE actuaría, pues, como un *ectoenzima,* por tres razones: 1) la Big ET-1, la ET-1 y el fragmento C-terminal de Big ET han sido detectados en sobrenadante de células y en plasma, 2) la administración intravenosa de Big ET-1 condiciona un incremento de ET-1 y 3) la NEP con estructura similar a ECE también degrada sustratos en la superficie de la membrana.

Al comparar las propiedades enzimáticas de las isoformas ECE-1 a, b y c, no se encuentran diferencias significativas en los parámetros cinéticos K_m, K_{cat}, V_{max}, y *h,* esperable teniendo en cuenta que el centro catalítico está en el dominio carboxilo-terminal que es común a todas las isoformas. La actividad específica máxima promedio de las tres isoformas es de 0.75 ± 0.14 µmol de ET-1/min por mg de ECE-1 a 37°C (43). A pesar de esto, la conversión intracelular de Big ET-1 es más eficaz que la extracelular. Ante el estímulo de una señal aún desconocida, se sugiere que la isoforma ECE-1b puede regular esta conversión citoplasmática en la vía secretora, y ECE-1a y ECE-1c se encargarían de la maduración de Big ET-1 en la superficie celular (59).

- **Inhibidores**

La actividad de ECE-1 se inhibe con agentes quelantes como EDTA o fenantrolina y con fosforamidón (inhibidor inespecífico de las endopeptidasas neutras), pero no con tiorfano (inhibidor selectivamente específico de NEP). La inactivación con EDTA se restaura parcialmente con cobalto, manganeso o cobre y totalmente con zinc, pero no con hierro, magnesio o calcio. Pequeñas concentraciones de cationes como Cu^{2+}, Zn^{2+}, Fe^{2+}, Co^{2+}, pero no Ca^{2+}, Mn^{2+} y Mg^{2+} inhiben la actividad del enzima. El captopril, metaloproteinasa neutra e inhibidor de la enzima de conversión de angiotensina, no inhibe la actividad de ECE, y tampoco los inhibidores de la serina, ácido aspártico o proteasas del grupo tiol. La actividad de ECE-1 también se inhibe más o menos selectivamente con compuestos químicos como FR-901533 (Fujisawa

Co.) y CGS-26303 o CGS-35066 (Novartis) (60). El fosforamidón y FR-901533 inhiben la actividad de forma dosis dependiente, con una IC_{50} de 1 μM y 1,6 μM, respectivamente. La amidación del Trp^{21} causa una marcada disminución de la actividad de ECE-1, sin alcanzar a inhibirla completamente (61).

Se ha demostrado que a pesar de que tanto el fosforamidón como el FR-901533 inhiben eficazmente la actividad de ECE-1 in vitro, solo el fosforamidón es capaz de hacerlo en células doblemente transfectadas con los plásmidos de prepro-ET-1 y ECE-1, requiriendo concentraciones más altas para ello. Sin embargo, la conversión de la Big ET-1 exógena es inhibida a bajas concentraciones tanto por FR-901533 como por fosforamidón, sugiriendo que las células que expresan ECE-1 convierten la Big ET-1 endógena dentro de la célula, seguramente durante su tránsito por el sistema secretor. Por esa razón, FR-901533 es incapaz de bloquear el efecto al no poder ingresar a la célula (57).

En conclusión, actualmente se considera que existen tres clases de inhibidores de ECE: Los inhibidores de acción dual sobre ECE/NEP (ej. fosforamidón, CGS-26303), los de acción triple sobre ECE/NEP/ACE (ej. CGS-35601(62) y los inhibidores ECE más selectivos (ej. CGS-35066, FR-901532); algunos de los cuales han mostrado efectos benéficos al controlar enfermedades cardiovasculares, renales y del sistema nervioso central en estudios preclínicos en modelos animales (63).

3.3. Localización

- **Celular**

A nivel molecular, los resultados sugieren que ECE-1 se encuentra mayoritariamente en la superficie celular siguiendo un patrón con disposición en parches o tipo “clusters” (64); existiendo un porcentaje menor localizado en la superficie nuclear cerca del complejo de Golgi (65).

Por medio de inmunofluorescencia indirecta ha sido posible determinar que éste comportamiento no es aleatorio, sino que por el contrario está determinado por una localización subcelular específica para cada isoforma. De esta manera, ECE-1a y ECE-1c se localizan predominantemente en la membrana plasmática, mientras que ECE-1b (95 %) y ECE-1d lo hacen en el citosol (38, 43, 66). Encontrándose, que pequeñas proporciones de ECE-1c pueden estar intracelularmente en el aparato de Golgi y que cantidades similares de ECE-1b lo están en la región yuxtanuclear, en el sistema trans-Golgi o en estructuras compatibles con endosomas y vesículas. Todo indica que la disposición intracelular de ECE-1b es posible gracias a una señal de retención contenida en los 17 aminoácidos ubicados en el extremo amino-terminal, única diferencia con la ECE-1c (Figura 3) (43, 59). El motivo específico de di-Leucina

para ECE-1b y el motivo Val^8-Leu^9 para ECE-1d son los responsables de que esta retención intracelular ocurra (59). Además, ECE-1b tiene la capacidad de formar heterodímeros con ECE-1a provocando la translocación de esta última a los endosomas tardíos y a los cuerpos multivesiculares intracelulares (38), sin que la expresión de ECE-b en la membrana plasmática se vea aumentada con esta unión.

Las variaciones en la localización intracelular de las distintas isoformas no solo dependen de sus características fenotípicas, sino también del tipo celular en el cual se expresen, así como de la menor o mayor cantidad de sitios de fosforilación y de residuos de tirosina que contengan. En particular, ECE-1c resultó ser la más abundante, excepto en HuVEC, donde ECE-1a es la fracción mayoritaria.

Se ha demostrado en células endoteliales la presencia de ECE-1a en la membrana nuclear (67) y de ECE-1c en el retículo endoplásmico (66). Por otra parte, en células neuroendocrinas se ha descrito que ECE-1b se encuentra en vesículas de gran tamaño dispersas por todo el citoplasma, mientras que ECE-1d se encuentra en la región perinuclear. En el sistema endosomal, ECE-1b se encuentra en endosomas de reciclaje, endosomas tardíos y en los cuerpos multivesiculares, mientras que ECE-1d está presente en los endosomas de reciclaje y en la región perinuclear (38).

Recientemente, se ha demostrado que la translocación específica a la membrana nuclear de ECE-1a, en un medio con alta concentración de glucosa (25 mM), se produce por la existencia de sitios de fosforilación para PKC en el extremo amino terminal. Así mismo, se confirmó la fosforilación de ECE-1b a través de sitios para PKA ubicados en la misma región sin conocerse aún el efecto que pueda tener está característica sobre la actividad de la proteína (67).

La internalización de pequeñas cantidades de ECE-1c, sugiere la presencia de otra señal en el dominio citosólico o transmembrana común a ECE-1b y ECE-1c. Resultados preliminares indican que un residuo de tirosina participa en este fenómeno. Esta tirosina puede interactuar con AP-2 y redireccionar cierta cantidad de proteínas a los compartimentos intracelulares. A pesar de esto, se piensa que debe ser un sistema de baja capacidad y por esta razón la mayor parte de ECE-1c permanece en la membrana citoplasmática (59).

- **Orgánica**

Existe una amplia expresión del gen de ECE-1 en tejidos humanos, así como en otras especies, lo que sugiere que tiene un papel biológico importante. Se ha detectado ECE-1 en la glándula adrenal, corteza renal, glándula paratiroides, próstata y pulmón, así como en la aorta abdominal, la arteria carótida común y en la arteria gástrica (68). Sin embargo, se encuentra mayoritariamente expresada en pulmón,

placenta y páncreas, y en menor cantidad en cerebro, corazón, hígado y músculo esquelético (41, 46, 68). En pulmón, la inmunoreactividad se confirmó en las células endoteliales que recubren el lumen de las arterias, en venas pequeñas y en arterias bronquiales. Sin embargo, la tinción fue débil en capilares, en las paredes alveolares y en las células epiteliales bronquiales. En el riñón, está presente en las células endoteliales de las arterias interlobulares (65). Los niveles relativos de expresión y localización de cada isoforma de ECE-1 varían entre los diferentes tejidos y tipos celulares.

Estas diferencias en la distribución de las distintas isoformas también son observadas en las patologías relacionadas con disfunción endotelial, así lo demuestran los resultados de Jackson et al. (69), los cuales indicaron que las isoformas aparecían en las lesiones ateroscleróticas con un patrón específico. Se observó, por ejemplo, ECE-1c en macrófagos, mientras que ECE-1a estaba tanto en macrófagos como en células musculares lisas.

3.4. Importancia en situaciones fisiopatológicas del enzima de conversión de la endotelina-1 (ECE-1)

- **Hipertensión arterial**

En la actualidad la hipertensión arterial (HTA) es considerada como uno de los problemas de salud pública más importantes, que a pesar de cursar en forma asintomática durante la mayor parte de la vida adulta, no deja de ser uno de los factores de riesgo más relevantes para el desarrollo de enfermedades cardiovasculares.

La HTA aparece cuando se pierden los mecanismos fisiológicos que regulan la presión arterial, presentándose una variación en el gasto cardiaco y un aumento en la resistencia vascular periférica. Puesto que el tono vascular es regulado por los factores vasoactivos endoteliales, en particular por el sistema renina-angiotensina, NO y ET-1, el estudio de los diferentes mecanismos de regulación es un aspecto crucial para el desarrollo de nuevos tratamientos.

La ET-1, por ejemplo, ha mostrado tener un papel relevante en la elevación de la presión arterial en algunos modelos de hipertensión, como los dependientes de ingesta de sal, en los que se encuentran aumentadas las concentraciones de éste péptido (70). Su efecto vasoconstrictor contribuye al aumento de la presión sanguínea, pero adicionalmente, su acción como promotor del crecimiento celular estimula la hipertrofia de la pared vascular.

En ratas, con activación del sistema ECE/ET-1, se ha observado un aumento mayor de ET-1 en las arterias coronarias, lo que sugiere su implicación en la aparición

de isquemia miocárdica secundaria a hipertensión. En estos casos, el uso de antagonistas selectivos de los receptores de la ET-1 (ETA/B y ETA) produce una ligera disminución de la presión arterial y una reducción significativa del crecimiento vascular, particularmente de la microvasculatura (70, 71).

Ante el resultado favorable obtenido, actualmente existen muchos estudios enfocados en esclarecer los mecanismos de regulación del sistema ET-1/ECE-1, así como, de diseñar nuevos antagonistas farmacológicos capaces, no solo, de inhibir selectivamente la unión de la ET-1 a sus receptores, sino también de descubrir nuevas moléculas que inhiban selectivamente la actividad de ECE-1 (70, 72).

La existencia de polimorfismos de un único nucleótido para los genes de la preproET-1, de ECE-1 y de los receptores para ET-1 han generado opiniones contrarias sobre su importancia en la regulación del sistema; sin embargo, no hay ninguna evidencia convincente que demuestre que afecten las diferentes estrategias terapéuticas antihipertensivas (72, 73).

Por otra parte, recientemente se ha descrito que el acortamiento de los telómeros está relacionado con el desarrollo de disfunción endotelial e hipertensión (74). Estos ratones deficientes en el componente ARN para la telomerasa, presentan mayores concentraciones plasmáticas y urinarias de ET-1 y una mayor expresión de ARNm de ECE-1 en presencia de especies reactivas de oxígeno. Los autores observaron que cuando se administraba a los ratones FR-901533, un antagonista de ECE-1, las cifras de presión arterial disminuían, sugiriendo que la hipertensión desarrollada por los ratones deficientes en telomerasa dependía, entre otros factores, de la sobreexpresión de ECE-1 (74).

A pesar de esto, aún existen muchas preguntas por resolver, sobre todo porque la comprensión de los mecanismos de regulación de ECE-1 no está del todo clara y porque en modelos deficientes en ECE-1 y ECE-2 se ha detectado ET-1, lo que sugiere que deben existir proteasas alternativas involucradas en el proceso de biosíntesis de ET-1.

- **Aterosclerosis**

La aterosclerosis es la primera causa de muerte e incapacidad en el mundo occidental. Se han identificado varios factores de riesgo (Tabla 2) que contribuyen a su desarrollo, pero aún existen muchos mecanismos de su fisiopatología que se desconocen.

La aterosclerosis se describe como una inflamación crónica que se presenta como respuesta a una lesión endotelial. La lesión característica es el ateroma, también conocida como placa ateromatosa o fibroadiposa. Aparece inicialmente en zonas

donde el endotelio se mantiene morfológicamente intacto, por tanto, es indispensable que exista una disfunción endotelial que incluya un aumento en la permeabilidad, un incremento en la adherencia leucocitaria y alteraciones en la generación de productos génicos y reguladores de las células endoteliales (75, 76).

Tabla 2. FACTORES DE RIESGO DE LA ATEROSCLEROSIS

Riesgos Principales	Riesgos secundarios, inciertos o no cuantificados
No modificables	
Envejecimiento	Obesidad
Género masculino	Inactividad física
Antecedentes familiares	Estrés (personalidad tipo A)
Anomalías genéticas	Deficiencia posmenopáusica de estrógenos
	Consumo elevado de hidratos de carbono
Potencialmente controlables	Lipoproteína
Hiperlipidemia	Consumo de grasas insaturadas endurecidas
Hipertensión arterial	(*trans*)
Tabaquismo	*Chlamydia pneumonie*
Diabetes	

Tabla I. Factores de riesgo de la aterosclerosis.

(Extraída del texto “Patología humana” de Robbins. Kumar, Cotran and Robbins. 7ª edición)

La acumulación de lípidos en la íntima del vaso da origen a la estría grasa, durante la fase inicial de la lesión. Estas lipoproteínas son más susceptibles de ser modificadas por procesos de glicosilación y oxidación lo que altera su función y acelera la aterogénesis.

Altos niveles plasmáticos de LDL han sido relacionados con el daño endotelial al generar OxLDL que poseen un efecto citotóxico. Además, las OxLDL intervienen en la diferenciación de monocitos a macrófagos (2, 77, 78) requiriendo la presencia de receptores celulares no regulados tipo scavenger I y II para ser fagocitadas. Sin embargo, las células endoteliales expresan apenas este tipo de receptores; así en 1997, Sawamura et al., descubrieron un nuevo receptor tipo lectina que denominaron receptor tipo lectina para OxLDL (LOX-1) (79) presente en las células endoteliales, macrófagos, CML y plaquetas, cuya expresión está aumenta en presencia de OxLDL (80) y en enfermedades como diabetes, hipertensión arterial y dislipidemias (81-86).

Concomitantemente, se produce un reclutamiento de leucocitos (monocitos y linfocitos) mediado por la expresión de moléculas de adhesión (VCAM-1, ICAM-1 o MCP-1) aumentando de esta forma el edema y la inflamación local. Estos monocitos trasformados en macrófagos fagocitarán más moléculas de colesterol y lípidos dando origen a las células espumosas, un rasgo característico de las placas ateroscleróticas complicadas (70). El acumulo de colesterol en las células espumosas (87, 88), hace que éstas mueran por apoptosis, liberando su contenido (IL-1, TNF-α y MCP-1) empeorando, de ésta forma, el proceso aterosclerótico al estimular la proliferación de CML, la producción de la matriz extracelular y al atraer más leucocitos a la lesión (76). (Figura 6).

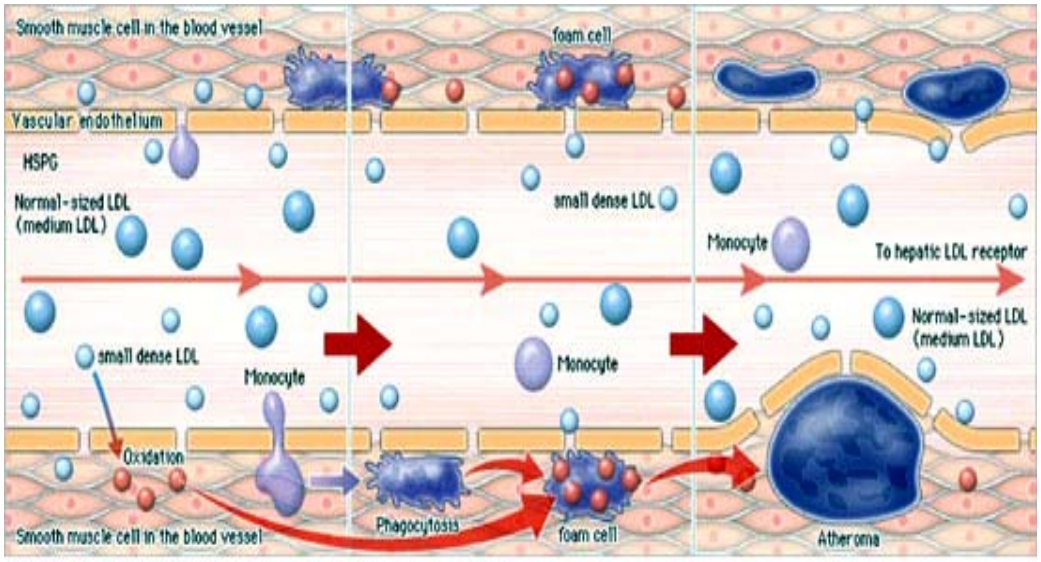

Figura 6. Proceso de aterogénesis.

Como se ha descrito anteriormente, algunos estudios han demostrado un papel relevante del sistema ET-1/ECE-1 en la aparición de disfunción endotelial y en procesos de remodelado vascular (89). De ahí que en los últimos años haya tomado especial importancia la descripción y la comprensión de la regulación de los elementos involucrados en este sistema, en especial ECE-1.

Desde 1997, Minamino et al., demostraron que ECE-1 está presente en las CML de la neointima de arterias de rata lesionadas con balón y, en CML y macrófagos de lesiones ateroscleróticas humanas. Además, al inhibir la actividad de ECE-1 con fosforamidón disminuyeron los efectos adversos sobre la neointima de los vasos lesionados (90).

En vasos coronarios humanos con diferentes estadios de aterosclerosis, se ha observado que las placas ateroscleróticas tempranas presentan un aumento en la expresión de ECE-1 predominantemente en las CE localizadas en la luz del vaso y en CML de la intima. En contraste, en placas con estadios más avanzados existe un mayor contenido de ECE-1 en macrófagos y en CE ubicadas dentro del ateroma, mientras que las CE de la íntima muestran una pobre cantidad.

El aumento de ECE-1 podría contribuir a la proliferación de CML y a generar vasoconstricción en estadios tempranos, promoviendo la desestabilización de la placa en estadios avanzados (91). Además, ante la administración de Big ET-1 se ha evidenciado un incremento en la producción de ET-1 y de la vasoconstricción en pacientes con aterosclerosis (92). La co-expresión de ECE-1 y ET-1 en las lesiones ateromatosas demuestra que un aumento en la expresión de ECE-1 se refleja en su actividad. Más importante aún, es que los ateromas que están en estado inflamatorio contienen más ET-1 que aquellos que no lo están. Queda claro que una estimulación positiva del sistema ECE-1/ET-1 está estrechamente relacionada con la presencia de inflamación crónica y que este efecto aparece desde estadios muy tempranos cuando aún la lesión no es clínicamente perceptible (93).

1. HIPÓTESIS

1.1. Hipótesis General

El enzima de conversión de la endotelina, ECE-1, es una proteína clave en la regulación de la endotelina por parte de las células endoteliales. El estudio de su regulación, farmacológica o fisiológica, y el análisis de su importancia en modelos experimentales, pueden ayudar a identificar dianas terapéuticas para problemas cardiovasculares.

1.2. Hipótesis específicas

- Los inhibidores de la actividad de ECE-1, fármacos que se están desarrollando a nivel experimental, pueden inducir cambios no sólo en la actividad sino en el contenido enzimático celular, y estos cambios pueden condicionar respuestas biológicas complejas que pueden contribuir a explicar la regulación de la enzima y el nivel de respuesta farmacológica en condiciones fisiopatológicas.

- Existe una regulación cruzada entre los dos autacoides endoteliales más ampliamente estudiados, el NO y la endotelina, de forma que el primero de ellos actúa regulando el contenido celular y/o la actividad de ECE-1.

- ECE-1 puede jugar un papel relevante en la patogénesis de la arteriosclerosis, probablemente porque puede ser regulada por metabolitos activos, como las LDL oxidadas, implicados en el desarrollo de la enfermedad.

2. OBJETIVOS

2.1. Objetivo general

Estudiar la regulación del enzima conversor, fisiológica o farmacológica, y su importancia en el desarrollo de la arteriosclerosis.

2.2. Objetivos específicos

- Analizar el efecto de los inhibidores del enzima de conversión de la endotelina en la regulación del propio enzima, estudiando los posibles mecanismos implicados.

- Evaluar la capacidad del NO para modular el enzima de conversión de la endotelina, analizando los mecanismos celulares responsables de esta posible regulación y sus posibles repercusiones biológicas.

- Estudiar la importancia del enzima de conversión de la endotelina en un modelo de arteriosclerosis experimental, evaluando la importancia de las LDL oxidadas en la génesis de los cambios observados.

1. MATERIALES

1.1. Cultivos celulares

Se utilizaron células endoteliales de aorta bovina (CEAB), recogida en fresco del matadero (94) y aisladas por digestión con colagenasa I_A. Se crecieron en RPMI 1640, suplementado con 15% de suero bovino y antibióticos, a 37ºC en una atmósfera con el 5% de CO_2. Se utilizaron pases entre 2 y 5 cuando alcanzaron la confluencia. Ocasionalmente, se utilizaron células de una línea celular endotelial humana, EA.hy926 (EA), crecidas en DMEM suplementado con 10% de suero fetal bovino y antibióticos, hasta alcanzar la confluencia.

1.2. Animales de experimentación

- **Ratones "knockout" para la apolipoproteína E (ApoE)**

Se utilizaron ratones machos homocigotos deficientes en la apolipoproteína E (ApoE) y ratones con fenotipo salvaje *"wild type"* C57BL/6J (WT), ambos de 4 semanas de edad y obtenidos del laboratorio Jackson (Charles River España, Barcelona, España). Tanto los ratones WT como los ApoE fueron alimentados con una dieta normal o con una dieta grasa tipo Western (TD88137, Harlan Teklad) durante 8 semanas para inducir aterosclerosis. Los animales tuvieron acceso libre al agua y fueron mantenidos a 24ºC con ciclos de 12 h día/noche. Posteriormente, los animales fueron sacrificados para realizar los análisis de los tejidos y del plasma. Toda la experimentación animal siguió las normas de la "Guía del cuidado y uso de animales de laboratorio" publicada por el *"US National Institute of Health"* (NIH publication No. 85-23, 1996) y con las regulaciones de la Comunidad Europea.

- **Ratas Wistar**

Se utilizaron ratas Wistar macho de 2 meses de edad, a las cuales se les administraron dos donadores de óxido nítrico, uno por vía oral (300 mg/kg de dinitrato de isosorbide, DNI, disuelto en el agua de bebida durante 4 y 24h) y otro por vía intraperitoneal (7,5 μg/Kg de nitroprusiato sódico, SNP, durante 6 y 24h). Posteriormente, las ratas fueron sacrificadas para los análisis tisulares.

- **Ratones "knockout" para eNOS**

Se utilizaron ratones machos homocigotos deficientes en óxido nítrico sintetasa endotelial (eNOS) y sus correspondientes *"wild type"* C57BL/6J (WT), de 2 meses de edad y fueron obtenidos del laboratorio Jackson (Charles River España, Barcelona, España). Posteriormente, los ratones fueron sacrificados para analizar algunos tejidos.

1.3. Anticuerpos y Sondas

En el Western blot se utilizaron anticuerpos primarios para evaluar la expresión de distintas proteínas. Para detectar ECE-1 se utilizó el anticuerpo monoclonal mAb AEC32-236 (dilución 1:400) proporcionado por el Dr. Kohei Shimada (Biological Research Laboratories, Sankyo Co., Ltd. Tokyo, Japan). Para identificar LOX-1 se empleó el anticuerpo monoclonal mAb anti-LOX-1 #5-2 (dilución 1:1000) proporcionado por el Dr. Tatsuya Sawamura (National Cardiovascular Centre Research Institute, Osaka, Japan). Como control de carga se utilizó el anticuerpo monoclonal anti-β-Tubulina de Sigma-Aldrich (St. Louis, MO, USA). Como anticuerpos secundarios se utilizó el anticuerpo goat anti-mouse a una dilución 1:50000 de Cultek (Pierce, Rockford, USA) para ECE-1 y para LOX-1, y el anticuerpo rabbit anti-mouse a una dilución 1:3000 de Dako Cytomation (Denmark) para la tubulina.

En el Northern blot se utilizaron sondas de ADN específicas para detectar la expresión del ARN mensajero de ECE-1, prepro-ET-1 y como control interno de carga la banda ribosomal 18S. La sonda para ECE-1 fue un fragmento de 798 pb obtenido por digestión con EcoRI y XhoI (54), la sonda para prepro-ET-1 fue un fragmento de 1200 pb obtenido por digestión con EcoRI (95), y la sonda para la 18S fue un fragmento de 5,8 Kb obtenido por digestión con EcoRI (54).

En los ensayos de retardo en gel se utilizaron oligonucleótidos de ADN diseñados y producidos sintéticamente. Estos oligonucleótidos contenían la secuencia específica dentro del gen de ECE-1 que reconocía la unión de los factores de transcripción NF-kB y AP-1. Para NF-KB las secuencias fueron las siguientes: *"sense"*: 5'-GGC TGG AGG GAT TTT TCC TCC TTT CA-3' y *"antisense"*: 5'-TGA AAG GAG GAA AAA TCC CTC CAG CC-3', y para AP-1: *"sense"*: 5'-CAT GGC TGT GTC ACC CTT GTC CC-3' y *"antisense"*: 5'-CAT GGC TGT GTC ACC CTT GTC CC-3'.

1.4. Isótopos radiactivos

Para marcar radiactivamente las sondas de ADN en el Northern se usó el isótopo radiactivo α-[^{32}P]-dCTP, y para marcar los oligonucleótidos de ADN en el ensayo de retardo en gel se utilizó el isótopo radiactivo γ-[^{32}P]-ATP, con el kit *"Ready to Go"* de GE Healthcare (Buckinghamshire, UK). Los isótopos impresionaron una película MXB y X-OMAT de Kodak (Rochester, NY, USA), y así se detectó la expresión de ARN mensajero de ECE-1, prepro-ET-1 o 18S en el Northern, y la unión de NF-kB o AP-1 al ADN en los ensayos de retardo en gel.

1.5. Inhibidores de ECE-1

Como inhibidores de la actividad de ECE-1 se emplearon varios compuestos: el fosforamidón de Sigma (St Louis, MO, USA), como inhibidor eficaz de la actividad de ECE-1 pero bastante inespecífico, el tiorfano de Sigma (St Louis, MO, USA) como inhibidor de la NEP pero no de ECE-1, dos compuestos sintéticos de Novartis proporcionados por el Dr. Arco Y Jeng (Novartis Pharmaceuticals Corporation, USA), CGS-26303 y CGS-35066, ambos con distinta especificidad por ECE-1, y por último, FR-901533, un antagonista selectivo de la actividad de ECE-1, proporcionado por Dr. Yuriyo Yamamoto (Fujisawa Pharmaceutical Co).

1.6. Reactivos generales

Los medios de cultivo fueron de Lonza (Basel, Switzerland). Las placas de cultivo de Cultek (Pierce, Rockford, USA). El kit de cuantificar proteínas BCA fue de BioRad (Richmond, CA, USA). En general, todos los productos utilizados, a menos que se especifique lo contrario, fueron de Sigma-Aldrich (St. Louis, MO, USA). Los inhibidores de proteína kinasa G, DT3 y KT-5823, fueron de Biolog (Life Science Institute, Bremen, Germany) y de Cayman Chemical Company (Ann Arbor, MI, USA), respectivamente, y la ET-1 (1-31) de Peptides International (Louisville, Kentuchy, USA).

2. MÉTODOS

2.1. Aislamiento y caracterización de células endoteliales de aorta bovina (CEAB)

Las células fueron obtenidas mediante digestión de aortas bovinas frescas siguiendo métodos ya descritos (94). La toxicidad celular fue evaluada en todas las condiciones experimentales por el método de exclusión con azul trypan y por la determinación de la liberación de lactato deshidrogenasa (LDH) al medio de cultivo, sin encontrar toxicidad significativa en ninguno de los casos estudiados.

2.2. Determinación de los niveles de endotelina-1 y de Big-ET-1

La producción de ET-1 y de Big-ET-1 se determinó a partir de los sobrenadantes de las CEAB, tratadas con los distintas condiciones experimentales, que fueron recogidos, liofilizados y almacenados a -70ºC hasta la realización del ensayo específico para cada una de ellas, ELISA de ET-1 de GE Healthcare (Buckinghamshire, UK) y ELISA de Big-ET-1 de R&D Systems (GmbH, Vienna, Austria), respectivamente.

2.3. Métodos de análisis de ECE-1

- **Valoración de la actividad de ECE-1**

La actividad de ECE-1 fue evaluada indirectamente por la producción de ET-1, la cual fue cuantificada mediante un ELISA comercial para ET-1. Para ello, las proteínas de membrana de las CEAB confluentes y estimuladas con cada condición experimental, fueron homogeneizadas por ultrasonidos en 1 ml de buffer de homogeneización (20 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 0.1 mM PMSF, 20 µM leupeptina, 20 µM aprotinina) (58). El homogeneizado fue centrifugado a 100.000 g durante 45 min. La concentración de proteínas fue cuantificada por BCA. Posteriormente, 30 µg del homogeneizado se incubaron con una cantidad fija de Big-ET-1 bovina (100 ng) durante 4h a 37 °C, en 250 µl de buffer que contenía 50 mM Tris-HCl pH 7 (55). La reacción fue frenada añadiendo 600 µl de etanol frío, y posteriormente, los sobrenadantes fueron liofilizados. Tras reconstituir los residuos secos con el buffer de ensayo del ELISA, la producción de ET-1 en cada muestra fue determinada por ELISA de ET-1 de GE Healthcare.

- **Cuantificación de la proteína**

Los niveles de proteína se analizaron por técnicas de Western blot siguiendo métodos ya descritos (54, 96).

- **Cuantificación del ARNm y análisis de su regulación**

- Aislamiento ARNm

El ARN celular total fue aislado por el método tradicional de isotiocianato de guanidinio-fenol-cloroformo descrito por Chomczynski and Sacchi en 1997 (97).

- Northern blot

La expresión génica fue evaluada por Northern blot según métodos ya publicados (54, 96).

- Experimentos con inhibidores de la transcripción

Para analizar si el ARNm de ECE-1 era desestabilizado por los estímulos, se hizo un ensayo utilizando el inhibidor de la transcripción, *"5,6-dichlorobenzoimidazole 1-beta-ribofuranoside"* (DRB). Para ello, las CEAB fueron tratadas en presencia de DRB (10 µM) con o sin los estímulos durante diferentes tiempos. Las muestras fueron procesadas y analizadas mediante Northern blot para ECE-1.

- Experimentos transfectando la región 3'-UTR del gen de ECE-1

La región 3' del gen de ECE-1, contiene una región rica en secuencias AU llamada UTR, región no traducida *"untranslated region"*, que regula la estabilidad del

gen. Para comprobar si la desestabilización de ECE-1 era debida a la activación de esa región, se diseñaron dos plásmidos. El primer plásmido pSGG-3'UTR-ECE, contenía la región 3' completa del gen de ECE-1 incluyendo la región UTR, que fue clonada en el sitio XbaI-XhoI del vector pSGG inmediatamente antes del gen reportador de la luciferasa. El segundo plásmido pSGG-Vacio no contenía dicha región. Ambos plásmidos fueron crecidos y purificados con el kit *"Endofree Plasmid Maxi kit"* de Qiagen (Maryland, USA), para ser utilizados en las transfecciones.

Las CEAB fueron crecidas al 60-80% de confluencia en pocillos de 12 y posteriormente fueron transfectadas con el plásmido 3'UTR-ECE o con el plásmido vacío acoplados a la luciferasa y junto con la *Renilla*-luciferasa (vector pRL-SV40). Para ello, 0.1 μg/μl de cada plásmido se mezcló con 1 ng/μl del plásmido control *Renilla*-luciferasa, y 4 μg/ml de lipofectamina. Tras 24h de transfección con dicha mezcla, las células fueron lavadas y reincubadas con medio completo durante 24h, y después fueron añadidos los distintos estímulos a distintos tiempos. Finalizada la incubación las células fueron lavadas y recogidas en un buffer especial para medir la actividad luciferasa con el kit *"Dual Luciferase Reporter Assay System"* de Promega. La actividad luciferasa fue expresada en unidades relativas de luz corregida por mg de proteína de cada pocillo.

- **Medida de la actividad del promotor de ECE-1**

- Diseño del promotor humano de ECE-1

Se amplificó por PCR el promotor del gen de ECE-1 humano partiendo de ADN genómico de células HeLa, utilizando primers específicos y el kit *"Advantage Genomic PCR"* de Clonthec Lab (Palo Alto, CA, USA) (96). El promotor fue subclonado en el sitio XhoI-HindIII del vector pGL3, inmediatamente antes del gen reportador de la luciferasa. El plásmido pGL3-ECE-1 fue crecido y purificado con columnas de Qiagen, para ser utilizado en transfecciones.

- Deleciones seriadas de ECE-1 humana

Se usó la PCR del ADN genómico de células HeLa para crear fragmentos de delecciones seriadas del promotor del gen de ECE-1 humana con los extremos terminales 5' en los nucleótidos -650 (AP-1), -596 (NF-kappa B), -542 (*acute phase*), -483 (*CAAT box*), -444 (*Shear stress*), -328 (STAT), y -216 (Elemento receptor de glucocorticoides-GRE), y con los 3' en los nucleótidos +1 utilizando el kit para PCR *"Advantage Genomic"* de Clonthec Lab. Cada uno de los fragmentos fue subclonado en el sitio XhoI-HindIII del vector pGL3, inmediatamente antes del gen reportador de la

luciferasa (96). Los plásmidos fueron crecidos y purificados con columnas de Qiagen, para ser utilizados en las transfecciones.

- Transfección transitoria del promotor de ECE-1

Las CEAB fueron crecidas al 60-80% de confluencia en pocillos de 12 y posteriormente fueron transfectadas con el promotor completo de ECE-1 acoplado a la luciferasa (pGL3-ECE-1) junto con la *Renilla*-luciferasa (vector pRL-SV40). Para ello, 0.1 μg/μl del plásmido con ECE-1 se mezcló con 1 ng/μl del plásmido control *Renilla*-luciferasa, y 4 μg/ml de lipofectamina. Tras 3h de transfección con dicha mezcla, las células fueron lavadas y reincubadas con medio completo durante 24h, y después fueron añadidos los distintos estímulos a distintos tiempos. Finalizada la incubación las células fueron lavadas y recogidas en un buffer especial para medir la actividad luciferasa con el kit *"Dual Luciferase Reporter Assay System"* de Promega. La actividad luciferasa fue expresada en unidades relativas de luz corregida por mg de proteína de cada pocillo.

- **Ensayos de retardo en gel (*gel shift*)**

Las CEAB fueron incubadas con cada estímulo a tiempos cortos, y se procedió a la extracción de los núcleos siguiendo métodos ya publicados (98, 99). Tras la incubación, las CEAB fueron lavadas dos veces con PBS, y recogidas en 400 µl de Buffer A (10 mM HEPES pH 7,9, 10 mM KCl, 0,1 mM EDTA, 0,1 mM EGTA, 1 mM DTT, 0,5 mM PMSF), conteniendo 1 µg/ml de leupeptina, antipaína y pepstatina A como inhibidores de proteasas. Después de incubar los extractos 15 min en hielo, se añadió 25 µl de Nonidet P-40 al 10%, e inmediatamente fueron centrifugadas a 15.000 rpm para separar los núcleos. El precipitado con los núcleos fue disuelto en 50 µl de Buffer B (20 mM HEPES pH 7,9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, e inhibidores de proteasas a 1 µg/ml), y se mantuvo en agitación durante 15 min a 4 ºC. Finalmente, los residuos celulares fueron eliminados por centrifugación y el sobrenadante con los extractos nucleares fue recogido, cuantificado y almacenado a -80 hasta realizar el ensayo. Las secuencias de los oligonucleótidos *"sense y antisense"* para detectar la unión de NF-kappa B al promotor ECE-1 (100), y para detectar unión del factor AP-1, se describen en el apartado de materiales. Los oligonucleótidos fueron marcados con γ-[^{32}P]-ATP en el extremo 5' usando la enzima T4 polinucleótido kinasa, y posteriormente, incubados con los extractos nucleares. Los complejos de unión ADN-proteína fueron separados en un gel no desnaturalizado de poliacrilamida al 1%, con 0,25 x Tris buffer EDTA. Los geles fueron secados al vacío y expuestos en películas UV-X de Kodak. Para los experimentos de competición, un

exceso (125 veces) de cada oligonucleótido no marcado, AP-1 o NF-kappa B, fue coincubado con la sonda marcada, AP-1 o NF-kappa B, respectivamente.

2.4. Estudios en animales

- **Ensayos de inmunohistoquímica de tejidos para detectar ECE-1**

Porciones de distintos tejidos aorta, pulmón, riñón y corazón fueron recogidos en parafina con el fin de realizar estudios de inmunohistoquímica (101) para detectar expresión de ECE-1 en cada tejido, utilizando el anticuerpo monoclonal mAb AEC32-236 (Dr. Kohei Shimada). Los complejos proteína-anticuerpo fueron detectados con un anticuerpo secundario, usando un reactivo de diaminobenzidina para intensificar la señal de Dako Cytomation (Denmark).

- **Medida de la presión arterial**

Una semana antes del sacrificio, la presión arterial fue registrada en los animales conscientes utilizando un esfigmomanómetro de cola (LE 5001 “Pressure Meter”, Letica Scientific Instruments, Hospitalet, España). Para ello, los animales fueron entrenados durante 3 días antes de iniciar las medidas finales para evitar variaciones por estrés. Adicionalmente, los cepos fueron previamente calentados a 30º con un calefactor (LE5660/6, Letica Scientific Instruments). La presión arterial fue medida en ratones “*Knockout*” ApoE y en ratones WT, antes y después de administrarles por vía intraperitoneal 1mg/Kg de FR-901533, un antagonista de la actividad de ECE-1.

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RESULTADOS

La disfunción vascular es un fenómeno común a una serie de patologías de gran prevalencia en la población como son la aterosclerosis, la hipertensión, la diabetes mellitus o la insuficiencia renal.

Durante los últimos años nuestro grupo ha estudiado el papel de alguno de los factores vasoactivos endoteliales como el óxido nítrico (NO), la endotelina-1 (ET-1) y el enzima conversor de la endotelina-1 (ECE-1).

A continuación se exponen, en forma de artículos, tres de las contribuciones en el estudio de ECE-1 que tratan de profundizar en los mecanismos de regulación de esta enzima así como, de conocer algunas de sus funciones en situaciones patológicas.

ARTÍCULO 1

"The peptidase inhibitor CGS-26303 increases endothelin-converting enzyme-1 expression in endothelial cells through accumulation of big endothelin-1". Br. J. Pharmacol. 2007; 152: 313-322.

Numerosos estudios han demostrado la importancia de la ET-1 en el desarrollo de enfermedades cardiovasculares, renales, pulmonares y del sistema nervioso central, razón que ha justificado la búsqueda de fármacos que bloqueen sus efectos adversos. De esta manera, han sido sintetizadas múltiples moléculas, entre las que se incluyen los antagonistas selectivos de los receptores de ET-1 y los inhibidores de ECE-1.

Como ECE-1 comparte parte de la secuencia de aminoácidos con la endopeptidasa neutra (NEP) y con el enzima de conversión de la angiotensina (ACE), se han diseñado inhibidores de doble acción, como fosforamidón y CGS-26303, y de triple acción, como SCH 54470; siendo estos últimos menos selectivos en la inhibición de ECE-1. Adicionalmente, existe un tercer grupo de inhibidores de origen natural como FR-901532 o como resultado de modificaciones estructurales de los de acción dual o triple como el CGS-35066, con capacidad de bloquear selectivamente ECE-1 y con escasa o nula capacidad sobre NEP.

La evidencia clínica apoya la eficacia de los inhibidores de ECE-1. Sin embargo, quedan aún muchos interrogantes sobre la biodisponibilidad y efectos que tienen estos compuestos sobre el sistema ET-1/ECE-1. Por una parte, ECE-1 todavía no está completamente estudiada, y por otra, aún no han sido identificados todos los substratos de la enzima, lo que impide conocer completamente los efectos secundarios e indeseables que pueden llegar a tener sus inhibidores.

El fosforamidón parece ser poco específico como inhibidor, y algunos resultados han demostrado que aumenta los niveles intracelulares de ECE-1a y 1b, lo que podría disminuir su efectividad como tratamiento a largo plazo. También se sabe que el CGS-26303 administrado crónicamente disminuye la hipertrofia ventricular izquierda, así como la dilatación y fibrosis miocárdica; sin embargo, su efecto sobre ECE-1 se desconoce.

El objetivo de éste trabajo fue evaluar el efecto del CGS-26303 sobre la expresión de ECE-1, analizando los mecanismos implicados.

Los resultados en células tratadas con CGS-26303 mostraron un aumento dosis y tiempo dependiente del contenido proteico así como del ARNm de ECE-1. Este

efecto fue consecuencia de un aumento en la transcripción al aumentar la actividad del promotor de ECE-1. Este incremento de ECE-1 fue confirmado con otro inhibidor más específico para ECE-1, CGS-35066, mientras que no se encontró efecto al utilizar un inhibidor específico de NEP como es el tiorfano.

Con el fin de analizar los mecanismos implicados en dicho efecto, en primer lugar, se descartó que existiera un *feedback* negativo ante niveles bajos de ET-1. Para ello, se administró ET-1 exógena, o Bosentán, antagonista de los receptores de ET-1, y no se observó ningún cambio en los contenidos proteicos de ECE-1. Tampoco hubo ningún cambio utilizando ET-1 exógena (1-31).

En segundo lugar, se confirmó que el efecto era específico de la inhibición sobre ECE-1 y no de la posible inhibición de NEP, puesto que el CGS-35066, inhibidor específico de ECE-1, reproducía el efecto del CGS-26303; mientras que el Tiorfano, inhibidor específico de NEP, no.

En tercer lugar, se comprobó si el efecto era debido a una acumulación de Big-ET-1 al estar inhibida ECE-1. Para ello, se midieron los niveles de Big-ET-1 en el sobrenadante de las células incubadas con CGS-26303, y se encontró un acúmulo significativo de la misma respecto a los controles. Posteriormente, se procedió a añadir Big-ET-1 y ver el efecto directo sobre ECE-1. La Big-ET-1 fue capaz de aumentar los niveles proteicos de ECE-1, así como la actividad del promotor de ECE-1. Estos resultados sugerían que la acumulación de sustrato, Big-ET-1 era la principal causa de estimulación de ECE-1 por parte de los inhibidores. Sin embargo, el mecanismo de acción necesita ser estudiado con mayor profundidad.

Los resultados obtenidos pueden tener un carácter clínicamente relevante, teniendo en cuenta que este aumento en la proteína podría generar un efecto completamente opuesto al deseado en tratamientos crónicos ya sea durante la administración del inhibidor o una vez que éste sea suspendido.

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RESEARCH PAPER

The peptidase inhibitor CGS-26303 increases endothelin converting enzyme-1 expression in endothelial cells through accumulation of big endothelin-1

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Background and purpose: CGS-26303 inhibits endothelin converting enzyme (ECE)-1 more specifically than phosphoramidon. We have studied the effect of CGS-26303 on ECE-1 expression in bovine aortic endothelial cells.
Methods: ECE-1 activity and big endothelin (ET)-1 levels were measured by ELISA, ECE-1 expression using western and northern blot and promoter activity using transfection assays.
Key results: ECE-1 activity was completely inhibited by CGS-26303 25 μM and phosphoramidon 100 μM. CGS-26303 and phosphoramidon, though not thiorphan, a neutral endopeptidase (NEP) inhibitor, stimulated ECE-1 expression in cells (maximal effect at 16 h, 25 μM). Cycloheximide abolished that effect. CGS-26303 induced ECE-1 mRNA expression and ECE-1 promoter activity. CGS-35066, a selective ECE-1 inhibitor, mimicked the effects of CGS-26303, suggesting that the effect was specific to ECE-1 inhibition. Big ET-1 accumulated in the cells and in the supernatants after CGS-26303 treatment. Neither exogenously added ET-1 nor the blockade of their receptors with bosentan modified ECE-1 protein. When big ET-1 was added to cells, significant increases in ECE-1 protein content and ECE-1 promoter activity were found. Bosentan did not block those effects. CGS-26303 did not modify prepro-ET-1 expression. CGS-26303 and big ET-1 induced the same effects in human endothelial cells, at lower doses.
Conclusions: These results suggest that the accumulation of big ET-1 is responsible for the effects of CGS-26303 on ECE-1 and they did not depend on NEP blockade. Changes in ECE-1 protein after the administration of CGS-26303 could lead to a decreased response in long-term treatments.
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Keywords: endothelial cells; endothelin-1; big ET-1; endothelin converting enzyme-1; ECE-1 regulation; CGS-26303; CGS-35066

Abbreviations: BAEC, bovine aortic endothelial cells; ECE-1, endothelin converting enzyme-1; ET-1, endothelin-1; NEP, neutral endopeptidase

Introduction

Endothelin-1 (ET-1), the most powerful endothelium-derived vasoconstrictor known, is generated from the posttranslational processing of a precursor protein, prepro-ET-1 to a peptide precursor, big ET-1, which is further processed to biologically active ET-1 by a specific phosphoramidon-sensitive metalloprotease called endothelin converting enzyme (ECE) (Xu *et al*., 1994). Two ECEs, labelled ECE-1 and ECE-2, are transcribed from different genes, and have been cloned and functionally characterized (Xu *et al*., 1994; Emoto and Yanagisawa, 1995). Both enzymes are type II membrane proteins and belong to the family of zinc metalloproteases related to neutral endopeptidase (NEP). The regulation of ECE gene expression in cultured endothelial cells has still to be fully described. It has been reported that ECE is up-regulated by phorbol ester through the transcriptional factor Ets-1 (Orzechowski *et al*., 1998), hydrogen peroxide through the transcriptional factor STAT 3 (Lopez-Ongil *et al*., 2002), thrombin through Erk1/2 (Eto *et al*., 2001), glucose (Keynan *et al*., 2004), oxidized LDL

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(Niemann *et al*., 2005) and vascular endothelial growth factor (Matsuura *et al*., 1997). On the other hand, it is downregulated by shear stress (Morawietz *et al*., 2000; Masatsugu *et al*., 2003), angiotensin II (Lopez-Ongil *et al*., 2005) and ET-1 (Naomi *et al*., 1998), and superoxide anion inhibits ECE-1 activity (Lopez-Ongil *et al*., 2000).

Since ET-1 is believed to play a critical role in different pathological settings (Lüscher and Barton, 2000; Aharinejad *et al*., 2005; Attina *et al*., 2005), the regulation of ECE synthesis or ECE activity could prove to be very helpful in controlling a number of cardiovascular or renal diseases. Phosphoramidon, the first reported ECE inhibitor, blocks the biological actions of big ET-1 both *in vitro* and *in vivo* (McMahon *et al*., 1991; Turner *et al*., 2001). However, it seems to be a rather nonspecific compound and it has been reported to increase the intracellular expression of ECE-1a and 1b (Isaka *et al*., 2003), an effect that could lead to a decreased response for long-term drug treatments. For these reasons, new ECE inhibitors are being developed and one of these, CGS-26303, has been shown to inhibit ECE-1 with an IC_{50} of 410 nM. *In vivo*, CGS-26303 was able to inhibit the pressor response to big ET-1 by 50% (Jeng *et al*., 2000). Long-term treatment with CGS-26303 decreases both preload and afterload, increases cardiac output, and diminishes left ventricular hypertrophy, dilatation and cardiac fibrosis (Mulder *et al*., 2004). The effect of CGS-26303 cannot be completely explained by its anti-ECE activity, as this drug also inhibits NEP (Jeng, 1997), subsequently increasing vasoactive peptides such as atrial natriuretic peptide (van der Zander *et al*., 1999; Horio *et al*., 2000).

In contrast to previous studies with phosphoramidon, the effect of CGS-26303 on ECE-1 expression has not been yet analysed. Changes in ECE-1 expression after the administration of CGS-26303 could determine an increased or decreased therapeutic response in long-term treatments. Present experiments were designed to analyse the effect of CGS-26303 on the cell content of ECE-1. Because a significantly increased expression of this protein was detected after treatment of cells with CGS-26303, the mechanisms responsible for these changes were explored.

Methods

Cell culture

Bovine aortic endothelial cells (BAEC) were isolated from bovine thoracic aortas, using the previously described methods (Marsden *et al*., 1990). The cells were maintained in Rosewell Park Memorial Institute 1640 (RPMI 1640) supplemented with 15% calf serum, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin, in an atmosphere of 95% air and 5% CO_2. The human endothelial cell line (EA.hy926) was grown in DMEM supplemented with 10% foetal bovine serum, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin, in an atmosphere of 95% air and 5% CO_2. Experiments were routinely performed on confluent monolayers between passages 2–5, that were made quiescent by serum deprivation. The cellular toxicity of CGS-26303 and CGS-35066 were excluded in every experimental condition using the trypan blue dye exclusion method and measuring lactic dehydrogenase (LDH) activity.

Western blot analysis of immunoreactive ECE-1

BAEC were grown to confluence and then homogenized in 1 ml of RIPA buffer (50 mM Tris-HCl pH 7.2, 150 mM NaCl, 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100) containing 1 mM phenylmethylsulphonyl fluoride (PMSF) and 0.2 mM orthosodium vanadate. The homogenates were centrifuged at 10 000 *g* for 30 min. The protein concentration in each supernatant was determined with the Bio-Rad Protein Assay Kit. Proteins were separated on sodium dodecyl sulphate-polyacrylamide gel electrophoresis 6% (30 μg protein per lane) and transferred onto nitrocellulose membranes using the Universal buffer (25 mM Tris pH 8.3, 192 mM glycine, 20% methanol, 0.05% SDS). The nitrocellulose membranes were blocked with 5% (w/v) non-fat dried milk in phosphate-buffered saline (PBS) 1 h at room temperature, and then incubated with 10 μg ml^{-1} of the monoclonal antibody against bovine ECE-1 (Dr Kohei Shimada). After being washed in tween tris buffered saline (TTBS) (20 mM Tris-HCl, 0.9% NaCl, 0.05% Tween 20), the blots were incubated with 200-fold-diluted horseradish peroxidase-conjugated goat anti-mouse IgG. The blots were then reblotted with a monoclonal anti–tubulin antibody in order to normalize ECE-1 levels. The immunoreactive bands were visualized with the supersignal detection system.

RNA isolation and northern analysis

Total cellular RNA was isolated from the BAEC with the guanidinium thiocyanate–phenol–chloroform method (Chomczynski and Sacchi, 1987). For northern analysis, 10 μg per lane of total RNA were subjected to electrophoresis in 1% agarose gels containing 0.66 M formaldehyde, transferred to Hybond nylon membranes, UV crosslinked and hybridized with labelled specific bovine probes (Lopez-Ongil *et al*., 2005) in hybridization solution (50% formamide, 5 × Denhardt's solution, 5 × SSPE, 0.5% SDS and 100 μg herring sperm DNA) at 42°C. The blots were washed at final stringency conditions of 50°C, 0.2 × SSC, 0.5% SDS for 30 min, and exposed on UV-X film for 24–48 h using intensifying screens at −80°C. The filters were stripped by boiling in 0.1% SDS solution and reprobed with a ^{32}P-labelled 18 S cDNA (5.8 kb fragment digested by *Eco*RI). The densitometric analysis of the film was performed with an image scanner using the public domain software package National Institutes of Health Image 1.55 (Bethesda, MD, USA). Levels of ECE-1 and prepro-ET-1 were normalized using 18 S expressions in the same lane and expressed in relative densitometric units with respect to the control values.

Measurement of ET-1 synthesis and ECE activity

Supernatants of confluent monolayers of BAEC were collected, lyophilized and stored at −70°C until assay. ET-1 was measured by enzyme-linked immunosorbent assay

(ELISA) according to the kit instructions. Membrane proteins of confluent monolayers of BAEC were homogenized in 1 ml of homogenization buffer (20 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 0.1 mM PMSF, 20 μM leupeptin, 20 μM aprotinin) using a Polytron homogenizer (Lopez-Ongil *et al*., 2000). The homogenates were centrifuged at 100 000 *g* for 45 min. The resultant membranes were washed three times by resuspension in homogenization buffer and recentrifuged after homogenization using a teflon/glass homogenizer. The protein concentration in each supernatant was determined using the Bio-Rad Protein Assay kit. Then 30 μg of this homogenate was incubated with bovine big ET-1 (100 ng) for 4 h at 37°C, in 250 μl of a reaction mixture containing 50 mM Tris-HCl buffer pH 7 (Ohnaka *et al*., 1990). The reaction was stopped by adding 600 μl of cold ethanol (−20°C). After centrifugation at 10 000 *g* for 10 min, supernatants were lyophilized. The dry residues were reconstituted with assay buffer, and ET-1 production in each sample was measured using ELISA.

Measurement of big endothelin-1 in cells and incubation media
Confluent monolayers of BAEC were treated with different ECE-1/NEP inhibitors for 16 h at 37°C. After this period, supernatants were collected and stored at −20°C. To assess whether big ET-1 was really able to cross the cell membrane, intracellular big ET-1 was measured in cells after adding CGS-26303 and exogenous big ET-1 for 16 h at 37°C. Afterwards, cells were sonicated in order to release the intracellular big ET-1. A commercial ELISA measured big ET-1 using a 96-well microtitre plate reader. To generate a standard curve for big ET-1, serial dilutions of big ET-1 stock ranging from 0.625–10 fmol ml^{-1} were used. A 4PL algorithm curve was automatically fitted to the standard and unknown values interpolated from the standard curves.

Transfection of BAEC with promoter/reporter constructs
We used the polymerase chain reaction (PCR) of HeLa cell genomic DNA to create the human ECE-1 α gene promoter with the Advantage Genomic PCR kit. Promoter was subcloned in the *Xho*I–*Hin*d III site of the pGL3 vector, upstream from a luciferase reporter gene. The plasmid was then grown in *Escherichia coli* DH5α and purified with Qiagen columns.

BAEC were grown in RPMI 1640 supplemented with 15% calf serum and antibiotics, and the cells were maintained in 5% CO_2 and plated approximately 24 h before transfection at a density of 60–80% of confluence in six-well plates with promoter/luciferase constructs. Transfections were performed by mixing 0.1 $\mu g\,\mu l^{-1}$ of plasmid DNA (pGL3-ECE-1) with 1 ng μl^{-1} of plasmid control from *Renilla* luciferase (pRL-SV40 vector) and 4 $\mu g\,ml^{-1}$ of Lipofectamine. The cells were washed with PBS 24 h after transfection, re-fed with RPMI 1640 and serum, and CGS-26303, big ET-1, or CGS-35066 was added at different times. The cells were harvested and assayed for luciferase activity using the Dual Luciferase Reporter Assay System. Luciferase activity was expressed as relative light units of plasmid DNA per milligram protein for each well.

Statistical analysis
Unless otherwise indicated, data are expressed as mean value ± s.e. mean obtained in at least three independent experiments, and are usually expressed as a percentage of the control values. Since the number of data in each distribution was never greater than 10, non-parametric statistics, in particular the Wilcoxon (two groups) or Friedman (more than two groups) tests, were selected to compare the paired results with the different experimental groups. The level of statistically significant difference was defined as $P<0.05$.

Materials
Bovine big ET-1, bovine ET-1, phosphoramidon, thiorphan, cycloheximide, actinomycin D, Triton X-100, PMSF, monoclonal anti–tubulin antibody, cell culture media RPMI 1640, calf serum, trypsin-EDTA (0.02%) and penicillin/streptomycin were purchased from Sigma Chemical Co. (St Louis, MO, USA). ET-1 (1–31) was purchased from Peptides International (Louisville, Kentuchy, USA). Dual Luciferase Reporter Assay System, pGL3 vector and pRL-SV40 vector were purchased from Innogenetics (Walkersville, MD, USA). Lipofectamine reagent was purchased from GIBCO-Invitrogen (Barcelona, Spain). Acrylamide–bisacrylamide was purchased from Hispanlab-Pronadisa (Madrid, Spain). MXB film was purchased from Kodak (Rochester, NY, USA). The supersignal detection system, secondary horseradish peroxidase-conjugated goat anti-mouse IgG, and NucleoBond PC 500 EF kits were purchased from Pierce (Rockford, USA). Protein markers, Bio-Rad protein assay kits, nitrocellulose membranes, plates and electrophoresis equipment were purchased from Bio-Rad Laboratories (Richmond, CA, USA). The endothelin-1 ELISA system, α-[^{32}P]dCTP and Hybond nylon membranes were from GE Healthcare (Buckinghamshire, UK). The Advantage Genomic PCR kit was purchased from Clontech Lab (Palo Alto, CA, USA). Qiagen columns were purchased from IZASA (GmbH, Hamburg, Germany). The big ET-1 ELISA system was purchased from BIOMEDICA (GmbH, Vienna, Austria).

Results

Effects of CGS-26303 on ECE-1
The ability of CGS-26303 to inhibit ECE activity was tested in cell extracts. When CGS-26303 was used at concentrations over 10 μM, the inhibition of the enzyme was maximal and comparable to that induced by 100 μM phosphoramidon (Figure 1a). In contrast, CGS-26303 induced a significant increase in ECE-1 protein content after 16 h, an effect that was also observed with phosphoramidon, but not with thiorphan, a specific NEP inhibitor (Figure 1b). Cells incubated with 25 μM CGS-26303 showed a time-dependent increase of ECE-1 protein content that was detectable at 4 h of treatment and maximal at 16 h (Figure 2a). A dose–response curve confirmed that the maximal effect of CGS-26303 on ECE-1 protein content took place at 25 μM, and was observable at concentrations between 5 and 50 μM (Figure 2b).

To analyse the molecular pathways involved in the CGS-26303-dependent ECE-1 upregulation, three kinds of

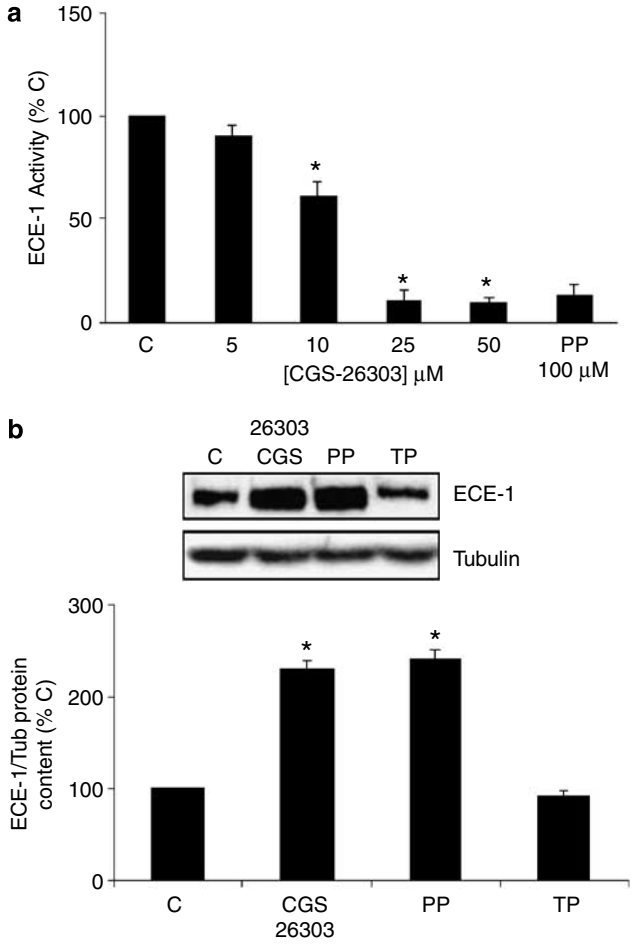

Figure 1 Effects of CGS-26303 on ECE-1 activity and ECE-1 protein content. (**a**) Cell extracts from BAEC were incubated with different concentrations of CGS-26303 for 6 h and ECE-1 activity was measured. 100 μM phosphoramidon (PP) was used as an internal control. Results are the mean value ± s.e. mean of four independent experiments. *$P<0.05$ vs control values (C). (**b**) BAEC were incubated for 16 h with 25 μM CGS-26303, 100 μM PP, and 10 μM thiorphan (TP). A representative western blot is shown in the upper part of the panel, whereas in the lower part, the densitometric analysis of three independent experiments is shown (mean value ± s.e. mean). *$P<0.05$ vs control values (C). BAEC, bovine aortic endothelial cells; ECE-1, endothelin converting enzyme-1; PP, phosphoramidon; TP, thiorphan.

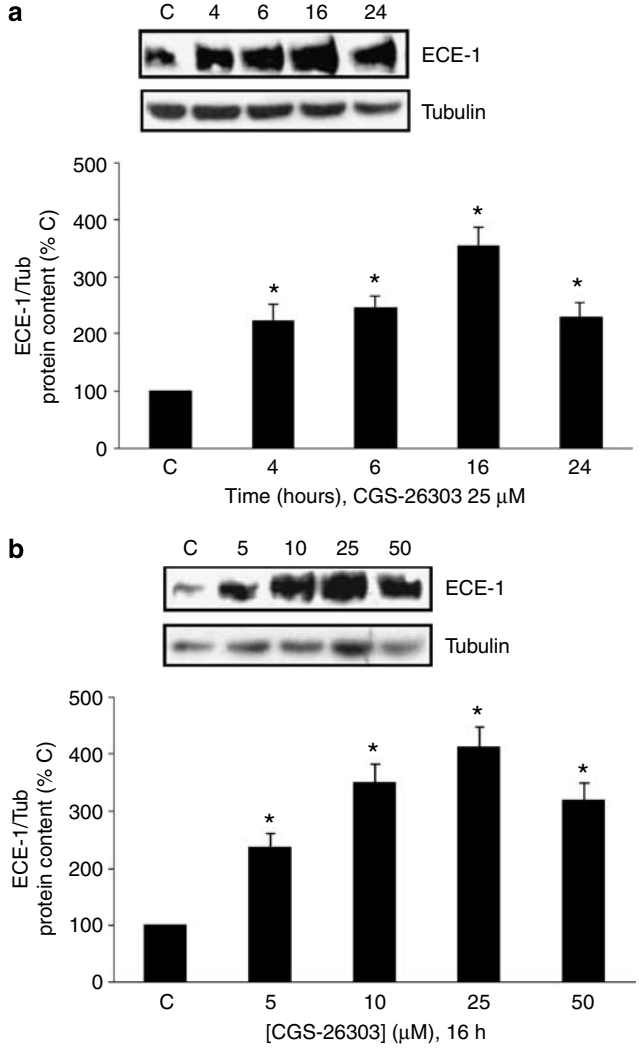

Figure 2 Effect of CGS-26303 on ECE-1 protein content. BAEC were incubated for different periods of time with 25 μM CGS-26303 (**a**), or with different concentrations of CGS-26303 for 16 h (**b**). A representative western blot is shown in the upper part of each panel, whereas in the lower part, the densitometric analysis of five independent experiments is shown (mean value ± s.e. mean). *$P<0.05$ vs control values (C). BAEC, bovine aortic endothelial cells; ECE-1, endothelin converting enzyme-1.

experiments were performed. First, the importance of *de novo* synthesis of proteins was tested using cycloheximide. As shown in Figure 3a, incubation with cycloheximide abolished the stimulation of ECE-1 induced by CGS-26303. The effects of CGS-26303 on ECE-1 mRNA content in BAEC were then considered. A statistically significant, dose–response induction of ECE-1 mRNA was elicited with CGS-26303 treatment (Figure 3b). This mRNA increase was not due to mRNA stabilization, as ECE-1 mRNA expression levels were comparable in cells treated with and without CGS-26303, when mRNA synthesis was blocked with actinomycin D (Figure 3c). Finally, the drug induced a time- and dose-dependent induction of ECE-1a promoter activity, with a similar pattern to the one observed in the mRNA ECE-1 expression (Figure 4).

To confirm the specific effect of CGS-26303 on ECE-1 upregulation, various experiments with a more selective ECE-1 inhibitor, CGS-35066, were performed. A time- (Figure 5a) and dose-dependent (Figure 5b) increase in ECE-1 protein content was also observed, appearing after 4 h of cell incubation and reaching its peak at 24 h (Figure 5a). The stimulation was maximal at 5 μM CGS-35066 (Figure 5b). Increased ECE-1 promoter activity was also observed in BAEC incubated with CGS-35066, as also happened with CGS-26303, but its maximal effect appeared at lower concentrations (Figure 5c).

Mechanisms involved in CGS-26303-induced upregulation of ECE-1

Considering the pharmacological activity of CGS-26303, there are two main mechanisms that could account for the

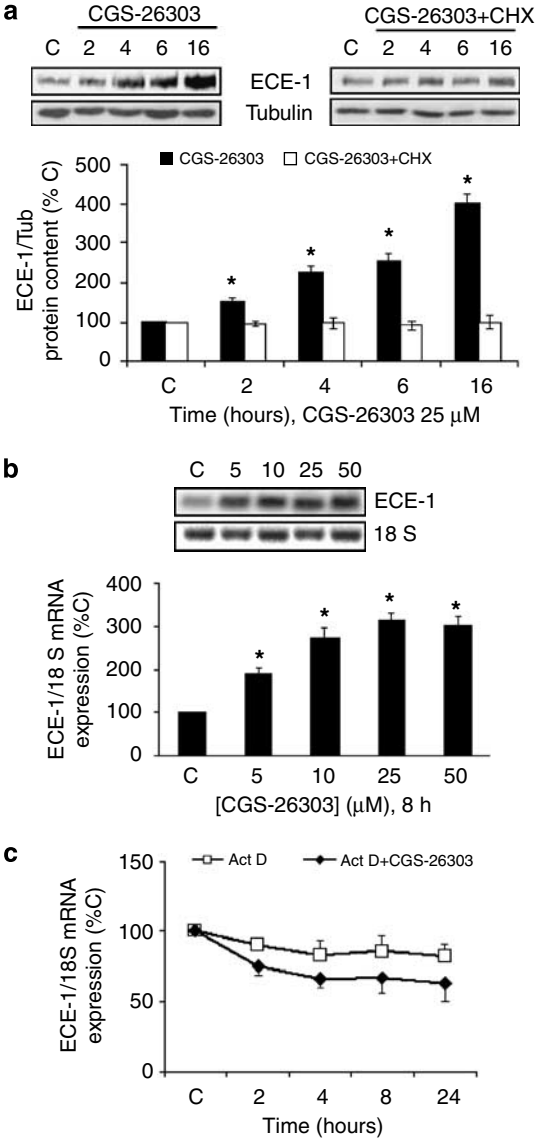

Figure 3 Mechanisms involved in the CGS-26303-dependent upregulation of ECE-1. Importance of protein synthesis, mRNA expression and mRNA stability. (**a**) BAEC were incubated with 25 μM CGS-26303 for different periods of time in basal conditions or in the presence of 1 μM cycloheximide (CHX). A representative western blot is shown above the densitometric analysis of three independent experiments (mean value $\pm$ s.e. mean). *$P<0.05$ vs control cells (C). (**b**) BAEC were incubated with different concentrations of CGS-26303 for 8 h. A representative northern blot is shown in the upper part of the panel, whereas in the lower part, the densitometric analysis of three independent experiments is shown (mean value $\pm$ s.e. mean). *$P<0.05$ vs control cells (C). (**c**) BAEC were incubated with actinomycin D (Act D) (10 μg ml^{-1}) with and without 25 μM CGS-26303, at different times. The mean values of mRNA levels in three independent experiments are shown (mean value $\pm$ s.e. mean). Act D alone; Act D + CGS-26303. Act D, actinomycin D; BAEC, bovine aortic endothelial cells; CHX, cycloheximide; ECE-1, endothelin converting enzyme-1.

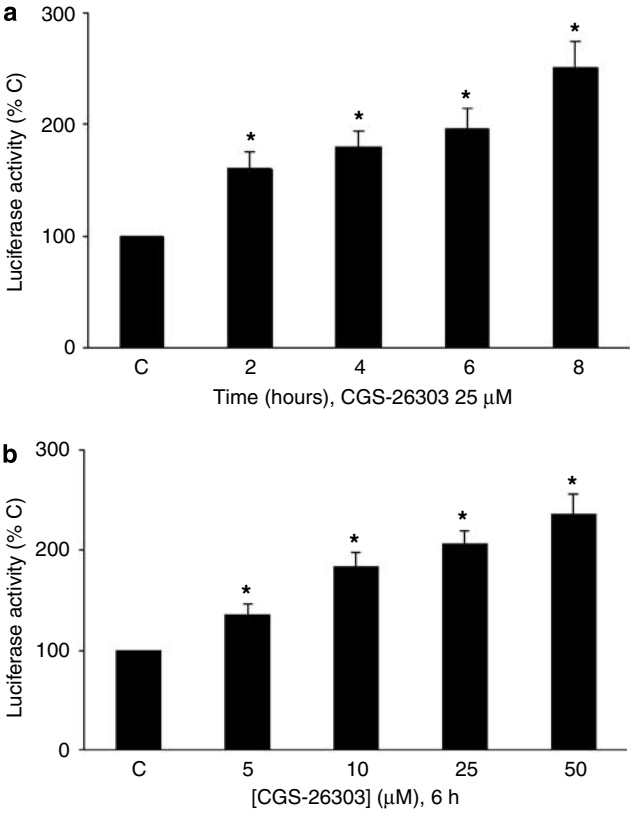

Figure 4 Mechanism involved in the CGS-26303-dependent upregulation of ECE-1. Importance of ECE-1 promoter activation. BAEC were incubated for different periods of time with 25 μM CGS-26303 (**a**), or with different concentrations of CGS-26303 for 6 h (**b**). Results are the mean value $\pm$ s.e. mean of four independent experiments. *$P<0.05$ vs control values (C). The stimulation observed with phorbol myristate acetate (PMA; 0.3 μM) for 6 h, which was used as a positive control, was 283 $\pm$ 38% ($n=4$). BAEC, bovine aortic endothelial cells; ECE-1, endothelin converting enzyme-1; PMA, phorbol myristate acetate.

upregulation of ECE-1: the inhibition of the ET-1 synthetic pathway or the NEP blockade. As Figure 1b shows, NEP blockade was not the cause of this effect, since thiorphan was unable to induce any change in ECE-1 protein content. The hypothesis that lower levels of ET-1, or that the accumulation of big ET-1, could be involved in this activation, was then tested. Big ET-1 did accumulate in cell supernatants (Figure 6a) and extracts (Figure 6b) after CGS-26303 treatment. Neither exogenously added ET-1 nor the blockade of their receptors modified ECE-1 protein content (Figure 7a). Different ET-1 concentrations (5–50 nM) at different times were tested and no changes in this protein were found (results not shown). Similar results were observed with ET-1 (1–31) (Figure 7b). In contrast, cell treatment with big ET-1 induced a significantly increased amount of ECE-1 protein in cells (Figure 7c). This effect, as well as CGS-26303-dependent stimulation, continued in the presence of bosentan, the dual ET-1 receptor blocker. Moreover, the stimulation induced by big ET-1 showed a time- and dose-dependent pattern (Figure 8), appearing after 6 h of cell incubation with big ET-1 and reaching its maximum between 16 and 24 h (Figure 8a). The minimum dose of big ET-1 that elicited this stimulation was 10 nM (Figure 8b). As can be seen in Figure 9, these changes in ECE-1 protein content reflect the modulation of ECE-1a promoter activity by big ET-1. Exogenously added big ET-1 was able to cross the cell membrane, as

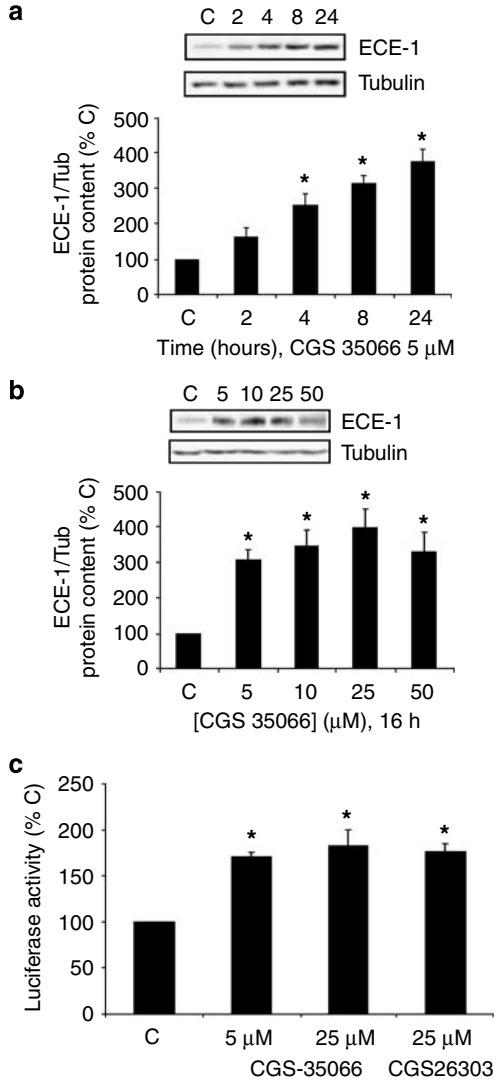

Figure 5 Effects of CGS-35066 on ECE-1 protein content and ECE-1 promoter activity. (**a**, **b**) BAEC were incubated for different periods of time with 5 μM CGS-35066 (**a**), or with different concentrations of CGS-35066 for 16 h (**b**). A representative western blot is shown in the upper part of each panel, whereas in the lower part the densitometric analysis of five independent experiments is shown (mean value$\pm$s.e. mean). *$P<0.05$ vs control values (C). (**c**) BAEC were incubated with two doses of CGS-35066 and with 25 μM of CGS-26303 for 6 h. Results are the mean value$\pm$s.e. mean of three independent experiments. *$P<0.05$ vs control values (C). The stimulation observed with PMA (0.3 μM) for 6 h, which was used as a positive control, was 349$\pm$42% ($n=3$). BAEC, bovine aortic endothelial cells; ECE-1, endothelin converting enzyme-1; PMA, phorbol myristate acetate.

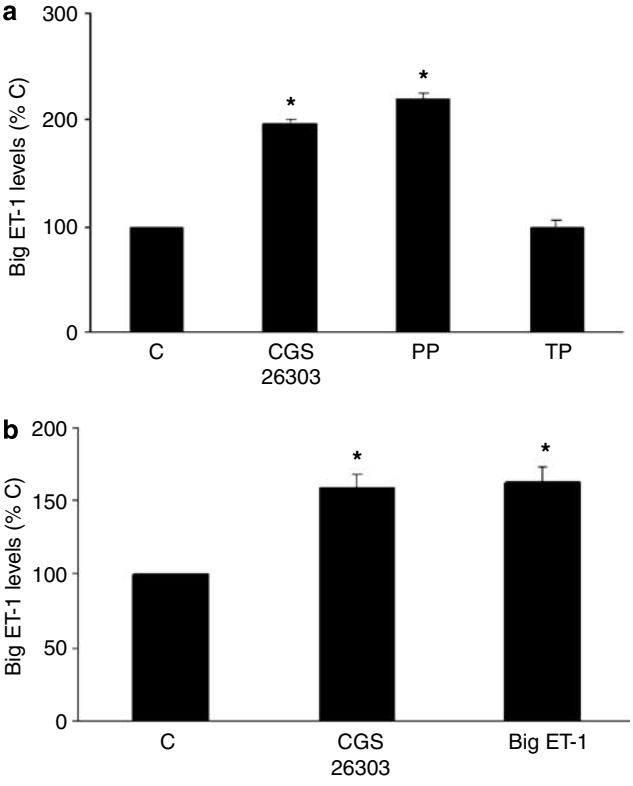

Figure 6 Effect of CGS-26303 on big ET-1 levels in cells and supernatants. (**a**) BAEC were incubated for 16 h with 25 μM CGS-26303, 100 μM phosphoramidon (PP) or 10 μM thiorphan (TP), and big ET-1 was measured in cell supernatants. Results are the mean value$\pm$s.e. mean of five independent experiments. *$P<0.05$ vs control values (C). Big ET-1 concentration was 29$\pm$3 fmol ml^{-1} in control cells. (**b**) BAEC were incubated for 16 h with 25 μM CGS-26303 and 25 nM big ET-1, and intracellular big ET-1 was measured in cells. *$P<0.05$ vs control values (C). Intracellular big ET-1 concentration was 25$\pm$4 fmol ml^{-1} mg^{-1} protein in control cells. BAEC, bovine aortic endothelial cells; ECE-1, endothelin converting enzyme-1; ET-1, endothelin-1; PP, phosphoramidon; TP, thiorphan.

increased concentrations of this protein were detected in cell extracts after big ET-1 incubation (Figure 6b).

The ECE-1 upregulation observed in endothelial cells treated with CGS-26303 may have relevant functional consequences. In fact, ET-1 concentration in cell supernatants after incubation for 6 h with 25 μM CGS-26303 decreased between 25 and 50% (mean inhibition 33$\pm$6%, $n=5$), a value significantly lower than the *in vitro* enzyme inhibition, which approached 100%. This dissociation may be explained by ECE-1 upregulation, since prepro-ET-1 expression was not modified by CGS-26303 treatment (Figure 10).

To study the effect of CGS-26303 on a different endothelial cell type, a human endothelial cell line (EA.hy926) was used. Cells were treated with 25 nM big ET-1, 25 μM CGS-26303 and 5 μM CGS-35066 for 16 h. Figure 11a shows the effect of these compounds on ECE-1 protein content. The stimulatory effect was similar for all of them. Moreover, lower doses of CGS-26303 were needed to induce ECE-1 protein content in human cells (Figure 11b).

Discussion

The present results clearly demonstrate that the cellular content of ECE-1 was increased by incubation with the dual ECE/NEP inhibitor CGS-26303, as well as with the more specific ECE-1 inhibitor CGS-35066, in both bovine and human endothelial cells. These inhibitors also define some of the primary mechanisms responsible for this stimulation. In short, in the presence of CGS-26303, a rapid increase in

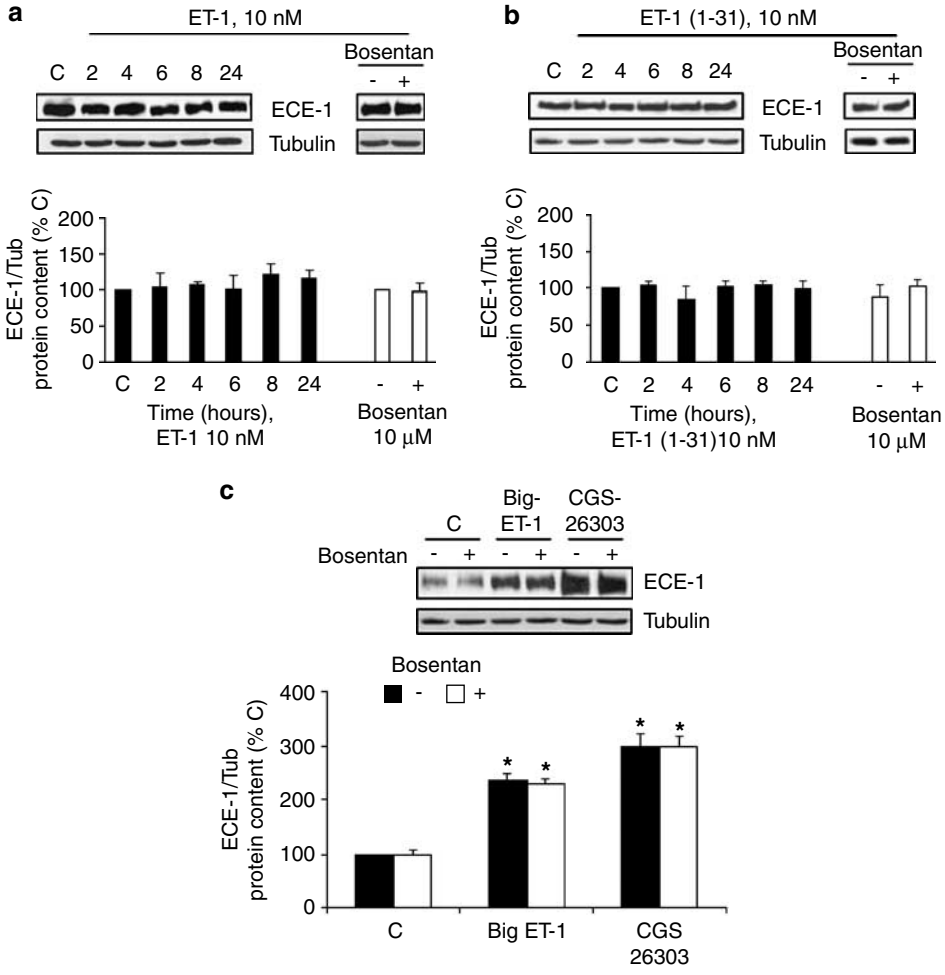

Figure 7 Role of ET-1, ET-1 (1–31) and big ET-1 in the CGS-26303-dependent ECE-1 upregulation. (**a**) BAEC were incubated for different periods of time with 10 nM ET-1 alone, or with 10 nM ET-1, in the absence or presence of 10 μM bosentan for 16 h. A representative western blot is shown in the upper part of the panel, whereas the densitometric analysis of four independent experiments is shown below (mean value $\pm$ s.e. mean). (**b**) BAEC were incubated for different periods of time with 10 nM ET-1 (1–31) alone or with 10 nM ET-1 (1–31) for 16 h, in the absence or presence of 10 μM bosentan. A representative western blot is shown in the upper part of the panel, whereas the densitometric analysis of three independent experiments is shown below (mean value $\pm$ s.e. mean). (C) BAEC were incubated for 16 h in control conditions (C), or with 25 nM big ET-1 or 25 μM CGS-26303 in the absence or presence of 10 μM bosentan. A representative western blot is shown above, whereas in the lower part the densitometric analysis of four independent experiments is shown (mean value $\pm$ s.e. mean). *$P<0.05$ vs control values (**c**). BAEC, bovine aortic endothelial cells; ECE-1, endothelin converting enzyme-1; ET-1, endothelin-1.

ECE-1a promoter activity was detected, with a subsequent rise in ECE-1 mRNA steady-state levels. The translation of this mRNA determined the increase in protein content that was observed in the cells. Although alternative mechanisms such as protein or mRNA stabilization could also be proposed to explain the changes observed in ECE-1, our different experimental data, including the analysis of the steady-state mRNA levels, and the promoter activity, as well as the cycloheximide and actinomycin D studies, allow us to reasonably exclude these possibilities.

The consequences of this upregulation may be clinically relevant, since such a compensatory mechanism could induce a reduced response to enzyme inhibition after the chronic administration of an inhibitor drug. In fact, in our hands, CGS-26303 in a dose that almost completely inhibited ECE activity *in vitro* only partially decreased ET-1 synthesis in cultured cells. Although cultured cells and cellular extracts are not completely comparable when interpreting the results with drugs, these discrepancies ought to be considered when analysing the biological response to a particular treatment. Differences between the experimental approaches could also explain the apparent discrepancies between the dose-response curves shown in Figures 1 and 2. For instance, 5 μM CGS-26303 did not modify ECE-1 activity in cell extracts, but it did increase ECE-1 protein content in BAEC. In addition to differences in the types of measurements, it could be suggested that the balance between the moderate increase in ECE-1 and the presence of the inhibitor at this concentration might not lead to any changes in ECE-1 activity.

Considering the pharmacological activity of CGS-26303, at least three main mechanisms could account for the ECE-1 upregulation detected: reduced ET-1 synthesis, the accumulation of big ET-1 and the increased local concentration of

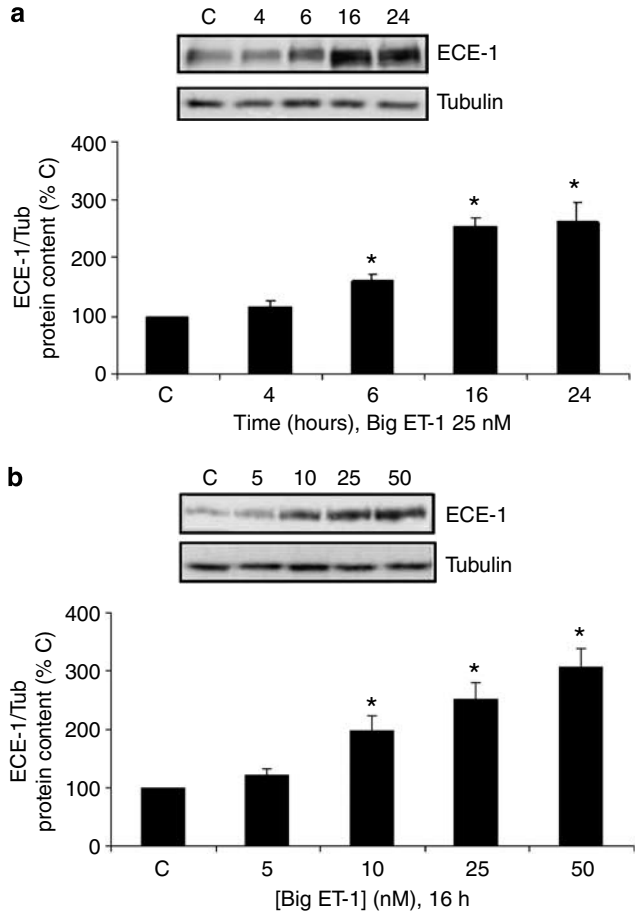

Figure 8 Effect of big ET-1 on ECE-1 protein content. BAEC were incubated for different periods of time with 25 nM big ET-1 (**a**) or with different doses of big ET-1 for 16 h (**b**). A representative western blot is shown in the upper part of each panel, whereas in the lower part the densitometric analysis of four independent experiments is shown (mean value$\pm$s.e. mean). *$P<0.05$ vs control values (C). BAEC, bovine aortic endothelial cells; ECE-1, endothelin converting enzyme-1; ET-1, endothelin-1.

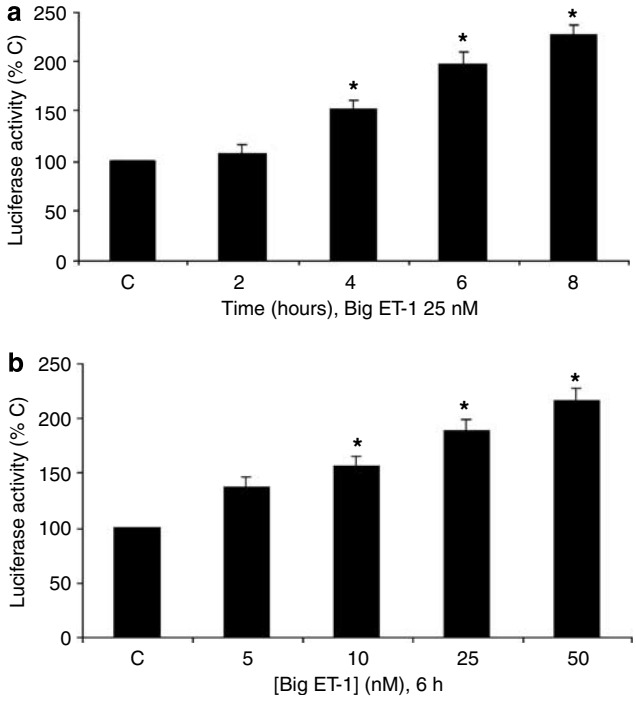

Figure 9 Effect of big ET-1 on ECE-1 promoter activity. BAEC were incubated for different periods of time with 25 nM big ET-1 (**a**) or with different doses of big ET-1 for 6 h (**b**). Results are the mean value$\pm$s.e. mean of four independent experiments. *$P<0.05$ vs control values (C). The stimulation observed with PMA (0.3 μM) for 6 h, which was used as a positive control, was 272$\pm$25% ($n=4$). BAEC, bovine aortic endothelial cells; ECE-1, endothelin converting enzyme-1; ET-1, endothelin-1; PMA, phorbol myristate acetate.

peptides degraded by neutral endopeptidases. Reduced ET-1 synthesis, by activating a negative feedback loop, could increase ECE-1 expression. In fact, it is already known that ET-1 downregulated ECE-1 gene expression in cultured rat pulmonary endothelial cells (Naomi *et al.*, 1998). In our cells, however, the results obtained do not support this hypothesis; neither cell incubation with ET-1 at different doses nor the blockade of the ET-1 receptor with bosentan (10 μM) modified ECE protein content in endothelial cells. The differences between our results and those of Naomi *et al.* (1998) could be attributed to a difference in species or ECE-1 isoforms. Similar results were obtained with ET-1 (1–31). In contrast, when endothelial cells were incubated directly with big ET-1 at different doses, a significant increase in ECE-1 protein content, as well as in ECE-1a promoter activity, was detected, suggesting that the accumulation of this substrate was the main factor in the up-regulation of ECE-1. Neither the thiorphan experiments nor the results obtained with CGS-35066, a specific ECE blocker, support a role for NEP inhibition in the genesis of the observed effects.

The mechanism by which big ET-1 modulates ECE-1 synthesis was not extensively explored; however, some indirect data allows hypotheses to be made about this mechanism. The effect of big ET-1 was not blocked by high concentrations of bosentan, suggesting that it is not dependent on the synthesis of new ET-1 or the interaction of this molecule with ET-1 receptors. Moreover, the big ET-1-dependent upregulation of ECE-1 was first observed after 6 h of incubation, whereas upregulation induced by CGS-26303 was already clear after 4 h of incubation. This would suggest that the exogenous big ET-1 must be transferred to the intracellular compartment in order to elicit its actions. At this point, results concerning the direct measurement of big ET-1 concentrations in cells and supernatants must be considered. These experiments were performed for two reasons. First, it was necessary to confirm the ability of CGS-26303 to induce big ET-1 accumulation. Big ET-1 concentration was significantly higher in cell extracts and supernatants after incubation with CGS-26303, probably reflecting the accumulation that takes place in the intracellular compartments after ECE-1 inhibition and the subsequent release in the culture media. Second, to demonstrate that exogenously added big ET-1 moves through the cell membrane and into the intracellular compartment.

There are no previous references to the possible regulatory role of big ET-1. The possibility that big ET-1 elicits specific actions has remained almost completely unexplored. Salvati

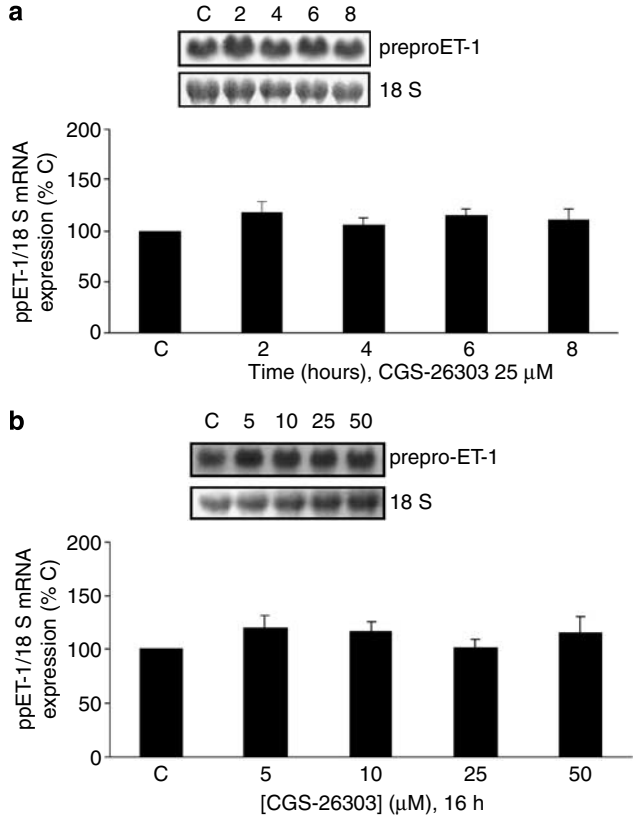

Figure 10 Effect of CGS-26303 on prepro-ET-1 (ppET-1) mRNA expression. BAEC were incubated for different periods of time with 25 μM CGS-26303 (**a**) or with different concentrations of CGS-26303 for 16 h (**b**). A representative northern blot is shown in the upper part of each panel, whereas in the lower part the densitometric analysis of four independent experiments is shown (mean value $\pm$ s.e. mean). BAEC, bovine aortic endothelial cells; ppET-1, prepro-ET-1.

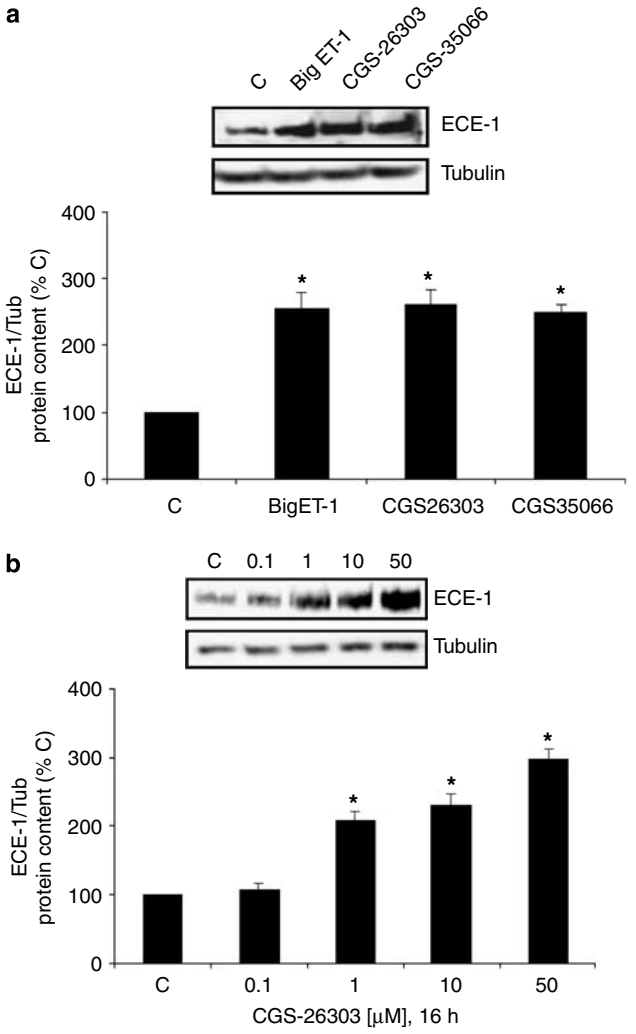

Figure 11 Effects of CGS-26303, big ET-1 and CGS-35066 on ECE-1 protein content in human endothelial cells (EA.hy926). (**a**) EA.hy926 were incubated with different compounds, 25 nM big ET-1, 25 μM CGS-26303 and 5 μM CGS-35066 for 16 h. (**b**) EA.hy926 were incubated with different doses of CGS-26303 for 16 h. A representative western blot is shown in the upper part of each panel, whereas in the lower part the densitometric analysis of four independent experiments is shown (mean value $\pm$ s.e. mean). *$P<0.05$ vs control values (C). ECE-1, endothelin converting enzyme-1; ET-1, endothelin-1.

et al. (1992) proposed a direct vasoconstrictor effect of big ET-1 on renal vascular resistance in the rat kidneys. Tirapelli *et al*. (2006) demonstrated that rabbit aortas generate ET-1 (1–31) from exogenously administered big ET-1 when ECE and NEP are inhibited by phosphoramidon. A chymase-like enzyme is probably involved in this process and synthesis of the 31-amino-acid peptide could explain the effects attributed to big ET-1. However, our results did not support that. Even so, no studies have analysed the possibility that big ET-1 acts directly in the intracellular compartment and that it regulates ECE-1 protein content.

With respect to ECE-1 upregulation by CGS-26303, previous studies have reported similar results. Thus, phosphoramidon, the first known ECE inhibitor, induced an increase in the expression levels of ECE-1a and ECE-1b, though not ECE-1c, in CHO-K1 cells (Isaka *et al*., 2003). On the other hand, the pharmacological inhibition of the angiotensin converting enzyme by lisinopril or captopril also induced the expression of this enzyme in porcine pulmonary artery endothelial cells (King and Oparil, 1992). However, these previous reports did not explore the mechanisms responsible for the effects observed. Our results stress the importance of the accumulation of substrate, in this case big ET-1, in the upregulation of the protein. Moreover, they point to a transcriptional mechanism, without changes in mRNA stability, as being primarily responsible for increased ECE-1 protein content after cell treatment with CGS-26303 or big ET-1.

Acknowledgements

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Conflict of interest

The authors state no conflict of interest.

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ARTÍCULO 2

"Nitric oxide decreases the expression of endothelin-converting enzyme-1 through mRNA destabilization". Circ. Res. (enviado a publicación)

En la actualidad se acepta que el endotelio participa activamente en múltiples procesos fisiológicos, regulados por sustancias vasoactivas endoteliales, que se encuentran en equilibrio para mantener la homeostasis. Cuando este equilibrio se rompe surge la disfunción endotelial. Los dos factores vasoactivos más estudiados son el NO y la ET-1, y se sabe que se regulan uno a otro. Sin embargo, nunca se ha estudiado si el NO regula directamente a ECE-1, enzima clave en la síntesis de ET-1.

El objetivo de este trabajo fue estudiar el efecto del NO sobre ECE-1, evaluando los posibles mecanismos implicados.

El NO administrado a células endoteliales (CEAB) en forma de dos donadores de NO, nitroprusiato sódico (SNP) y dietilamina complejo de NO (DEA), fue capaz de inducir una reducción en la expresión proteica y génica de ECE-1. Este efecto del NO dependía de la guanilato ciclasa soluble porque al preincubar con ODQ, un inhibidor de la enzima, se revirtió el efecto observado con el donador de NO. Por otro lado, al añadir exógenamente GMP cíclico, producto resultante de la unión del NO a la guanilato ciclasa soluble, en forma de un análogo estable llamado diburil-GMP (dB-GMP), se observó el mismo efecto que con el donador de NO. Este resultado indicaba que el NO, al unirse con su receptor guanilato ciclasa soluble y convertirse en GMPc, o el dB-GMP administrado directamente, provocaban una reducción en la expresión proteica y génica de ECE-1. La disminución del ARNm de ECE-1 inducida por el dB-GMP dependía, fundamentalmente, de un desestabilización del mensajero.

Para estudiar la región del gen de ECE-1 que podría estar modulando la estabilidad del ARNm en respuesta al GMP cíclico, se construyeron dos plásmidos, uno con la región completa de UTR del gen de ECE-1 (UTR-ECE), región no traducida responsable de la estabilidad, y otro plásmido que no contenía dicha región. Después de transfectar las CEAB con dichos plásmidos, se midió la actividad luciferasa en presencia o no del dB-GMP. Los resultados indican que el dB-GMP es capaz de unirse a la región UTR-ECE completa, desestabilizando el ARNm de ECE-1.

En la misma línea, se analizó si la vía PKG estaba implicada. Para ello se preincubaron las CEAB con dos inhibidores de PKG, el DT3 y el KT-5823, y posteriormente se añadió el dB-GMP. Los resultados indican que el dB-GMP es capaz de reducir la expresión proteica de ECE-1 y que dichos inhibidores revierten el efecto del dB-GMP de forma dosis dependiente.

Estos resultados indican que la vía PKG está implicada en el efecto de los donadores de NO sobre la regulación de ECE-1, y el efecto parece estar mediado por la unión a la región UTR.

Para comprobar la importancia fisiológica del efecto del NO se realizaron cocultivos de CEAB con macrófagos estimulados con lipopolisacárido (LPS), con el fin de que los macrófagos activados indujeran la síntesis de NO, y poder estudiar su acción sobre ECE-1 endotelial. Las CEAB, incubadas con LPS o con macrófagos por separado, mostraron pocos cambios en la expresión basal de ECE-1, pero el contenido endotelial de esta enzima disminuyó significativamente al incubar CEAB con macrófagos y LPS durante 24h, un efecto que se acompañó de un incremento de endotelina en el medio de cultivo y que fue revertido por ODQ, el inhibidor de la guanilato ciclasa soluble. Este resultado sugiere que la síntesis de NO en una situación fisiopatológica también provoca una reducción de ECE-1.

Una nueva aproximación experimental para estudiar la importancia funcional de estos efectos se llevó a cabo en un modelo animal. A ratas de dos meses de edad se les administraron dos donadores de NO a tiempos cortos, SNP y dinitrato de isosorbide (DNI), y posteriormente, se estudió la expresión proteica de ECE-1 en aorta y pulmón. Se encontró una reducción similar al efecto causado por el donador de NO en células *in vitro*.

En resumen, los resultados sugieren que el NO reduce la expresión de ECE-1, desestabilizando el ARNm vía PKG.

TITLE OF THE ARTICLE:

NITRIC OXIDE DECREASES THE EXPRESSION OF ENDOTHELIN-CONVERTING ENZYME-1 THROUGH mRNA DESTABILIZATION.

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SHORT TITLE:

Nitric oxide reduces endothelin-converting enzyme

SUBJECT CODES:

[95] Endothelium/vascular type/nitric oxide

ABSTRACT:

Endothelial function depends on the equilibrium in the synthesis of vasoactive endothelial factors. It is well known that endothelin (ET-1) and nitric oxide (NO) modulate one to each other. The aim of this study was to assess the ability of NO to regulate endothelin-converting enzyme-1 (ECE-1) content in bovine aortic endothelial cells (BAEC). BAEC were incubated with two different NO donors as well as with an cyclic-GMP analogue, dibutiryl-GMP. ECE-1 protein content and mRNA expression were evaluated by Western blot and Northern blot respectively, promoter activity by transfection experiments, ECE-1 activity by ELISA and cyclic GMP production by radioimmunoassay. Both NO donors decreased ECE-1 protein content, mRNA expression, and ECE-1 activity. These effects were completely blocked by ODQ, an inhibitor of soluble guanylate cyclase (sGC). NO donors raised cGMP levels and dibutiryl-GMP mimicked their effects on ECE-1 expression. A specific PKG inhibitor, KT5823, blocked the effect of dibutiryl-GMP. The changes on ECE-1 were due to a destabilization of its mRNA, and the untranslated region (UTR) of this mRNA seemed to play a relevant role in the destabilization process. The co-transfection of BAEC with plasmids over expressing an active or inactive PKG plus a plasmid containing UTR of ECE showed as active PKG reproduced the results obtained with dibutiryl-GMP. The biological relevance of this regulation was confirmed in BAEC coincubated with macrophages in the presence of lipolyssacharide, in eNOS deficient mice, and in Wistar rats treated with NO donors. In any case, an inverse relationship was observed between NO and ECE-1 protein content. Our results suggest that NO donors, through cGMP synthesis and PKG activation, provoke a destabilization of the ECE-1 mRNA, and point to the UTR of this mRNA as a key element in the destabilization.

Key words: nitric oxide, endothelin-converting enzyme-1, protein kinase G, endothelial cells; cyclic GMP.

ABBREVIATIONS:

ECE-1: Endothelin-converting enzyme-1

BAEC: Bovine aortic endothelial cells

dB-GMP: N'2-O-Dibutyrylguanosine 3',5'-cyclic monophosphate sodium salt hydrate

DEA: Diethylamine/nitric oxide complex sodium salt, 2-(N,N-Diethyl-amino)-diazenolate 2-oxide

DRB: 5,6-Dichlorobenzoimidazole 1-beta-d-ribofuranoside

IBMX: 3-Isobutyl-1-methylxanthine

ISDN: Isosorbide dinitrate

LPS: lipopolyssacharide

NO: Nitric oxide

ODQ: 1H-(1,2,4) Oxadiazolo (4,3-a) quinoxalin-1-one

SNP: Sodium nitroprusside

TTBS: Tween Tris buffer saline

VASP: Vasodilator-stimulated phosphoprotein

1- INTRODUCTION

Nowadays, it is well known that endothelium participates in the regulation of multiple functions in order to maintain a normal vascular function. Vasoactive endothelial factors such as nitric oxide (NO) or endothelin-1 (ET-1) are involved in that regulation. An unbalanced production of these factors provokes endothelial dysfunction, a main factor in the development of renal and cardiovascular diseases like diabetes, hypertension or atherosclerosis (1-4). ET-1 discovered in 1988 (5) is the most powerful vasoconstrictor peptide known and NO is a vasodilator produced mainly by endothelial cells (6, 7). Both of them are able to regulate one to each other (8, 9) in order to maintain such essential equilibrium. NO donors have been used therapeutically when the endothelial dysfunction is present in an environment lacking of NO availability (10-13).

The synthesis of ET-1 is the result of two main steps, one of them converts the long precursor prepro-ET-1 into the inactive short precursor big ET-1, and the second one, convert big ET-1 specifically in ET-1 through the action of the endothelin-converting enzyme (ECE-1) (14, 15). ECE-1 is a zinc type II membrane protein, an enzyme which regulation is been actively investigated (16-18). Four different isoforms of ECE-1 have been identified. They are generated by alternative splicing of a unique promoter (19, 20).

No previous work has explored the possibility that the cross-regulation between NO and ET-1 could take place through the modulation of ECE-1. Thus, the present experiments were devoted to assess the effect of NO on ECE-1 levels in bovine aortic endothelial cells (BAEC), as well as to analyze the mechanisms involved in this regulation.

2- MATERIALS AND METHODS

2.1.- Cell culture

BAEC were isolated as described (21, 22). Murine derived RAW L704 macrophage cell line was incubated as described (23, 24). Co-culture of BAEC and Raw were designed as described (25) (see supplemented data to detail). NO donors: DEA (2-N,N-Diethyl-amino-diazenolate 2-oxide) and SNP (sodium nitroprusside), as well as an active analogue of cyclic GMP, dB-GMP (dibutyril-GMP) were added to confluent cells for different times.

2.2.- Animal studies

Male homozygous eNOS-deficient (KO eNOS) and C57BL/6J control (WT) mice were obtained from The Jackson Laboratory (Charles River España, Barcelona, Spain). Two-month old male Wistar rats were treated with NO donors, 300 mg/Kg weight isosorbide dinitrate (ISDN) in the drinking water or 7.5 μg/Kg weight sodium nitroprusside (SNP) intraperitoneal for different times. All animals had free access to water, were maintained at 24ºC, and kept at a 12 h light/dark cycle. After that, animals were anaesthetized with pentobarbital (50 mg/kg i.p.), and aortas and lungs were removed and stored until analysis. The study design and the experimental protocols were according with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No.85-23, revised 1996) and to the European Union regulations.

2.3.- Analytical procedures

Immunoblot analysis: Protein extraction from BAEC and tissues, and the protein immunoblots were performed as described previously (18). ECE-1 protein expression was analyzed using a monoclonal anti-ECE-1 antibody (mAb AEC32-236 from Dr. Kohei Shimada).

RNA isolation and Northern blot analysis**:** Total RNA from BAEC was isolated as described (26, 27). For Northern analysis, a 2,900-bp fragment of bovine ECE-1 (28), and an 18S RNA probe were radiolabelled (Ready to Go, GE Healthcare Bio-Sciences (Buckinghamshire, UK).

Measurement of ECE-1 activity**:** Membrane proteins from BAEC treated with dB-GMP were homogenized as described (29). Then, 30 µg of the homogenate was incubated with bovine big ET-1 (100 ng) for 4 h at 37ºC in 50 mmol/L Tris-HCl buffer pH 7.0 (29, 30). ET-1 production was measured by ELISA.

Measurement of cyclic GMP**:** Cyclic GMP was extracted from BAEC following published methods (31); cGMP production in each sample was measured by radioimmunoassay, and corrected by protein amount (see supplemented data to detail).

Transient transfection experiments**:** Several transitory transfections were done (see supplemented data to detail). First, promoter activity of ECE-1 was studied using a human ECE-1 promoter/luciferase reporter gene plasmid (22, 28). Second, untranslated region (UTR) of ECE-1 was analyzed using a human UTR-ECE-1/luciferase reporter gene plasmid (pSGG-UTR-ECE-1). Third, PKG involvement was studied with two plasmids: a dominant-negative (fcGK-1αR) and -positive (fcGK-1αC) of PKG-1α (32, 33).

2.4.- Statistical analysis

Unless otherwise specified, data are expressed as means ± SEM as a percentage of the control values, and experiments were performed at least three times in duplicate. Comparisons were made by non-parametric statistics, particularly the Wilcoxon (two groups) or Friedman (more than two groups). Animal studies were analyzed by ANOVA, followed by the Scheffe multiple comparison test, after confirming the normality of the data distribution. The level of statistically significance was defined as $p < 0.05$.

3- RESULTS

Confluent BAEC monolayers were incubated with two different NO donors, SNP and DEA at different times and doses, in order to evaluate their effects on ECE-1 expression. Figure 1 shows how both NO donors were able to decrease ECE-1 protein content in a dose and time dependent fashion. The reduction was maximal at 8 h, an effect that remained for 24 h (Fig. 1B, 1D), and with minimal concentrations of 1 μmol/L for SNP (Fig. 1A) and 250 μmol/L for DEA (Fig. 1C). This effect depended on soluble guanylate cyclase (sGC) through cyclic GMP generation, as supported by different experiments. First, NO donors induced cyclic GMP production (Fig 2A). Second, an active analogue of cyclic GMP such as dibutyril-GMP (dB-GMP) induced a significant reduction of ECE-1 protein content (Fig. 2B), that was dose (Fig. 2C) and time dependent (Fig. 2D), mimicking the effects observed with SNP and DEA. The effect of dB-GMP took place before the NO donors effect, being maximal at 6 h with 10 μmol/L. Third, the decreased ECE-1 content induced by NO donors was completely blocked by ODQ, a specific inhibitor of sGC (Fig. 2B). These changes in protein content were paralleled by changes in enzyme activity. When BAEC were incubated with 10 μmol/L dB-GMP for 6 h, the total ECE-1 activity in cell extracts decreased about 50 % (Control values: 11.25 ± 2.68 fmol ET-1 per well; dB-GMP values: 4.75* ± 1.32 fmol ET-1 per well; *$p<0.05$).

At this point, different experiments were designed to analyze the mechanism involved in the cGMP-dependent ECE-1 down regulation. The role of PKG was studied by pretreating BAEC with a specific PKG inhibitor, KT5823, and measuring ECE-1 protein content by Western blot. KT5823 completely blocked the down regulation of ECE-1 induced by dB-GMP (Fig. 3A), at concentrations that inhibited phosphorylation of vasodilator-stimulated phosphoprotein (VASP) (Fig. 3B). Thereafter, the ability of dB-GMP to modulate ECE-1 mRNA expression was tested. A significant decrease of ECE-1 mRNA was found in cells incubated with several doses (Fig. 4A) and for different times (Fig. 4B) of dB-GMP. To assess if this mRNA ECE-1 down regulation was due to transcriptional changes, the ECE-1 promoter activity was measured by transitory transfection assays using the whole ECE-1 promoter linked to a luciferase expression reporter plasmid. When dB-GMP was added for

different times to transfected BAECs no significant changes were found on ECE-1 promoter activity (Fig. 4C). In consequence the stability of ECE-1 mRNA in cells treated with dB-GMP was checked. For this purpose, BAEC were treated with 10 µmol/L DRB, a transcriptional inhibitor, in the presence or not of 10 µmol/L dB-GMP and ECE-1 mRNA expression was studied by Northern blot at different times. Figure 4D shows that dB-GMP reduced ECE-1 mRNA expression, an effect that was not observed in control cells. In order to assess if the untranslated region (UTR) of ECE-1 gene was implied in the destabilization of mRNA, luciferase activity was measured after BAEC transfection with the whole UTR from ECE (pSGG-UTR-ECE) constructed into a luciferase expression reporter plasmid. Transfected cells were incubated with dB-GMP at different times. As shown in the Fig. 4E dB-GMP reduced the luciferase activity when pSGG-UTR-ECE plasmid was used, but not with the control plasmid pSGG-empty (Fig. 4E). Overexpression of the catalytic region of PKG-1α (fcGK-1αC), which retains kinase activity in the absence of cGMP, reproduced the effects of dB-GMP on UTR-ECE activity (Fig. 4F). Transfection with the regulatory region of PKG-1α (fcGK-1αR), which acts as a dominant negative for PKG-1α activity, led to the abrogation of dB-GMP effects on UTR-ECE activity (Fig. 4F). Transfection efficiency was verified by immunological detection with anti-Flag antibody (data not shown).

To evaluate the biological relevance of the NO/cGMP-dependent ECE-1 down regulation, BAEC were incubated with macrophages (Raw L704 cells), in the presence of lipopolysaccharide (LPS), a known stimulus of NO production. Figure 5A shows that BAEC incubated with LPS-activated macrophages exhibited a reduction in ECE-1 protein levels and ET-1 production, an effect that was not observed in BAEC incubated with macrophages or LPS separately. Indeed, when BAEC was treated with LPS alone, a slight up-regulation of ECE-1 protein content and ET-1 production were observed, as previously described (34, 35). Moreover, lung tissues from 8-wks old eNOS deficient mice expressed higher levels of ECE-1 protein than their wild-type counterparts (Fig. 5B). Finally, rats treated with NO donors showed a significant reduction of ECE-1 protein content in aorta and lung tissues (Fig. 6).

4. DISCUSSION

Our data suggest a new role for the NO/cGMP/PKG pathway in vascular endothelium. NO donors, through soluble guanylate cyclase, cGMP synthesis and PKG activation, down-regulate ECE-1 expression. A destabilization of ECE-1 mRNA, in which the UTR region is involved, seems to be a key element in the decreased ECE-1 protein content.

ECE-1 is the key enzyme in ET-1 synthesis, and this peptide is involved in the pathogenesis of different cardiovascular diseases. Our results demonstrate that NO donors decrease the ECE-1 protein content in BAEC, suggesting that this enzyme could be considered as a target for NO in the local cross regulation that seems to take place at endothelial level. Previous work from Kelly et al. (8) were unable to find any effect of NO on ECE-1, and they suggested that the ET-1 down-regulation observed in the presence of NO was due to a decreased expression of pre-pro-ET-1. Those experiments were performed in pulmonary endothelial cells from ovine origin, and probably the differences in animal species and in the origin of endothelial cells could explain the discrepancies observed.

Most of cellular actions of NO are mediated by a classical pathway in which the activation of soluble guanylate cyclase, the synthesis of cGMP, and the activation of PKG are involved (34-39). We tested the possibility that this pathway could be the responsible for the decreased ECE-1 protein content observed in cells incubated with NO donors. As expected, NO donors increased cGMP, and this stimulation was blocked by ODQ, a soluble guanylate cyclase inhibitor. ODQ also inhibited the effect of NO donors on ECE-1 protein content. Moreover, exogenously added cGMP reproduced the NO effects on ECE-1, and activated PKG. Again, the blockade of this enzyme abrogated the inhibitory effect of cGMP on ECE-1 protein content. Taking together, these results strongly support that the regulation of ECE-1 by NO depends on the classical sGC/cGMP/PKG pathway.

After studying the intracellular pathways involved in the NO-dependent ECE-1 protein down-regulation, the mechanisms responsible for the decreased protein content were evaluated. The cyclic GMP analogue significantly decreased the ECE-1 steady-state mRNA levels, without changes in the promoter activity. Moreover, it increased the degradation rate

of the mRNA when transcriptional activity was inhibited. Transfection experiments, in which a construction containing the UTR of the ECE-1 mRNA linked to a reporter system was used, demonstrated the relevance of this region in the increased ECE-1 mRNA degradation. The activity of the ECE-1 mRNA UTR depended on PKG activity, as demonstrated by using genetic material that modulates enzyme activity (31-33). Considering together these results, a general mechanism may be proposed to explain the reduction in ECE-1 protein content in the presence of NO donors. After activation of the classical sGC/cGMP/PKG pathway, any protein or group of proteins could be phosphorylated, and under this conformation could interact with the UTR of the ECE-1 mRNA in a different way. These changes in the UTR would determine a decreased stability of the mRNA, with the subsequent reduction in the steady-state levels and a decreased synthesis of the protein.

This is not the first report about the relevance of ECE-1 mRNA stability in the regulation of ECE-1 protein content. Previous studies in models of hepatic wound healing demonstrated that the increased ECE-1 content observed in this model was the consequence of an increased mRNA stability, and the UTR was critical in the maintenance of this stability (40). Additional information that may be obtained from the present results concerns the PKG isoform involved in the regulation of ECE-1. In the transfection experiments, plasmids over-expressing the catalytic and the regulatory regions of the PKG-1alpha isoform were used. This isoform mediates several effects of NO in vascular beds (32, 41, 42), and probably it also regulates ECE-1 mRNA stability.

The addition of NO donors is a pharmacological intervention that tries to reproduce the effect of endogenous NO. To assess the biological relevance of the NO-dependent ECE-1 regulation, we checked ECE-1 protein levels in BAEC co-cultured with a macrophage cell line treated with LPS for 24h. It is well known that LPS induces NO production in macrophages (43), and it has been also reported that LPS induces ECE-1 expression in endothelial cells (44, 45). BAEC incubated with LPS alone showed an increased ET-1 production and ECE-1 expression. In contrast, BAEC co-cultured with macrophages plus LPS showed a significantly decreased ET-1 production and ECE-1 expression, an effect that

was blocked by ODQ. Another experimental approach to test the biological relevance of the *in vitro* studies was to treat animals with NO donors and to analyze ECE-1 content in aorta and lung tissues. A similar reduction to that demonstrated in cells was observed in rats receiving NO donors. Furthermore, lungs extracted from deficient eNOS knockout mice presented higher expression of ECE-1 than their wild type counterparts, supporting the relevance of NO on this effect.

In consequence, present results clearly demonstrated that NO down-regulates ECE-1 in cultured cells and in animals, through a mechanisms in which is involved the sGC/cGMP/PGK pathway. The activation of this system induces a destabilization of the ECE-1 mRNA, and the UTR of this mRNA seems to be involved in the genesis of this effect. These findings support the biological relevance of the cross-regulation between the NO and the endothelin systems, and point to ECE-1 as a target of NO.

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7. DISCLOSURES

None.

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9- LEGENDS TO THE FIGURES

Figure 1. Effect of NO donors on ECE-1 protein content. BAEC were incubated with sodium nitroprusside (SNP) (panels A and B) or diethylamine complex (DEA) (panels B and D), with different doses for 24 h (panels A and C) and at different times using 1 µmol//L SNP (panel B) or 250 µmol/L DEA (panel D). ECE-1 protein content was measured by Western blot. In the upper part of each panel a representative Western-blot is shown, whereas in the lower part the densitometric analysis of 5 independent experiments is given (mean ± SEM). Values are expressed as the percentage of control cells (C); *p<0.05 vs. C.

Figure 2. Role of cyclic GMP in the NO-dependent ECE-1 down-regulation. Panel A) BAEC were incubated with 250 µmol/L diethylamine complex (DEA) for 15 min, in the presence or not of 1 µmol/L ODQ, a specific guanylate cyclase inhibitor, and then cyclic GMP production was measured with radioimmunoassay. Values are expressed as pmol per mg protein, and are the mean ± SEM of 3 independent experiments in triplicate; *p<0.05 vs. the other groups. Cyclic GMP synthesis by control cells (C) was 4.96 ± 1.96 pmol/mg protein. **Panel B)** BAEC were incubated with 250 µmol/L DEA for 8 h in presence or not of 1 µmol/L ODQ, and with an analogue of cyclic GMP (dB-GMP, 10 µmol/L for 8 h). ECE-1 protein content was measured by Western blot. In the upper part of the panel a representative Western-blot is shown, whereas in the lower part the densitometric analysis of 5 independent experiments is given (mean ± SEM). Values are expressed as the percentage of control cells (C); *p<0.05 vs. the other groups. **Panels C, D)** BAEC were incubated with dB-GMP at different concentrations for 8 h (panel C) and at different times with 10 µmol/L dB-GMP (panel D). ECE-1 protein content was measured by Western blot. In the upper part of each panel a representative Western-blot is shown, whereas in the lower part the densitometric analysis of 6 independent experiments is given (mean ± SEM). Values are expressed as the percentage of control cells (C); *p<0.05 vs. C.

Figure 3. Role of PKG in the cyclic GMP-dependent ECE-1 down-regulation. Panel A) BAEC were incubated 10 µmol/L dB-GMP (dB) for 6 h in the presence or not of a specific PKG inhibitor, KT5823 (KT), 250 nmol/L. ECE-1 protein content was measured by Western

blot. **Panel B)** BAEC were incubated 10 µmol/L dB-GMP (dB) for 15 min in the presence or not of 250 nmol/L KT5823 (KT). Phosphorylation of vasodilator-stimulated phosphoprotein (P-VASP) was measured by Western blot. In both panels, the upper part shows a representative immunoblot, whereas in the lower part a densitometric analysis of 3 independent experiments is shown. Values are expressed as the percentage of control cells (C); *p<0.05 vs. the other groups.

Figure 4.- Effect of dB-GMP on mRNA expression, promoter activity and UTR activity of ECE-1. Panels A-B) BAEC were incubated with different concentrations of dB-GMP for 8 h (panel A) and with 10 µmol/L of dB-GMP at different times (panel B). ECE-1 mRNA expression was analyzed by Northern blot. In the upper part of each panel a representative Northern-blot is shown, whereas in the lower part the densitometric analysis of 6 independent experiments is given (mean ± SEM). Values are expressed as the percentage of control cells (C); *p<0.05 vs. C. **Panel C)** BAEC were transfected with a reporter vector consisting of ECE-1 5' flanking region driving transcription of the luciferase gene. Transfected BAEC were then incubated with 10 µmol/L dB-GMP at different times, and the ECE-1 promoter activity was measured using a Luciferase Assay. Mean ± SEM of 3 independent experiments, in triplicate, are expressed as the percentage of control cells (C). In the transfection experiments, phorbol myristate acetate (3×10^{-7} mol/L, 6 h) was used as positive control (Stimulation: 283 ± 18 %, n = 4). **Panel D)** BAEC were incubated with 10 µmol/L DRB (transcriptional inhibitor), in the presence (open square) or not (closed square) of 10 µmol/L of dB-GMP at different times. ECE-1 mRNA expression was analyzed by Northern blot. In the upper part of the panel a representative Northern-blot is shown, whereas in the lower part the densitometric analysis of 6 independent experiments is given (mean ± SEM). Values are expressed as the percentage of control cells (C); *p<0.05 vs. C. **Panel E)** BAEC were transfected with a reporter vector consisting of ECE-1 3'-untranslated region driving transcription of the luciferase gene, pSGG-UTR-ECE-1 (open bars) and pSGG-empty as control plasmid (closed bars). Transfected BAEC were then incubated with 10 µmol/L dB-GMP for 6 h, and 3'UTR-ECE activity was measured using a Luciferase Assay.

Mean ± SEM of 4 independent experiments, in triplicate, are expressed as the percentage of control cells (C); with *p<0.05 vs. the other groups. **Panel F)** BAEC were co-transfected with pSGG-UTR-ECE-1 plasmid (open bars) or pSGG plasmid (closed bars) as control, and with a dominant-negative of PKG (fcGK-1αR) or a constitutively active PKG (fcGK-1αC). Co-transfected BAEC were treated or not with 10 µmol/L dB-GMP (dB) for 6 h, and 3'UTR-ECE activity was measured using a Luciferase Assay. Mean ± SEM of 3 independent experiments, in triplicate, are expressed as the percentage of control cells (C); *p<0.05 vs. other groups.

Figure 5. Biological relevance of the nitric oxide-dependent ECE-1 down regulation. Panel A) BAEC were coincubated with a macrophage line (Raw L704) and 0.1 µg/mL lipopolisacharide (LPS) for 24 h, in the presence or not of 10 µmol/L ODQ. ECE-1 protein content from BAEC was measured by Western blot. ET-1 production was measured in the supernatants by ELISA. In the upper part of the panel a representative Western-blot is shown, whereas in the lower part of the panel a densitometric analysis of 4 independent experiments is given, join to ET-1 values. Western values are expressed as the percentage of BAEC alone. *p<0.05 vs. the other groups. **Panel B)** Lung tissues from eNOS knockout (KO) mice were isolated to study ECE-1 protein content. The upper part of the panel shows a representative immunoblot, whereas the lower panel shows the densitometric analysis of 6 animals per group. *p<0.05 vs. wild type animals (WT).

Figure 6. *In vivo* effect of NO donors on ECE-1 protein content. Wistar rats were treated with sodium nitroprusside (SNP, 7.5 µg/Kg) or isosorbide dinitrate (ISDN, 300 mg/Kg) at different times. ECE-1 protein content was studied in aorta (panel A, B) and lung (panel C, D) tissues by Western blot. In the upper part of each panel a representative immunoblot is shown whereas the lower part shows the mean ± SEM of the densitometric analysis of 12 animals per group. *p<0.05 vs. Control group (C).

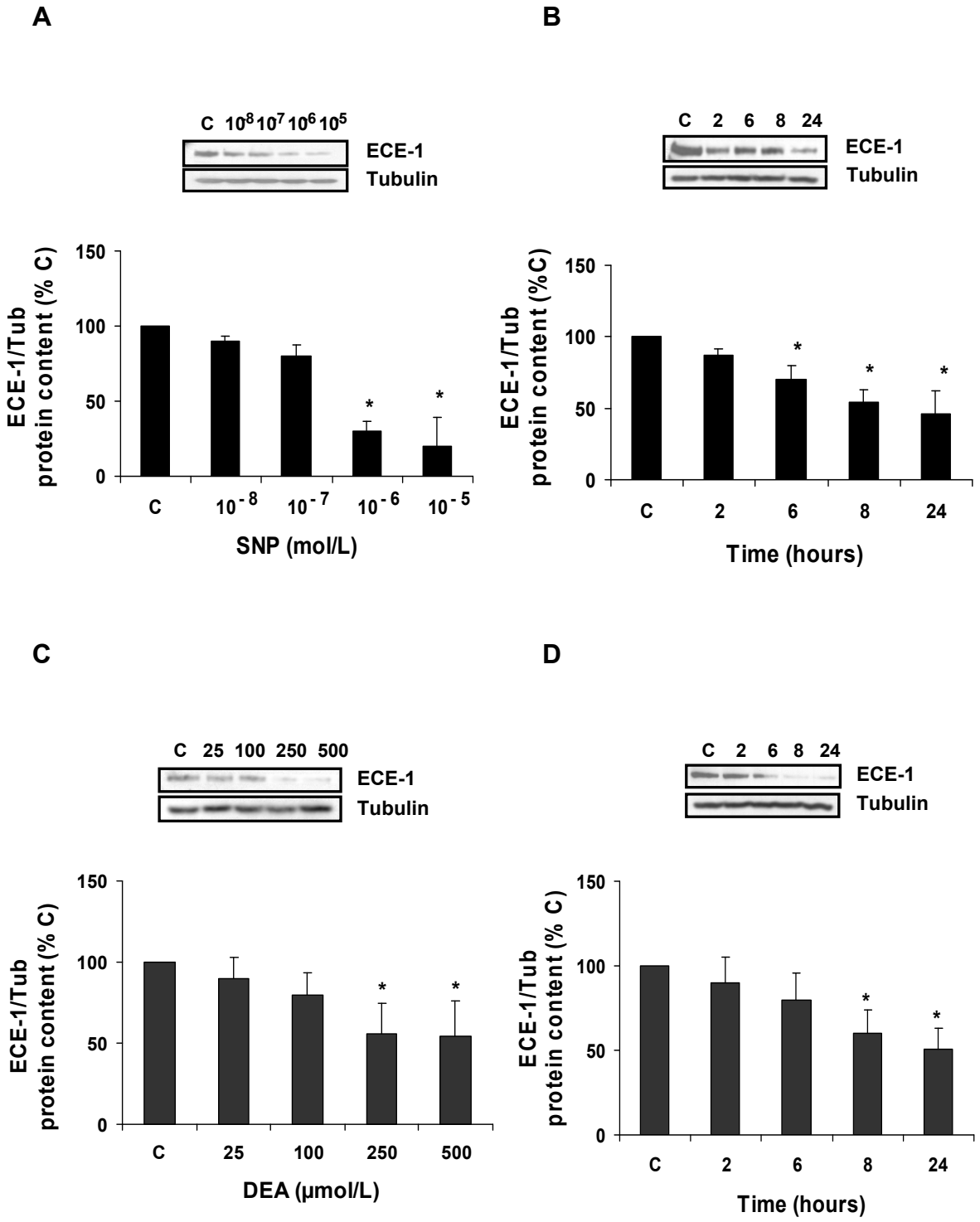

A
C 10^8 10^7 10^6 10^5
ECE-1
Tubulin
ECE-1/Tub protein content (% C)
150
100
50
0
C
10^-8
10^-7
10^-6
10^-5
SNP (mol/L)
B
C 2 6 8 24
ECE-1
Tubulin
ECE-1/Tub protein content (%C)
Time (hours)
C
D
C 25 100 250 500
DEA (µmol/L)
C 2 6 8 24
Time (hours)

Figure 1

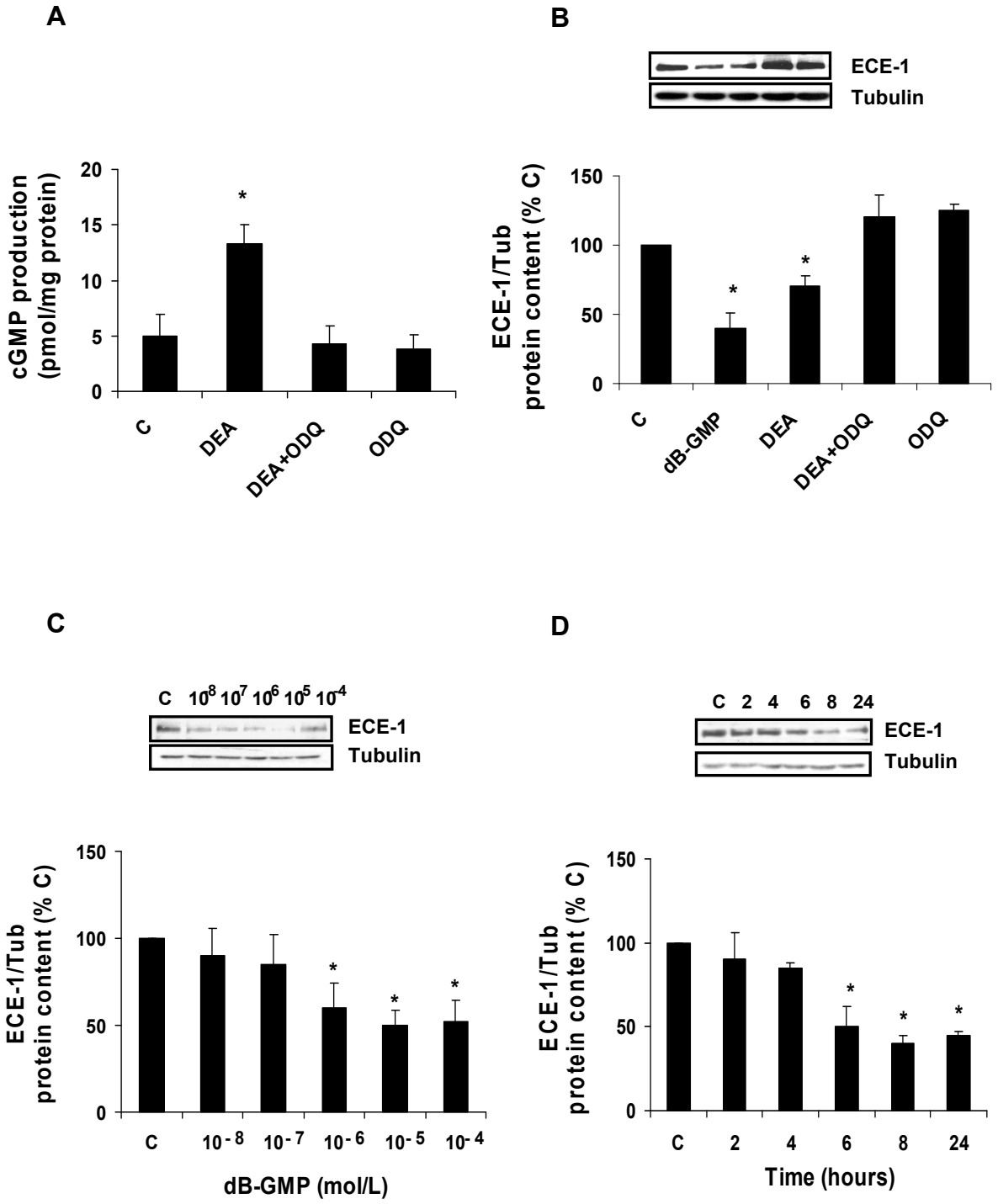

A
cGMP production (pmol/mg protein)
20
15
10
5
0
*
C
DEA
DEA+ODQ
ODQ
B
ECE-1
Tubulin
ECE-1/Tub protein content (% C)
150
100
50
0
*
*
C
dB-GMP
DEA
DEA+ODQ
ODQ
C
C 10^8 10^7 10^6 10^5 10^-4
ECE-1
Tubulin
ECE-1/Tub protein content (% C)
150
100
50
0
*
*
*
C
10^-8
10^-7
10^-6
10^-5
10^-4
dB-GMP (mol/L)
D
C 2 4 6 8 24
ECE-1
Tubulin
ECE-1/Tub protein content (% C)
150
100
50
0
*
*
*
C
2
4
6
8
24
Time (hours)

Figure 2

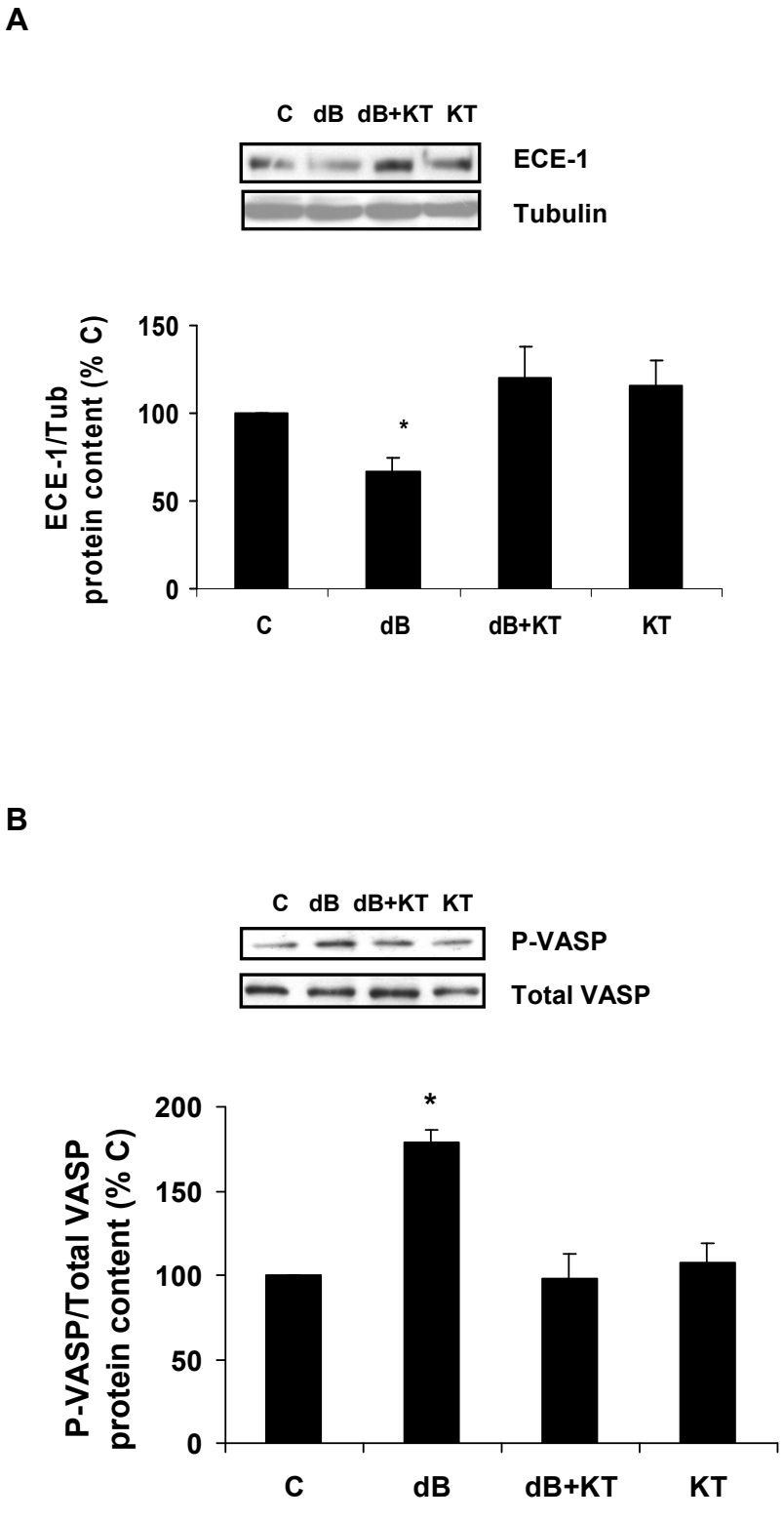

A
C dB dB+KT KT
ECE-1
Tubulin
ECE-1/Tub protein content (% C)
150
100
50
0
*
C
dB
dB+KT
KT
B
C dB dB+KT KT
P-VASP
Total VASP
P-VASP/Total VASP protein content (% C)
200
150
100
50
0
*
C
dB
dB+KT
KT

Figure 3

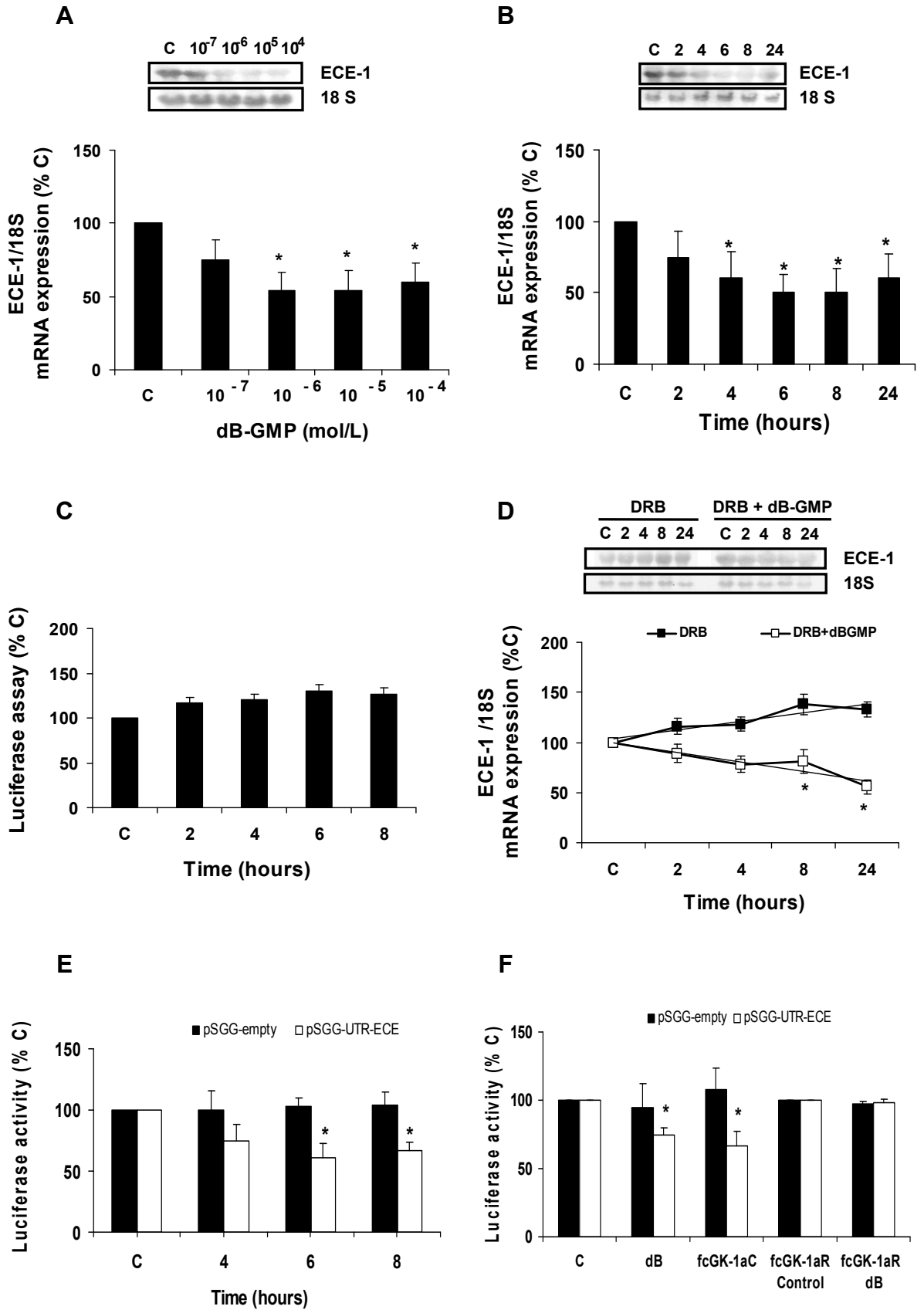
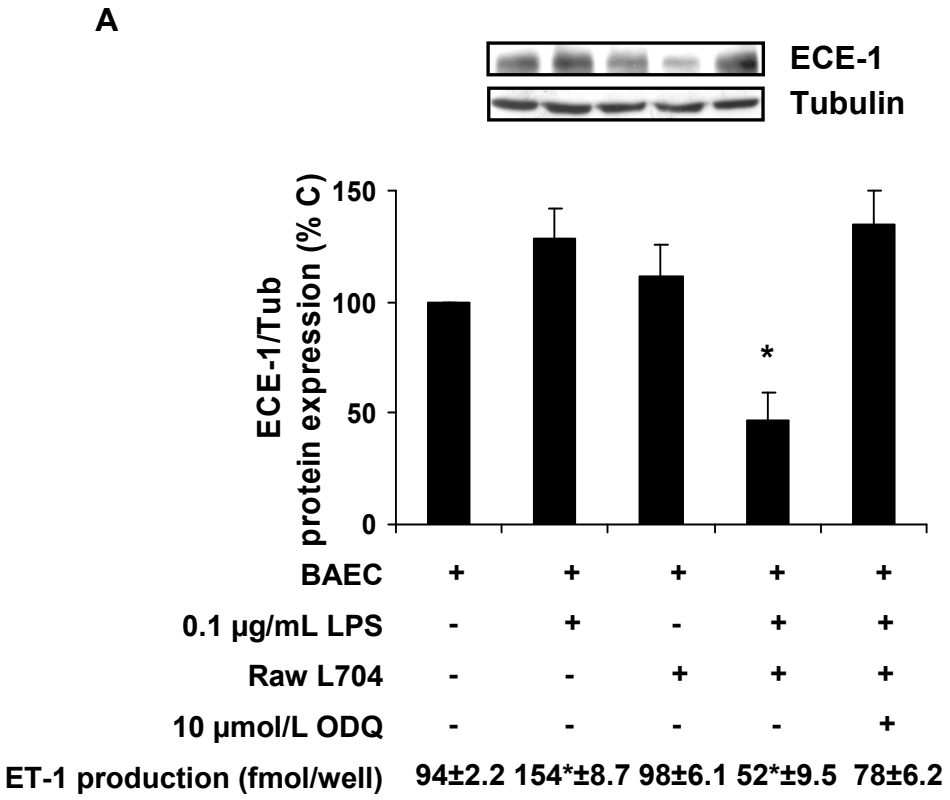

A
C 10-7 10-6 10-5 10-4
ECE-1
18 S
ECE-1/18S mRNA expression (% C)
150
100
50
0
C 10 -7 10 -6 10 -5 10 -4
dB-GMP (mol/L)
B
C 2 4 6 8 24
ECE-1
18 S
ECE-1/18S mRNA expression (% C)
C 2 4 6 8 24
Time (hours)
C
Luciferase assay (% C)
200
150
100
50
0
C 2 4 6 8
Time (hours)
D
DRB
DRB + dB-GMP
C 2 4 8 24 C 2 4 8 24
ECE-1
18S
DRB
DRB+dBGMP
ECE-1 /18S mRNA expression (%C)
C 2 4 8 24
Time (hours)
E
pSGG-empty
pSGG-UTR-ECE
Luciferase activity (% C)
C 4 6 8
Time (hours)
F
pSGG-empty
pSGG-UTR-ECE
Luciferase activity (% C)
C
dB
fcGK-1aC
fcGK-1aR Control
fcGK-1aR dB

Figure 4


A
ECE-1
Tubulin
ECE-1/Tub protein expression (% C)
150
100
50
0
*
BAEC + + + + +
0.1 µg/mL LPS - + - + +
Raw L704 - - + + +
10 µmol/L ODQ - - - - +
ET-1 production (fmol/well) 94±2.2 154*±8.7 98±6.1 52*±9.5 78±6.2

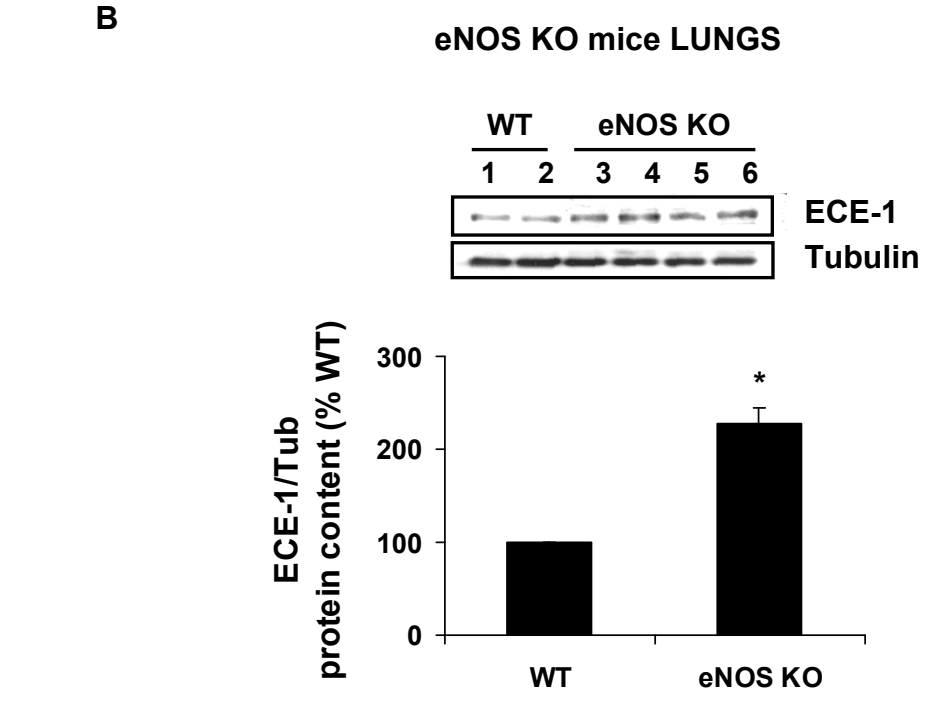

B
eNOS KO mice LUNGS
WT
eNOS KO
1 2 3 4 5 6
ECE-1
Tubulin
ECE-1/Tub protein content (% WT)
300
200
100
0
*
WT
eNOS KO

Figure 5

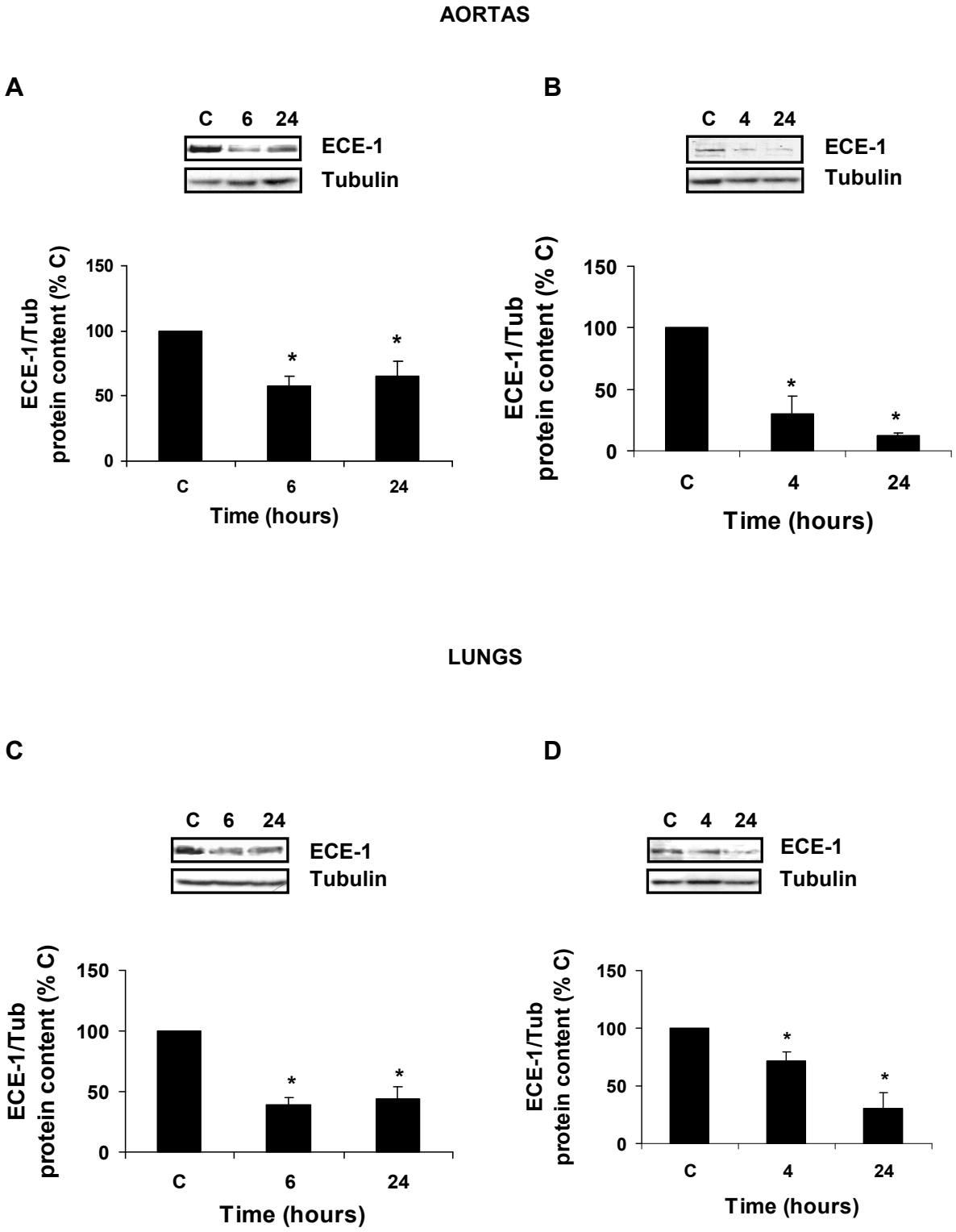

AORTAS
A
C 6 24
ECE-1
Tubulin
ECE-1/Tub protein content (% C)
150
100
50
0
*
*
C
6
24
Time (hours)
B
C 4 24
ECE-1
Tubulin
ECE-1/Tub protein content (% C)
150
100
50
0
*
*
C
4
24
Time (hours)
LUNGS
C
C 6 24
ECE-1
Tubulin
ECE-1/Tub protein content (% C)
150
100
50
0
*
*
C
6
24
Time (hours)
D
C 4 24
ECE-1
Tubulin
ECE-1/Tub protein content (% C)
150
100
50
0
*
*
C
4
24
Time (hours)

Figure 6

SUPPLEMENTAL DATA

EXPANDED MATERIAL AND METHODS

Materials

NO donors, 2-(N,N-Diethyl-amino)-diazenolate 2-oxide (DEA) and sodium nitroprusside (SNP), as well as an active analogue of cyclic GMP, dibutyril-GMP (dB-GMP) were form Sigma-Aldrich-Fluka Chemical Co. (St. Louis, MO, USA). Culture plates, supersignal detection system and secondary horseradish peroxidase-conjugated goat anti-mouse IgG were from Cultek (Pierce, Rockford, USA). Dual Luciferase Reporter Assay System, pGL3 vector and pRL-SV40 vector were from Innogenetics (Walkersville, MD, USA). Lipofectamine reagent and OptiMEM I media were from GIBCO-Invitrogen (Barcelona, SPAIN). Acrylamide-bisacrylamide was from Hispanlab-Pronadisa (Madrid, Spain). MXB films were from Kodak (Rochester, NY, USA). Protein markers, BioRad protein assay kit, plates and electrophoresis equipment were from Bio-Rad Laboratories (Richmond, CA, USA). Protease inhibitor cocktail tablets were from Roche Diagnostics (Madrid, Spain). The alpha-[^{32}P]-dCTP were from GE Healthcare Bio-Sciences (Buckinghamshire, UK). The ET-1 ELISA system was from R&D Systems (Abingdon, United Kingdom). Cyclic GMP [^{125}I] RIA kit was from PerkinElmer Life Sciences, INC. (Boston, USA). Unless other is indicated, the rest of drugs, culture media, antibodies and reagents were from Sigma-Aldrich-Fluka Chemical Co. (St. Louis, MO, USA).

Cell culture

BAEC were isolated from bovine thoracic aortas, using previously described methods (1, 2). Characterization was based on their typical cobblestone appearance and uniform uptake of fluorescent acetylated LDL. Cells were maintained in Rosewell Park Memorial Institute 1640 (RPMI 1640) supplemented with 15 % calf serum, 100 U/mL penicillin and 100 µg/mL streptomycin in an atmosphere of 95% air and 5% CO_2. Experiments were routinely performed on confluent monolayers between passages 2-5, made quiescent by serum deprivation. Cellular toxicity was evaluated in all experimental conditions using the trypan

blue dye exclusion method and by measurement of lactyc dehydrogenase (LDH) activity in the incubation media, without finding any toxicity with all drugs studied.

When BAEC got confluent, were washed and maintained 24 h on serum free-RPMI and then, BAEC were treated with different times and doses of two different NO donors, Diethylamine/nitric oxide complex sodium salt: 2-(N,N-Diethyl-amino)-diazenolate 2-oxide (DEA) and sodium nitroprusside (SNP), as well as an active analogue of cyclic GMP, dibutyril-GMP (dB-GMP), in order to study the effect of NO on ECE-1 regulation.

Coculture of BAEC and RAW Cells.

BAEC were grown in gelatine-coated six-well plates, and RAW macrophages were grown on 24 mm coverslips, until confluence. The coverslips were place upside down over an adequate insert placed in BAEC six-well plates, so that both cells were near each other (< 2mm) but not in contact, sharing the same incubation media (RPMI without serum). Cocultured cells were maintained for 24 h in the presence or not of 0.1 µg/mL lipopolysaccharide (LPS). Supernatants were collected to measure ET-1 production by ELISA (3), and proteins from BAEC were extracted to evaluate ECE-1 expression.

Measurement of cyclic GMP

BAEC were grown to confluence, and then cells were washed twice with PBS in presence of 1 µmol/L IBMX, a phosphodiesterase inhibitor to avoid cyclic GMP degradation. After that cells were treated or not for 30 min with 10 µmol/L ODQ, a soluble guanylate cyclase inhibitor, and then NO donors were added for 15 min at 37ºC. Cells were washed twice with PBS and 300 µL 65 % ethanol was added to extract cGMP for 30 min on ice. Cells were scrapped, after centrifugation at 3,000 rpm for 10 min, supernatants were lyophilized. The dry residues were reconstituted with assay buffer, and cyclic GMP production in each sample was measured by radioimmunoassay assay (4), and then corrected by protein amount.

Transient transfection experiments

To determine whether the effect of NO donors on ECE-1 gene expression was mediated by the 5′-flanking region of the gene, a human ECE-1 promoter/luciferase reporter

gene plasmid was constructed (pGL3-ECE-1) (2, 5). We used the PCR of HeLa cell genomic DNA to create the human ECE-1 gene promoter with the 5′ end at nucleotide -682 and the 3′ end nucleotide +1 using the Advantage Genomic PCR Kit (Clontech Lab., Palo Alto, CA, USA). Whole promoter was subcloned in the Xho I-Hind III site of pGL3 vector (Promega Co., Walkersville, MD, USA), upstream from a luciferase reporter gene. The plasmid was grown in *Escherichia coli* DH-5α and purified with Qiagen Columns (GmbH, Hamburg, Germany).

To determine whether the effect of NO donors on ECE-1 gene expression was mediated by the 3′-flanking region of the gene, a human ECE-1 untranslated region (UTR)/luciferase reporter gene plasmid (pSGG-UTR-ECE-1) and also an empty plasmid as control were constructed (Switch Gear Genomics, CA, USA).

To evaluate the role of PKG on ECE-1 gene expression, plasmids containing Flag-tagged cGMP-dependent protein kinase 1α regulatory region, dominant negative (fcGK-1αR), and Flag-tagged cGMP-dependent protein kinase 1α catalytic region, dominant positive (fcGK-1αC) were used (6, 7), which were kindly donated by Dr. D. Browning (Medical College of Georgia, Augusta). The plasmid fcGK-1αC encodes the catalytic domain of human PKG giving a dominant positive of PKG-1α, whereas the plasmid fcGK-1αR encodes the regulatory domain of PKG giving a dominant negative of PKG-1α. In this case, both plasmids were co-transfected with UTR-ECE/luciferase reporter or with empty plasmid in the presence or not of dB-GMP.

In all cases, BAEC were grown in RPMI 1640 supplemented with 15% serum and antibiotic, and cells were kept in 5% CO_2 and plated approximately 24 h before transfection at a density of 60-80% of confluence in 12-well plates and transfected with each luciferase constructs, by mixing plasmids DNA (0.1 µg/µL of PGL3-ECE-1, pSGG-UTR-ECE-1 or pSGG-empty) or (1 µg/µL of fcGK-1αC or fcGK-1αR) with 1 ng/µL of plasmid control from *Renilla* luciferase (pRL-SV40 vector, Promega Co.) and 4 µg/mL of Lipofectamine into OptiMEM I media. Cells were washed with PBS 24 h after transfection, refed with RPMI and

serum for 24 h, and then dB-GMP was added or not at different times using RPMI without serum. Cells were harvested and assayed for luciferase activity using a Dual-Luciferase Reporter Assay System (Promega Co.). Luciferase activity was expressed as relative light units of each plasmid DNA per relative light units of plasmid control (Renilla) per mg protein of each well.

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ARTÍCULO 3

"Endothelin-converting enzyme-1 increases in atherosclerotic mice: potential role of oxidized low density lipoproteins". Journal Lipid Research 2009; 50(3):364-375.

La aterosclerosis es una patología que resulta, entre otras causas, de un proceso inflamatorio crónico asociado con alteraciones en la estructura y función vascular, así como con la presencia de disfunción endotelial. De los factores de riesgo relacionados con la aterosclerosis, uno de los más importantes es la presencia de niveles plasmáticos elevados de colesterol, y en particular de su componente LDL, que sufre modificaciones por oxidación formando LDL oxidadas (oxLDL). Las oxLDL son retiradas de la circulación al unirse específicamente a sus receptores específicos, LOX-1, presentes tanto en macrófagos como en células endoteliales. Se sabe que la expresión de LOX-1 se activa por citoquinas, fuerzas de rozamiento, productos de glicosilación avanzada e incluso por las propias oxLDL, induciendo apoptosis y disfunción endotelial.

Por otra parte, algunos estudios demuestran que ECE-1 está presente en las placas ateromatosas y que su actividad esta aumentada en pacientes con aterosclerosis. Sin embargo, hay mucha controversia en este sentido, puesto que otros estudios proponen que tanto el contenido como la actividad del enzima disminuyen en pacientes con hipercolesterolémia.

El objetivo de este trabajo fue analizar el papel de ECE-1 en el proceso de aterosclerosis utilizando un modelo animal, analizando los mecanismos involucrados.

En ratones deficientes en apolipoproteína E (ApoE), se observó un aumento en la expresión génica y proteica de ECE-1 en varios tejidos, aorta, pulmón y riñón. No se encontraron diferencias significativas en corazón. Estos ratones mostraron cifras elevadas de colesterol y de presión arterial. La mayor expresión de LOX-1 en aorta y pulmón sugería indirectamente que estos ratones además de tener altos niveles lipídicos, poseían altos niveles de oxLDL, capaz por su parte de estimular la expresión de LOX-1. La inyección puntual de FR-901533, un inhibidor de la actividad de la ECE-1, disminuyó la presión arterial media de los ratones ApoE, sugiriendo que la sobreexpresión de ECE-1 es uno de los factores relacionados con la hipertensión desarrollada en este tipo de ratones.

La sobreexpresión de ECE-1 y LOX-1 en aorta y pulmón de ratones ApoE, apunta indirectamente a las oxLDL como responsables del desarrollo de la placa de

ateroma observada en las aortas de estos ratones, y sugiere un papel de la endotelina en la disfunción endotelial de la arteriosclerosis.

Con el fin de estudiar los mecanismos implicados de la regulación de ECE-1, se realizaron estudios *in vitro* con células endoteliales de aorta bovina (CEAB) incubadas con oxLDL. La expresión proteica y génica de ECE-1 aumentó de forma dosis y tiempo dependiente en CEAB incubadas con oxLDL; sin embargo, las LDL nativas no indujeron ningún cambio significativo. Las oxLDL también indujeron activación del promotor de ECE-1. Estudios utilizando deleciones seriadas del promotor apuntan al factor NF-kappa B como responsable de dicha estimulación, hecho que fue confirmado con ensayos de retardo en gel (*gel shift*). El uso de antioxidantes previno los efectos producidos por oxLDL sobre la actividad del promotor, sugiriendo que la generación de especies reactivas del oxígeno es importante en este proceso.

En resumen, elevados niveles de colesterol LDL, junto con un aumento de especies reactivas del oxígeno, generarían altos niveles de oxLDL, responsables de aumentar la expresión de ECE-1 vía activación NF-kappa B. Este aumento de ECE-1 generaría secundariamente más ET-1, potencialmente capaz de producir hipertensión, agravando el problema de la aterosclerosis.

Endothelin-converting enzyme-1 increases in atherosclerotic mice: potential role of oxidized low density lipoproteins

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Research Unit and Nephrology Section,* Hospital Universitario Príncipe de Asturias, and Physiology and Medicine Departments,** Alcalá University, Madrid; and Centro Nacional de Investigaciones Cardiovascular (CNIC),[†] Madrid, and Hospital Universitario Arnau de Vilanova,[§] Lérida, Spain

Abstract The aim of our study was to analyze the relationships between atherosclerosis and endothelin-converting enzyme-1 (ECE-1). Four-week-old C57BL/6J [wild-type (WT)] and apolipoprotein E-deficient (apoE) mice were fed with a standard or Western-type fat diet for 8 wks. ApoE showed atherosclerotic lesions in the aorta, higher blood pressure and vascular lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) protein content than WT. ApoE showed a significant increase in ECE-1 protein content and mRNA expression in aorta, lung, and kidney, without changes in heart. When an ECE-1 inhibitor, FR-901533, was administered to them, blood pressure decreased in apoE on fat diet versus apoE on normal diet and WT. ECE-1 and LOX-1 protein content were elevated in peripheral blood mononuclear cells (PBMC) from hypercholesterolemic patients. In order to study the mechanism involved in this ECE-1 up-regulation, bovine aortic endothelial cells (BAEC) were treated with oxidized-low density lipoproteins (oxLDL). OxLDL, but not LDL, increased ECE-1 protein content, mRNA expression and promoter activity. Our results demonstrate that ECE-1 increases in different atherosclerosis situations. Up-regulation of ECE-1 could contribute, at least partially, to the development of hypertension seen in apoE mice, because FR-901533 avoided it. Probably, atherosclerotic situations course with an increase of oxLDL, which is able to induce ECE-1 expression with the subsequent potential pathological effects.—Martínez-Miguel, P., V. Raoch, C. Zaragoza, J. M. Valdivielso, M. Rodríguez-Puyol, D. Rodríguez-Puyol, and S. López-Ongil. **Endothelin-converting enzyme-1 increases in atherosclerotic mice: potential role of oxidized low density lipoproteins. *J. Lipid Res.* 2009.** 50: **364–375.**

Supplementary key words hypercholesterolemia • endothelial function • hypertension

Atherosclerosis is a slowly evolutive age-linked disease of large arteries, characterized by local lipid deposition associated with chronic inflammatory response, leading potentially to acute plaque rupture, thrombosis, and ischemic diseases (1, 2). Atherogenesis includes a complex sequence of events, such as over expression of adhesion molecules, recruitment of mononuclear cells to the endothelium, local activation of leukocytes and inflammation, lipid accumulation, and foam cell formation (2–4).

The mechanisms involved in atherogenesis have been extensively studied but incompletely defined. Oxidized low density lipoproteins (oxLDL), generated by the local oxidation of LDL (5), seem to play a role in the development of atherosclerosis, at least in part through the interaction with lectin-like oxidized low density lipoprotein receptor-1 (LOX-1) (6), the activation of the transcription factor NF-κB (7), and the subsequent up-regulation of proinflammatory gene expression (8). Endothelial dysfunction seems to be also an early event in atherosclerosis, and it predisposes to the development of the structural vascular changes (9–11).

Different mediators have been proposed to be involved in the development of the atherosclerosis-linked endothelial dysfunction. Endothelin (ET) has been one of these mediators (12). In fact, increased circulating levels of ET-1 have been detected in patients with hypercholesterolemia (13, 14). Moreover, in apoE mice, an experimental model of hypercholesterolemia and atherosclerosis,

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Abbreviations: apoE, apolipoprotein E-deficient; BAEC, bovine aortic endothelial cells; ET, endothelin; ECE-1, endothelin-converting enzyme-1; LOX-1, lectin-like oxidized low-density lipoprotein receptor-1; oxLDL, oxidized low density lipoproteins; PBMC, peripheral blood mononuclear cells; TTBS, Tween Tris buffered saline; WT, wild-type.

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chronic ET-A receptor blockade normalized endothelial dysfunction and reduced atheroma formation (15, 16).

This increased synthesis of ET-1 may be related to the overexpression of prepro-ET-1 mRNA, as increased steady-state levels of this messenger have been demonstrated in the aorta from hypercholesterolemic mice (15, 16). However, changes in prepro-ET-1 intracellular levels are not the sole mechanism involved in the regulation of vascular ET-1 synthesis. Endothelin-converting enzyme (ECE-1), the enzyme that regulates the conversion from big ET-1 to ET-1, seems to be increased in atherosclerotic plaques (17), and its activity may be enhanced in patients with atherosclerosis (18). However, other studies propose that ECE-1 content and activity are decreased in hypercholesterolemic patients (19).

LDLs have been suggested to play a role in the modulation of the ET-1 synthesis. The oxidized form of these proteins increased the prepro-ET-1 mRNA expression in different cell types, with the subsequent increased ET-1 synthesis (20, 21). Moreover, native and oxLDL seem to stimulate ECE-1 expression in cultured endothelial cells (22), although indirect evidence from human studies suggests an inverse relationship between LDL levels and ECE-1 vascular activity (19).

Considering this information, we planned to analyze the possible role of ECE-1 in the pathogenesis of atherosclerosis, as well as the relationship between oxLDL and ECE-1, in order to clarify the previously published scarce and contradictory information. By a combined in vivo and in vitro approach, we tried to obtain consistent data and perform an analysis of the mechanisms responsible for the observed changes.

MATERIALS AND METHODS

Materials

Culture plates, SuperSignal detection system and secondary horseradish peroxidase-conjugated goat anti-mouse IgG were from Cultek (Pierce, Rockford, IL). Paragon electrophoresis system was from Beckamn Coulter Inc. (Fullerton, CA). Dual Luciferase Reporter Assay System, pGL3 vector, pRL-SV40 vector and T4 polynucleotide kinase were from Innogenetics (Walkersville, MD. Lipofectamine reagent and OptiMEM I media were from GIBCO-Invitrogen (Barcelona, Spain). Acrylamide-bisacrylamide was from Hispanlab-Pronadisa (Madrid, Spain). MXB films were from Kodak (Rochester, NY). Protein markers, BioRad protein assay kit, plates and electrophoresis equipment were from Bio-Rad Laboratories (Richmond, CA, USA). Protease inhibitor cocktail tablets were from Roche Diagnostics (Madrid, Spain). The ET-1 ELISA system, α-[^{32}P]dCTP and γ-[^{32}P]ATP were from GE Healthcare Bio-Sciences (Buckinghamshire, UK). Advantage Genomic PCR Kit was from Clontech Lab (Palo Alto, CA). Unless otherwise indicated, the rest of the drugs, culture media, antibodies, and reagents were from Sigma-Aldrich-Fluka Chemical Co. (St. Louis, MO).

In vivo studies

Male homozygous apoE and C57BL/6J control [wild-type (WT)] mice from 4 wks old were obtained from The Jackson Laboratory (Charles River España, Barcelona, Spain). WT and apoE were fed with a normal or Western type diet (TD88137, Harlan Teklad) to induce atherosclerosis for 8 wks. Animals had free access to water, were maintained at 24°C, and kept at a 12 h light/dark cycle. One week before the sacrifice, arterial blood pressure was measured in conscious animals by means of a tail-cuff sphygmomanometer (LE 5001 Pressure Meter, Letica Scientific Instruments, Hospitalet, Spain). Animals were trained for 3 days before starting the measurement to prevent stress and were prewarmed at 30°C with a heater (LE5660/6, Letica Scientific Instruments). Blood pressure was recorded in 2 consecutive days, with at least 20 determinations by day. In a subgroup of animals, blood pressure was also recorded at 7 wks, before and after the intraperitoneal administration of 1 mg/kg weight of FR-901533, a rather selective ECE antagonist (kindly provided by Dr. Yuriyo Yamamoto, Fujisawa Pharmaceutical Co.). After the 8 wks, animals were anesthetized with pentobarbital (50 mg/kg i.p.), and a blood sample was collected through puncture of the right ventricle. Plasma was separated (3,500 rpm, 10 min) and stored until biochemical determination (Hitachi 917). Plasma lipoproteins (LDL and HDL choslesterol), total cholesterol and triglycerides were determined using a colorimetric-based assay on a Cobas Mira Plus autoanalyzer (Roche Diagnostics, Basel, Switzerland) as described (15). Aorta, lungs, kidneys, and heart were removed via a thoracic-abdominal incision and stored until analysis. Aorta, lung, kidney, and heart portions were collected in 4% paraformaldehyde for histological studies. Because of the scarce tissue it was impossible to assay ECE-1 mRNA expression in aortas. The investigation was conducted in conformity with the Public Health Service policy on the Humane Care and Use of Laboratory Animals incorporated in the Institute for Laboratory Animal Research (ILAR), Guide for the Care and Use of Laboratory Animals published by the US National Academies. All the studies were approved by our Institutional Committee of Alcala University.

Preparations of aorta, lung, kidney, and heart tissues were subjected to immunostaining (23) with anti-ECE-1 antibody (mAb AEC32-236, generous gift from Dr. Kohei Shimada) (24). Antibody-protein complexes were detected with anti-HRP-horseradish secondary antibodies using diaminobenzidine reagent following manufacturer's instructions (Dako Cytometrix, Fort Collins, CO). At least four sections per animal were analyzed for each immunostaining.

Blood samples were taken from six male patients, with ages between 52 and 70 years and hypercholesterolemia (range: 250–320 mg/dl), as well as from six normocholesterolemic males (range: 172–215 mg/dl) between 49 and 71 years of age. Everyone gave their informed consent to make the protocol approved by Institutional Committee from our hospital, in accordance with the Principles outlined in the Declaration of Helsinki (*Cardiovascular Research* 1997; 35: 2–4). Peripheral blood mononuclear cells (PBMC) were isolated from blood samples with Ficoll solution (Comercial Rafer, Madrid, Spain), in order to extract total proteins, as described below.

Cell culture

Bovine aortic endothelial cells (BAEC) were isolated from bovine thoracic aortas using previously described methods (25). Characterization was based on their typical cobblestone appearance and uniform uptake of fluorescent acetylated LDL. Cells were maintained in RPMI 1,640 supplemented with 15% calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in an atmosphere of 95% air and 5% CO_2. Experiments were routinely performed on confluent monolayers at passages 2–5, made quiescent by serum deprivation. Cellular toxicity was evaluated in

all experimental conditions by the trypan blue dye exclusion method and by measurement of lactic dehydrogenase (LDH) activity in the incubation media. No significant toxicity was detected.

Human endothelial cell line, EA.hy926 (EA) were from Dr. Cora-Jean S. Edgell (Yale University School of Medicine, New Haven, CT), and they were grown in DMEM with high glucose supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin in an atmosphere of 95% air and 5% CO_2. Mouse aortic endothelial cells (MAEC) were isolated from WT animals by Dr. Carlos Zaragoza (CNIC, Madrid, Spain), and they were grown in DMEM-F12 supplemented with 20% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin and endothelial growth factor.

Oxidation of LDL

Human LDL (d = 1.019–1.063) was isolated from fresh plasma of healthy humans by sequential ultracentrifugation at 4°C as described (26). Oxidative modification of LDL was performed by incubation with 25 μM $CuSO_4$ in PBS for 24 h at room temperature (27). Protein concentrations of lipoprotein preparations were determined using the BioRad protein assay kit. OxLDL was assessed by electrophoretic mobility under nondenaturing conditions using a Paragon electrophoresis system, showing a single band with a 2-fold faster migration rate than native LDL. A final concentration between 50–200 μg protein/ml of native LDL or oxLDL was used in cells.

Western blot assays

Proteins were obtained from tissue, human PBMC, and BAEC, by using the Lysis Buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 0.1% sodium deoxycholate, 1% Triton X-100, 10 mM sodium pyrophosphate) containing a protease inhibitor cocktail. Protein concentration was determined with BioRad protein assay kit. Proteins were separated on SDS-PAGE 6% (30 μg protein/lane), and transferred onto nitrocellulose membranes. Membranes were blocked with 5% (w/v) nonfat dry milk in Tween Tris buffered saline (TTBS) (20 mM Tris-HCl pH 7.5, 0.9% NaCl, 0.05% Tween 20) at room temperature, and then incubated for 90 min with 10 μg/ml of the monoclonal anti-ECE-1 antibody (mAb AEC32-236) or with 1:1,000 dilution of the monoclonal anti-LOX-1 antibody (mAb anti-LOX-1 #5-2 from Dr. Tatsuya Sawamura). After washing in TTBS, blots were incubated with 200-fold-diluted horseradish peroxidase-conjugated goat anti-mouse IgG. The immunoreactive bands were visualized with the SuperSignal detection system after 30 s of exposure to MXB film. Then blots were reblotted with a monoclonal anti-β-tubulin antibody for samples from mice tissues and BAEC or with a rabbit anti-actin antibody for human PBMC in order to normalize ECE-1 and LOX-1 levels.

Northern blot analysis

Total cellular RNA was isolated from tissues or BAEC with the guanidinium thiocyanate-phenol-chloroform method (28). For Northern analysis, blots of RNA were hybridized with α-[^{32}P]dCTP labeled specific ECE-1 mice or bovine probes (29, 30) in hybridization solution (50% Formamide, 5× Denhardt's solution, 5× SSPE, 0.5% SDS, and 100 μg herring sperm DNA) at 42°C. The filters were stripped by boiling in 0.1% SDS solution and reprobed with a ^{32}P-labeled 18 S cDNA (5.8 kb fragment digested by *Eco*RI). The densitometric analysis of the films was performed with an image scanner using the public domain software package National Institutes of Health Image 1.55 (Bethesda, MD). Levels of ECE-1 were normalized by using 18 S expressions within the same lane, and expressed in relative densitometric units with respect to control values.

Measurement of ET-1 levels and ECE-1 activity

Supernatants and membrane proteins from BAEC treated with LDL or oxLDL were collected as described (31, 32) in order to measure ET-1 production and ECE-1 activity, respectively, using an enzyme-linked immunosorbent assay (ELISA). The membrane proteins from BAEC treated for 24 h with 100 μg/ml OxLDL or LDL were isolated, and then 30 μg were incubated with a fixed amount of big ET-1 (100 ng) for 4 h at 37°C in the presence or not of 100 μM phosphoramidon. In order to generate a standard curve for ET-1 serial dilutions of ET-1 ranging from 1–16 fmol per well were used. A cubic-spline curve was fit to the standards and unknown values interpolated from the standard curves automatically. The cross-reactivity of the ELISA ET-1 antibody, as determined by the concentration giving 50% B/Bmax, was: ET-1 (100), ET-2 (100), ET-3 (<0.001), big-ET-1 human (<0.07), and atrial natriuretic peptide (<0.0006).

Transient transfection experiments

To determine whether the effect of oxLDL on ECE-1 gene expression was mediated by the 5′-flanking region of the gene, a human ECE-1 promoter/luciferase reporter gene plasmid was constructed (pGL3-ECE-1) (33). We used the PCR of HeLa cell genomic DNA to create serial deletion fragments of the human ECE-1 gene promoter with the 5′ ends at nucleotides −650 (AP-1), −596 (NF-κB), −542 (Acute phase), −483 (CAAT box), −444 (Shear stress), −328 (STAT), and −216 (Glucocorticoid receptor element), and the 3′ end nucleotide +1 using the Advantage Genomic PCR Kit. Fragments were subcloned in the Xho I-Hind III site of pGL3 vector, upstream from a luciferase reporter gene.

BAEC were grown at 60–80% of confluence in 12-well plates and transfected with promoter/luciferase constructs, by mixing 0.1 μg/μL of pGL3-ECE-1 with 1 ng/μL of plasmid control from *Renilla* luciferase (pRL-SV40 vector) and 4 μg/ml of Lipofectamine into OptiMEM I media. After 24 h of transfection, cells were refed with complete RPMI 1,640 for at least 16 h, and then native LDL or oxLDL was added at different doses and times using RPMI without serum. Luciferase activity was assessed using a Dual Luciferase Reporter Assay System, and expressed as relative light units of pGL3-ECE-1/*Renilla*/mg protein of each well.

Electrophoretic mobility shift assays

BAEC were incubated with oxLDL at different times and electrophoretic mobility shift assays was displayed to check on the activation of NF-κB, as previously described (34). Oligonucleotide sequences were based on the putative NF-κB binding element in the ECE-1 promoter (from nucleotides −617 to −591) as follow: *sense* 5′-GGC TGG AGG GAT TTT TCC TCC TTT CA-3′ and *antisense* 5′-TGA AAG GAG GAA AAA TCC CTC CAG CC-3′ (35). Oligonucleotides were labeled with γ-[^{32}P]ATP at the 5′ end with T4 polynucleotide kinase and then incubated with nuclear extracts. Protein-DNA complexes were separated in a 6% nondenaturing polyacrylamide gel in 0.25 × Tris buffer EDTA. The gels were dried under vacuum and exposed to X-ray film. For competition experiments, 125-fold molar excess of competitor DNA (AP-1 oligonucleotide, NF-κB oligonucleotide) was coincubated with the labeled oligonucleotide probe (NF-κB). Sequences of the oligonucleotides for AP-1 were: *sense* 5′-CAT GGC TGT GTC ACC CTT GTC CC-3′ and *antisense* 5′-GGG ACA AGG GTG ACA CAG CCA TG-3′.

Statistical analysis

Data are expressed as means ± SEM. Animal studies were analyzed by ANOVA, followed by the Scheffe multiple comparison test after confirming the normality of the distribution of data. Human studies were analyzed with the Mann-Whitney test. The in vitro studies include at least three separate experiments and are usually expressed as a percentage of the control values. Because the number of data in these experiments was never over 10, nonparametric statistics, particularly the Wilcoxon (two groups) or Friedman (more than two groups) tests, were selected to compare the paired results from the different experimental groups. The level of statistically significance was defined as $P < 0.05$.

RESULTS

ApoE mice and hypercholesterolemic patients show an increased expression of ECE-1

We used apoE mice, an animal model that resembles human atherosclerosis, to investigate the relationships between ECE-1 and vascular disease. ApoE mice developed atherosclerotic lesions in their aortas and had higher levels of cholesterol and lipids than WT animals (**Fig. 1A**). ApoE mice fed with the fat diet showed higher values of blood pressure than WT and apoE animals on the standard diet (Fig. 1A). LOX-1 protein content was increased in the aorta of apoE mice, a change that was also magnified by the fat diet (Fig. 1B).

In apoE mice, we found an increased ECE-1 protein content in aorta, lungs, and kidneys, with respect to their wild-type counterparts, as detected by immunohistochemistry (**Fig. 2**, left part) and immunoblot (Fig. 2, right part). However, no significant differences were found in the heart (Fig. 2). The fat diet induced a slight but significant increase of ECE-1 protein content in aorta and lungs from apoE animals (Fig. 2, right part). The analysis of ECE-1 mRNA in these animals revealed an increased expression in the same organs as above, without significant differences in heart tissue (**Fig. 3**). When FR-901533, an ECE-1 inhibitor (36), was administered to mice via intraperitoneal at 1 mg/kg, blood pressure was reduced significantly in apoE mice on the fat diet versus WT mice and apoE on the normal diet (**Fig. 4A**).

A

	WT		ApoE	
Fat diet	-	+	-	+
Total Cholesterol (mmol/L)	3.8 ± 0.3	7.6 ± 0.3*	16.5 ± 1.4**	18.9 ± 1.6*,**
LDL Cholesterol (mmol/L)	0.52 ±0.17	3.1 ± 0.21*	15.1 ± 1.2**	18.2 ± 0.6*,**
HDL Cholesterol (mmol/L)	2.9 ± 0.19	4.77 ± 0.24*	0.4 ± 0.1**	0.3 ± 0.03**
Triglycerides (mmol/L)	0.65 ± 0.07	0.59 ± 0.03	2.2 ± 0.54**	1.5 ± 0.14**
Systolic blood pressure (mmHg)	113 ± 3	112 ± 1	111 ± 4	136 ± 1*,**
Diastolic blood pressure (mmHg)	84 ± 4	78 ± 1	76 ± 6	102 ± 1 *,**
Body weight (g)	27.1 ± 0.3	28.3 ± 1.4	29.0± 0.8	29.6 ± 2.0

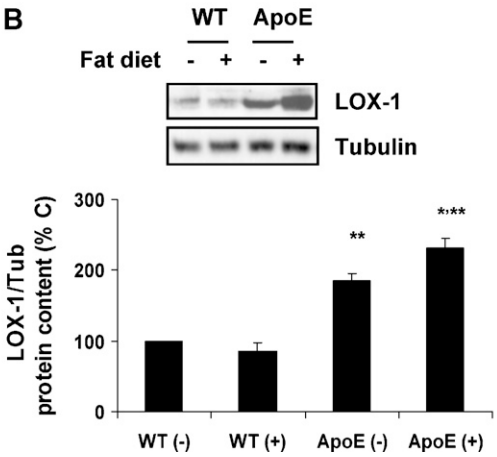


Fig. 1. Characteristics of wild-type (WT) and apolipoprotein E-deficient mice (apoE), on a normal (−) or fat diet (+). A: Body weight, lipids levels, and blood pressure in 12 animals per group. B: Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) content, assessed by Western blot, in the aorta. The upper part shows a representative experiment, whereas the lower part shows the mean ± SEM of the densitometric analysis of 12 animals per group (values are expressed as the percentage of WT animals on a normal diet). * $P < 0.05$ vs. animals of the same strain with a normal diet, ** $P < 0.05$ vs. WT mice on the same diet.

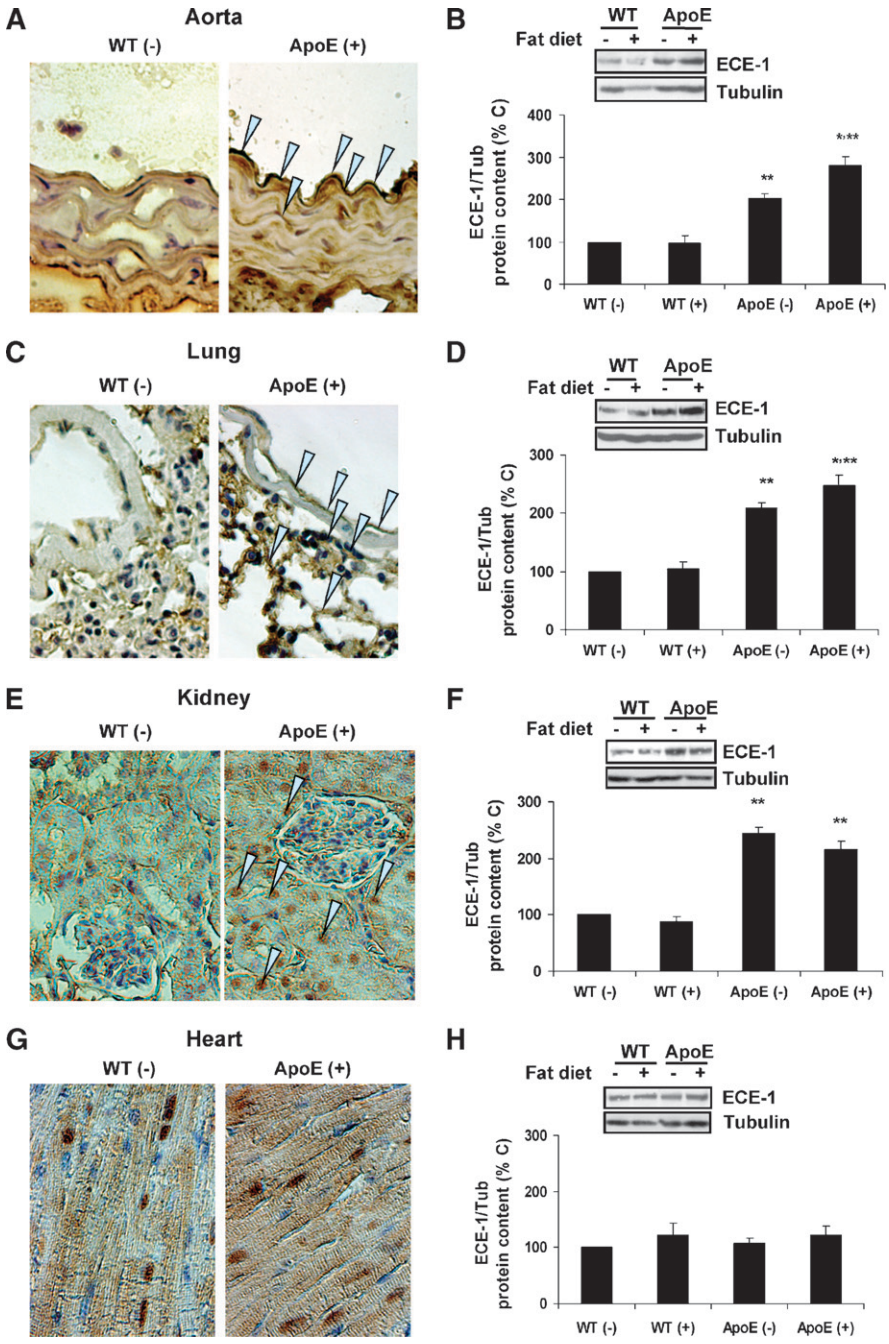


Fig. 2. Changes in endothelin-converting enzyme-1 (ECE-1) protein content in the aorta (A and B), lung (C and D), kidney cortex (E and F), and heart (G and H) from WT and apoE-deficient mice, on a normal (−) or fat diet (+). On the left part of the figure, photomicrographs of the immunostaining for ECE-1 in the different tissues (arrows point to positive ECE-1 immunostaining) are shown. On the right, their corresponding immunoblots are shown. At the top of each one, a representative Western blot is shown and at the bottom the mean ± SEM of the densitometric analysis of 12 animals per group (values are expressed as the percentage of WT animals on a normal diet) is provided. * $P < 0.05$ vs. animals of the same strain with a normal diet, ** $P < 0.05$ vs. WT mice on the same diet.

To evaluate these animal findings in human beings, we isolated PBMC from clinically diagnosed hypercholesterolemic patients and healthy people, and then, ECE-1 and LOX-1 protein content was measured. Both proteins significantly increased in hypercholesterolemic patients (Fig. 4B).

OxLDL up-regulates ECE-1 in cultured BAEC

To analyze the potential mechanisms involved in the up-regulation of ECE-1 found in hypercholesterolemic animals and patients, we used BAEC treated with LDL or oxLDL. ECE-1 protein content increased in BAEC incubated with oxLDL at different doses and times (**Fig. 5**).

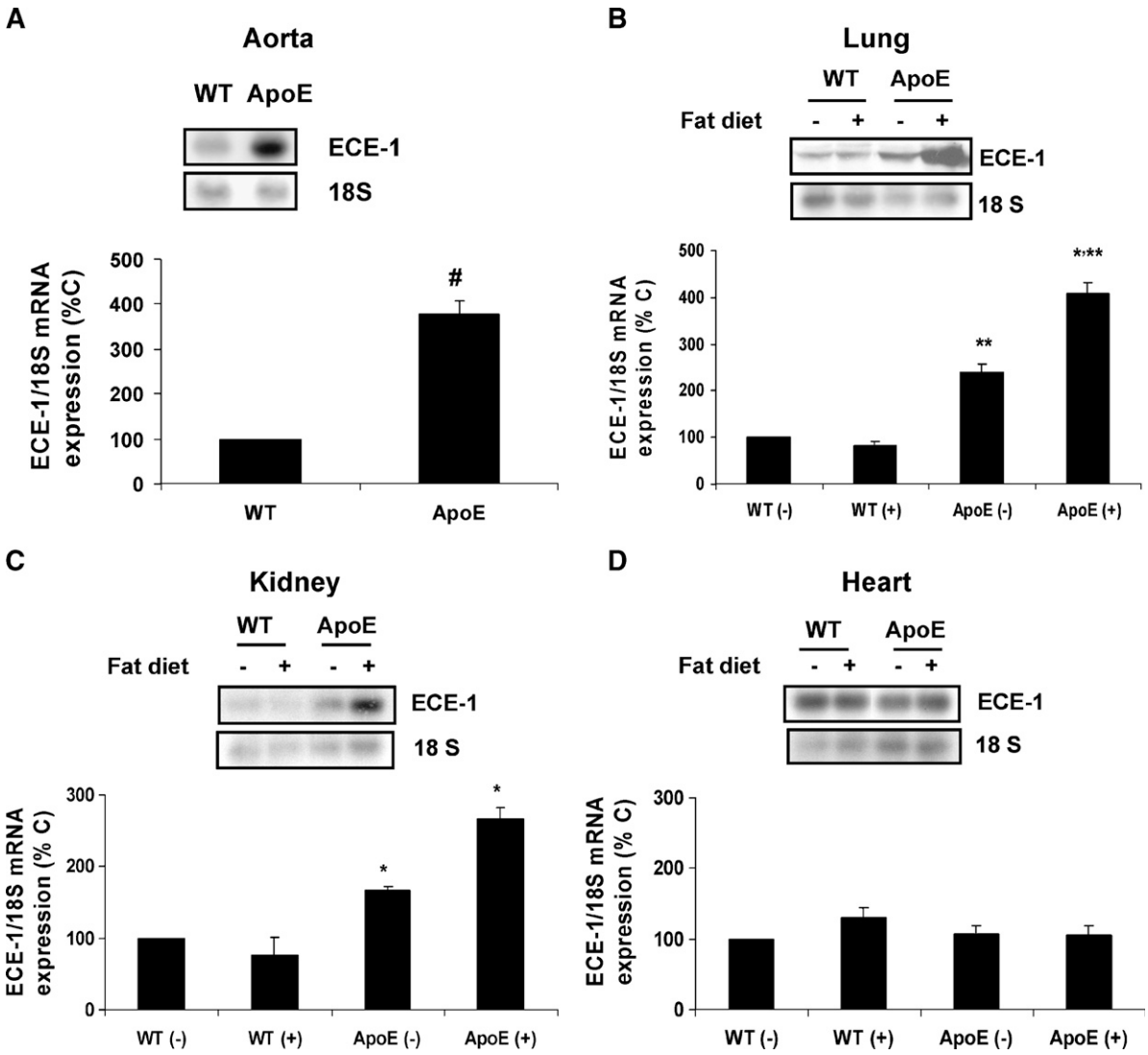


Fig. 3. Changes in ECE-1 mRNA expression in aorta (A), lung (B), kidney cortex (C), and heart (D) from wild-type (WT) and apoE-deficient mice, on a normal (−) or fat diet (+). The upper part of each panel shows a representative Northern blot experiment, whereas the lower part shows the mean ± SEM of the densitometric analysis of 12 animals per group (values are expressed as the percentage of WT animals on a normal diet). $^{\#}$ $P < 0.05$ vs. WT mice, * $P < 0.05$ vs. animals of the same strain with a normal diet, ** $P < 0.05$ vs. WT mice on the same diet.

The stimulatory effect started after 6 h of incubation and remained for 24 h (Fig. 5A). It was maximal at 100 μg/ml oxLDL (Fig. 5B). This same concentration of oxLDL significantly increased ECE-1 activity and ET-1 synthesis in cells (Fig. 5C). No significant changes were found with LDL (Fig. 5).

OxLDL regulates ECE-1 expression in endothelial cells through activation of NF-κB

To clarify the mechanisms involved in the oxLDL-dependent ECE-1 up-regulation, we tested the ability of oxLDL to modulate ECE-1 transcription. A significant increase of ECE-1 mRNA steady-state levels was detected in cells incubated for 8 h with 100 μg/ml oxLDL, an effect that was not observed with LDL (**Fig. 6A**). ECE-1 promoter activity was stimulated by oxLDL in a time- (Fig. 6B) and dose-dependent manner (Fig. 6C), reaching its maximum after 6 h of incubation with 100 μg/ml oxLDL. LDL incubation, by contrast, did not modify the promoter activity. On the other hand, incubation of transfected cells with the antioxidants catalase and N-acetyl cysteine prevented the stimulatory effect of oxLDL on ECE-1 promoter activity (Fig. 6D). To relate our in vitro results in BAEC to those obtained in animals and human beings, we transfected ECE-1 promoter also in human endothelial cells (EA) and in mouse aortic endothelial cells (MAEC) from WT animals. We found a significant increase in promoter activity after 6 h of incubation with 75 μg/ml oxLDL in both types of cells (Fig. 6E); in contrast LDL did not induce any change. To investigate the signaling that leads to ECE-1 promoter stimulation, serial deletions of the ECE-1 regulatory region were evaluated in a luciferase assay of

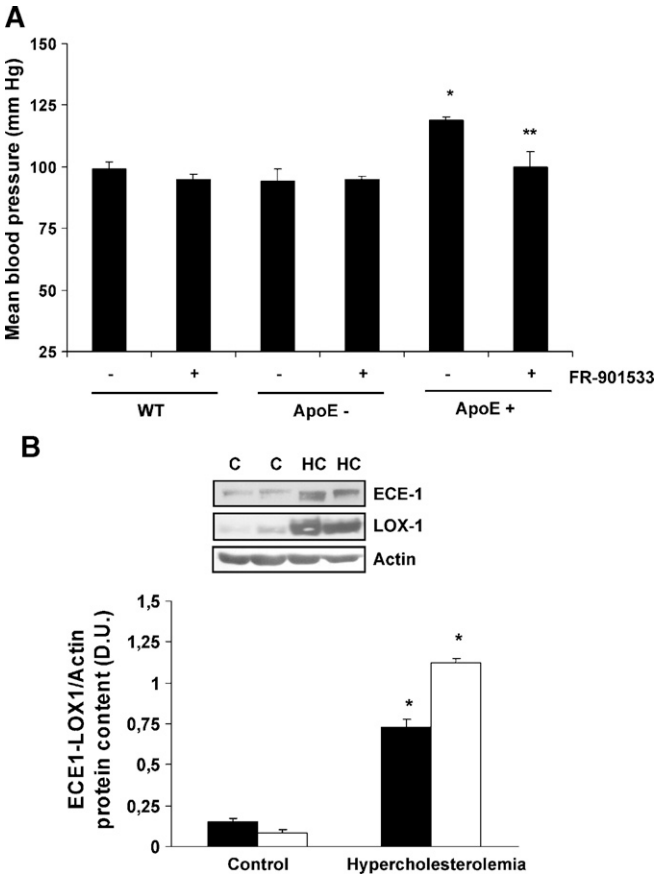


Fig. 4. A: Changes in the mean blood pressure of mice treated with the ECE-1 inhibitor FR-901533. The mean blood pressure was registered in three groups of animals, wild-type fed with a normal diet (WT), apoE-deficient fed with a normal diet (apoE−), and apoE fed with a fat diet (apoE +), in basal conditions (−) and after the intraperitoneal administration of 1 mg/kg FR-901533 (+). * $P < 0.05$ vs. other groups, ** $P < 0.05$ vs. basal apoE +. B: Changes in ECE-1 and LOX-1 protein content in peripheral blood mononuclear cells (PBMC) from normo- and hypercholesterolemic patients. The upper panel of the figure shows the representative Western blots, whereas the lower panel shows the densitometric analysis of six patients per group. Closed bars: ECE-1 values: Open bars: LOX-1 values. * $P < 0.05$ vs. control (normocholesterolemic people). Error bars represent SEM.

transiently-transfected BAEC. When the NF-κB responsive element was deleted (**Fig. 7A**, upper panel), the stimulatory effect of oxLDL fell down near control levels (Fig. 7A, lower panel). The involvement of NF-κB on ECE-1 transcriptional regulation was further confirmed by electrophoretic mobility shift assays, showing an increase of nuclear NF-κB binding after 30 min of incubation with oxLDL (Fig. 7B).

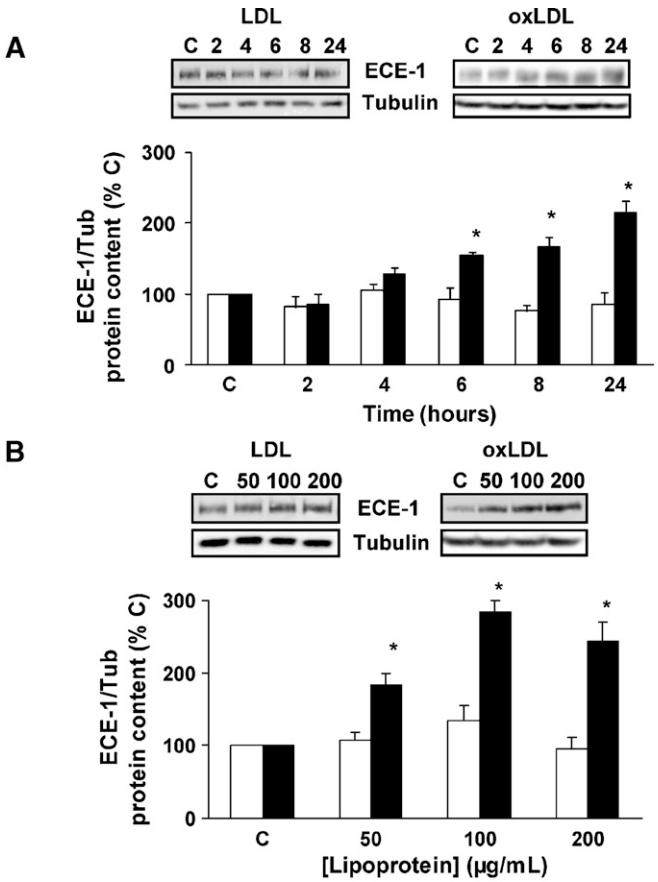
DISCUSSION

Present results demonstrate that apoE-deficient mice exhibit an increased content of ECE-1 in aorta, lungs, and kidneys, without changes in heart tissue. The changes observed seem to be the consequence of an increased gene expression, as steady-state ECE-1 mRNA levels in these tissues paralleled the protein content. These results confirm previous descriptions in human beings (17–19), suggesting a role for ECE-1 overproduction in the genesis of the endothelial dysfunction in atherosclerosis. Mitani et al. (37) also reported that rabbits with high levels of cholesterol showed higher levels of ECE-1. Additionally, our results provide the first demonstration of the tissue-specificity of the changes in ECE-1 over expression, where the most striking finding was the lack of changes in ECE-1 expression on heart tissue.

The mechanisms involved in the ECE-1 up-regulation in these animals have been indirectly explored in the present work. The relevance of hypercholesterolemia in this experimental model, the fact that the fat diet increased ECE-1 content in some tissues even more, and the general acceptance of the pathogenic role of LDL, particularly in its oxidized form, in atherosclerosis (38, 39), point to oxLDL as the possible intrinsic factor that stimulate ECE-1 expression. In spite of the fact that we did not measure plasma oxLDL levels in our mice, we found significant changes in LOX-1 protein content in the aorta of apoE mice. As LOX-1 expression can be stimulated by oxLDL (39–42), this finding could suggest that hypercholesterolemic animals could have increased levels of oxLDL.

Oxidized LDL induced an increased expression of the ECE-1 mRNA in cultured endothelial cells, with the subsequent increase in protein content. Protein changes were paralleled by an increase in the enzyme activity and the ET-1 synthesis. Previous work from Niemann et al. (22)reported a similar oxLDL-dependent stimulation of ECE-1 synthesis, but in that work LDL also increased the cellular content of ECE-1. Moreover, Ruschitzka et al. (19) proposed an inverse relationship between serum LDL levels and vascular ECE-1 activity; however, in our hands, LDL did not modify the synthesis of ECE-1 by cultured cells. OxLDL up-regulated the cellular content of ECE-1 in a concentration range between 50–200 μg/ml. These concentrations are similar to those previously measured in the plasma of hypercholesterolemic patients (43).

The mechanism responsible for the oxLDL-dependent ECE-1 up-regulation has been also partially explored in the present work in BAEC. The changes in the ECE-1 mRNA steady-state levels observed in endothelial cells seem to be the consequence, at least partially, of an increased activity of the ECE-1 promoter. Regulation of ECE-1 promoter by oxLDL was also confirmed in human and mouse endothelial cells, suggesting that our results are not specific of BAEC. These results demonstrate the relevance of oxLDL in the ECE-1 up-regulation not only in different species of cells but also in hypercholesterolemic mice. The analysis of the promoter activity after serial deletions and gel-shift assays support the relevance of NF-κB in this ECE-1 promoter-increased activity. The fact that antioxidants prevented the oxLDL-dependent changes in promoter activity also supports a role for reactive oxygen species in the genesis of the observed effects. Reactive oxygen species and NF-κB seem also to play a role



C

	Control	oxLDL	LDL	Phosphoramidon
ECE-1 activity (fmol ET-1 per well)	7.33 ± 0.86	12.67 ± 0.95*	8.06 ± 1.05	1.46 ± 0.32*
ET synthesis (fmol ET-1 per well)	640 ± 37	1055 ± 42*	698 ± 47	-

Fig 5. Effect of native (LDL) and oxidized (oxLDL) low density lipoproteins on ECE-1 protein content, ECE-1 activity, and endothelin (ET) synthesis. A, B: Bovine aortic endothelial cells (BAEC) were incubated with 100 μg/ml LDL (open bars) or oxLDL (closed bars) at different times (A) or at different concentrations for 24 h (B), and ECE-1 protein content was measured by Western blot. In the upper part of each panel a representative Western-blot is shown, whereas in the lower part the densitometric analysis of five independent experiments is given (mean ± SEM). Values are expressed as the percentage of control cells (C). * $P <$ 0.05 vs. C and LDL. C: ECE-1 activity in membrane proteins and ET concentration in supernatants from BAEC treated with 100 μg/ml LDL or oxLDL for 24 h were measured by ELISA. Values are expressed as fmol of ET-1 per well, and are the mean ± SEM of four independent experiments in triplicate. * $P <$ 0.05 vs. other groups. In the ECE-1 activity assay, phosphoramidon (100 μM) was used as negative control (ECE-1 activity inhibition: 80 ± 7%).

in the regulation of cell function by oxLDL in other pathophysiological conditions (7, 44).

The relevance of the changes detected in ECE-1 tissue content in the development of the alterations detected in apoE mice must also be discussed. If ECE-1 over expression were involved in the genesis of the morphological and functional changes observed in these mice, the blockade of the enzyme would improve these pathological changes. Previous experimental work demonstrated that the chronic blockade of the ET-A receptor prevented the vascular damage that characterizes this experimental model (15, 16). In our hands, acute blockade of ECE-1 with FR-901533 decreased blood pressure in apoE-deficient mice fed with a fat diet, but did not modify blood pressure in WT and apoE animals on a normal diet, suggesting that ECE-1 overexpression is only one of the factors involved in the high blood pressure maintenance in apoE mice.

Studies in experimental models and cultured cells must be considered critically when analyzing pathophysiological mechanisms in human beings. To perform a preliminary

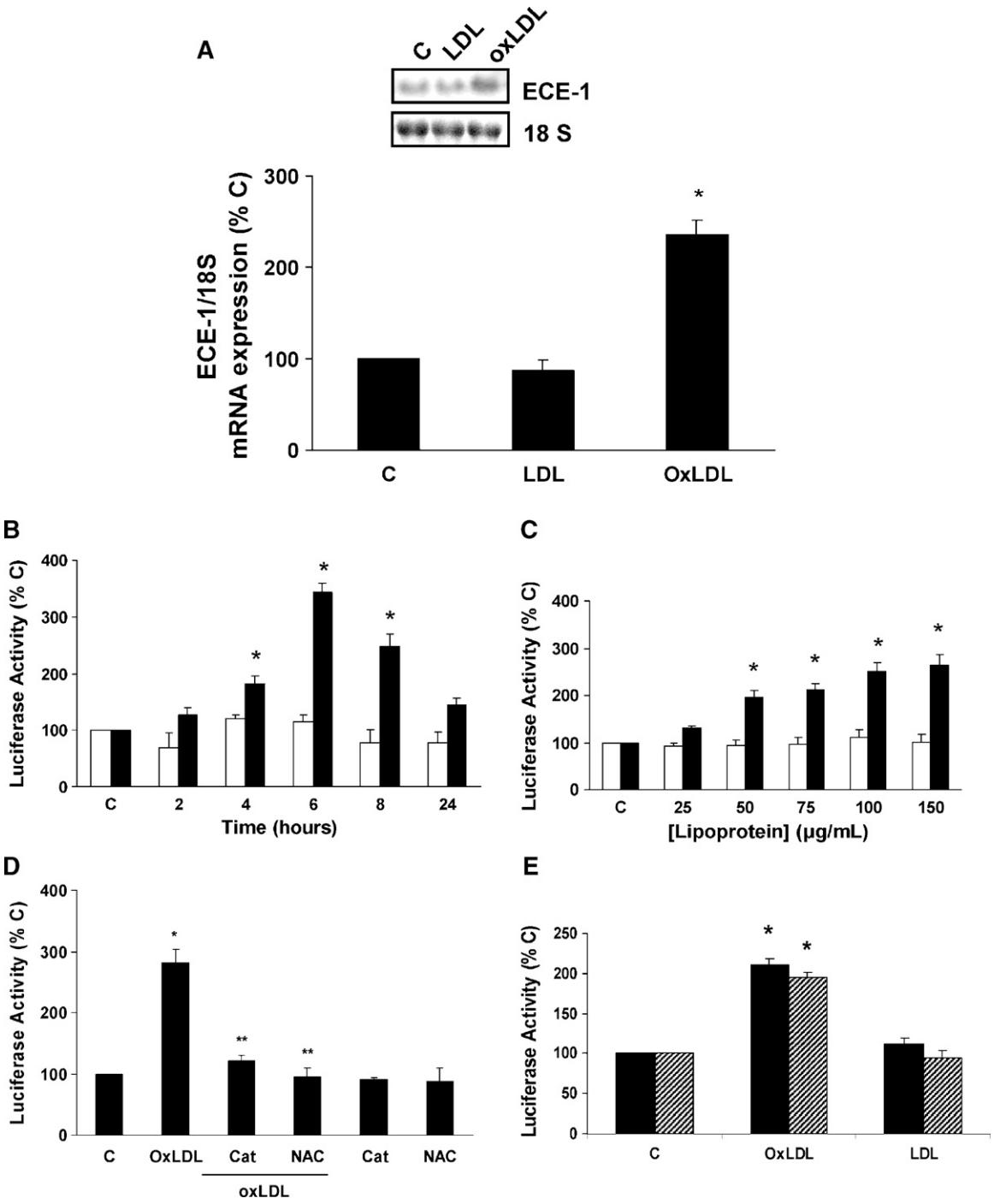


Fig. 6. Effect of native (LDL) and oxidized (oxLDL) low density lipoproteins on ECE-1 mRNA steady-state levels, and activity of ECE-1 promoter. A: BAEC were incubated with 100 μg/ml LDL or oxLDL for 8 h, and ECE-1 mRNA was measured by Northern blot. In the upper part of the panel, a representative Northern-blot is shown, whereas in the lower part the densitometric analysis of three independent experiments is given (mean ± SEM). Values are expressed as the percentage of control cells (C), * $P < 0.05$ vs. C and LDL. B, C: BAEC were transfected with an ECE-1 promoter/luciferase reporter gene plasmid. Transfected BAEC were then incubated with 100 μg/ml LDL (open bars) or oxLDL (closed bars) at different times (B) or concentrations for 6 h (C), and the ECE-1 promoter activity was measured. Values are expressed as the percentage of control cells (C), and are the mean ± SEM of three independent experiments in triplicate. * $P < 0.05$ vs. C and LDL. D: BAEC were transfected as above and then incubated with 100 μg/ml oxLDL for 6 h, in the presence or not of 80 U/ml catalase (Cat) or 100 μM N-acetyl cysteine (NAC), and then assayed for luciferase activity. Results are the mean ± SEM of four independent experiments in triplicate, and are expressed as the percentage of control cells (C). * $P < 0.05$ vs. the other groups, ** $P < 0.05$ vs. oxLDL. E: EA (closed bars) and MAEC (striped bars) were transfected with an ECE-1 promoter/luciferase reporter gene plasmid, incubated with 75 μg/ml oxLDL or LDL for 6 h and then assayed for luciferase activity. Results are the mean ± SEM of three independent experiments in triplicate, and are expressed as the percentage of control cells (C). * $P < 0.05$ vs. C and LDL. In the transfection experiments, phorbol myristate acetate (3×10^{-7} M, 6 h) was used as positive control (stimulation: 283 ± 18%).

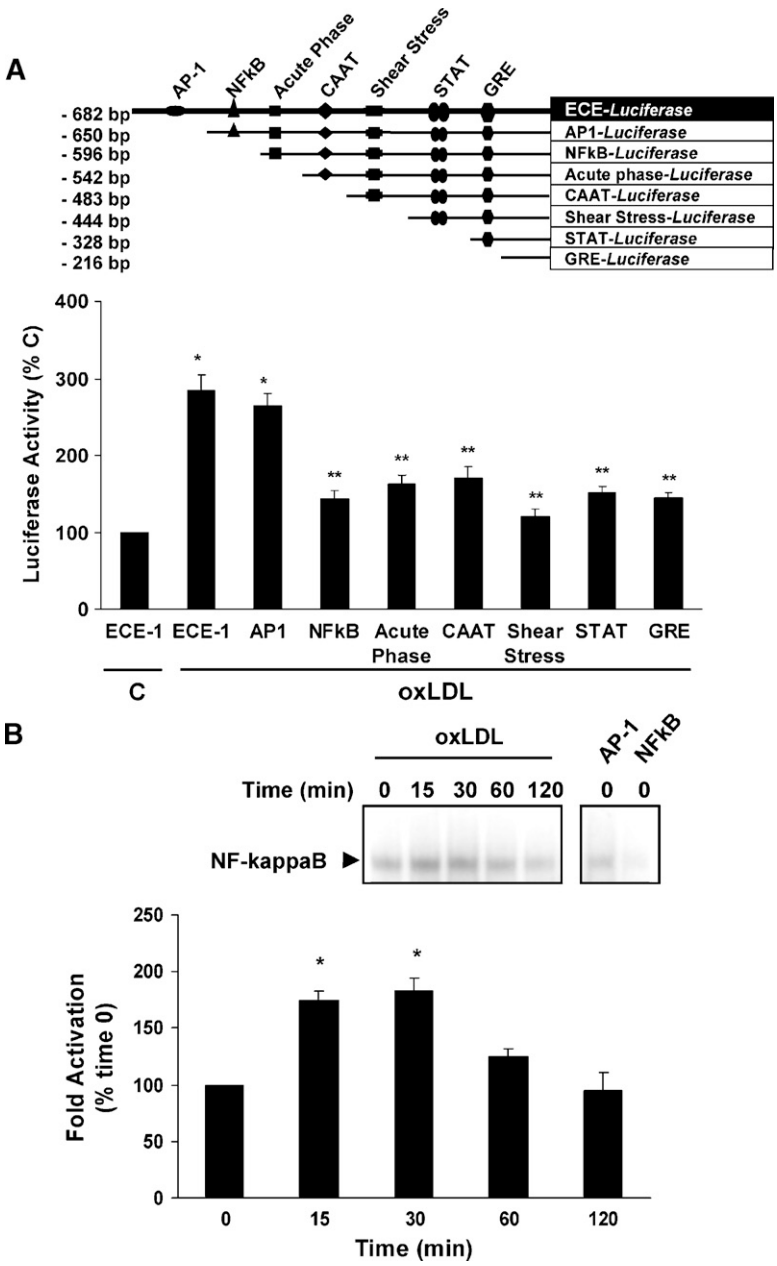


Fig. 7. Effect of oxLDL on transactivation of ECE-1 promoter deletions and on the binding of NFkB to nuclear extracts. A: BAEC were transfected with plasmids containing serial deletion fragments of ECE-1 promoter linked to luciferase, treated with 100 μg/ml oxLDL for 6 h, and assayed for luciferase activity. Top: Schematic of ECE-1 5′ flanking region deletions, showing some of the putative response elements in these regions: −650 (AP-1), −596 (NF-κB), −542 (Acute phase), −483 (CAAT box), −444 (Shear stress), −328 (STAT), and −216 [glucocorticoid receptor element (GRE)]. Bottom: The quantitative results are the mean ± SEM of four different experiments in triplicate, and are expressed as the percentage of induction with respect to control cells (C). * $P < 0.05$ vs. control (100%), ** $P < 0.05$ vs. ECE-1 and AP-1. Phorbol myristate acetate (3×10^{-7} M, 6 h) was used as positive control (stimulation: 258 ± 13%). B: Nuclear extracts were harvested from BAEC previously incubated with 100 μg/ml oxLDL for different times, and then incubated with the labeled oligonucleotide containing the NFkB consensus sequence. Top left: Effect of oxLDL on NFkB binding activity of BAEC nuclear extracts. Top right: Competition assay using unlabeled AP-1 and NFkB oligonucleotides. Bottom: Densitometric analysis of gel-shift experiments (mean ± SEM of four different experiments and are expressed as the percentage of time 0, with * $P < 0.05$ vs. time 0).

approach to the analysis of the relevance of the association between hypercholesterolemia and increased ECE-1 activity in humans, we hypothesized that ECE-1 could be expressed in PBMC, and we found a link between hypercholesterolemia and the increased content of ECE-1 in these cells. LOX-1 protein content also increased in PBMC of hypercholesterolemic patients, as it did in aortas of apoE-deficient mice. These findings point to the association between hypercholesterolemia and increased ECE-1 activity, suggesting that PBMC could be used as a valuable system to explore the changes in the enzyme in pathological conditions.

In summary, present results demonstrated a direct relationship between LDL and ECE-1 content, in contrast with Ruschitzka's results, and provide some information about the mechanisms involved in this relationship. ECE-1 expression increased in aorta, lungs, and kidneys of apoE mice, suggesting that ECE-1 could play a role in the hypertension found in some of these animals. Increased oxLDL levels may be proposed as one of the factors responsible for the ECE-1 up-regulation, throughout an increased reactive oxygen species synthesis, and NF-κB activation, with the subsequent stimulation of ECE-1 promoter activity and increased mRNA steady-state levels of ECE-1. Thus, ECE-1 could be proposed as a therapeutic target in atherosclerosis prevention.

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RESUMEN DE RESULTADOS

1. Los inhibidores de la actividad de ECE-1 aumentan la expresión proteica y génica de ECE-1 en células endoteliales bovinas, por una activación transcripcional inducida por la big-ET-1, que se acumula al estar inhibida la actividad de ECE-1.

2. Los donadores de NO, a través de una activación de la vía GCs-GMPc-PKG, inducen una reducción de la expresión de ECE-1, debido a una desestabilización del ARNm, en la que se encuentra implicada la región no traducida del mensajero.

3. Esta modulación de ECE-1 dependiente de NO se observa también en cultivos celulares complejos y en animales de experimentación.

4. Los niveles proteicos y génicos de ECE-1 están aumentados en aorta, pulmón y riñón de ratones ApoE deficientes respecto a sus controles. Estos ratones presentan también un aumento en el contenido tisular de LOX-1, probablemente secundario al incremento en LDL oxidadas, y son hipertensos.

5. ECE-1 es uno de los factores implicados en la hipertensión que presentan los ratones ApoE deficientes.

6. En células endoteliales, las oxLDL, pero no las LDL nativas, inducen un aumento de la expresión de ECE-1, al estimular la actividad de su promotor a través de la activación del factor NF-kappa B.

8. CONCLUSIONES FINALES

- La compleja regulación de ECE-1, en la que se encuentran implicados metabolitos de la cascada de síntesis de la propia endotelina y otros autacoides endoteliales, apoya la relevancia fisiológica de la enzima en la modulación de la función vascular.

- Existe una compleja interrelación entre los distintos metabolitos endoteliales bioactivos, de forma que no sólo condicionan respuestas celulares distintas destinadas a mantener la homeostasis vascular en situaciones diversas, sino que interaccionan entre ellos a nivel de su propia síntesis.

- ECE-1 puede jugar un papel patogénico en la generación de las alteraciones estructurales y funcionales de la arteriosclerosis, y puede constituir una diana terapéutica valiosa en el control de este proceso.

- La farmacología de distintos fármacos vasoactivos, como los donadores de NO o los propios antagonistas de ECE-1, debe ser interpretada no sólo en función de los efectos primarios atribuibles a los mismos sino también en el contexto de las respuestas compensatorias que se producen en los propios lechos vasculares.