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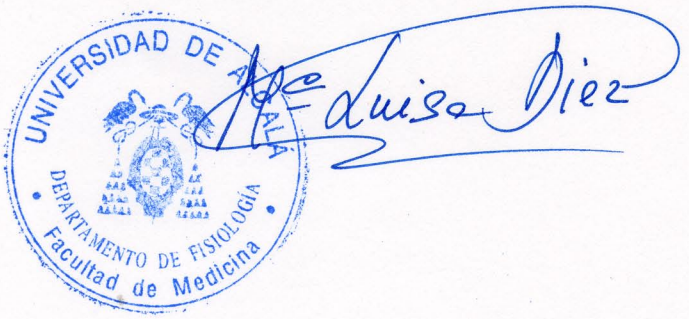
CERTIFICA

Que la Tesis Doctoral, que lleva por título "***Mecanismos de contracción - relajación vascular y celular: papel central de la guanilato ciclasa soluble y de la proteína kinasa G. Análisis de su modulación",*** que presenta Dña. **Aránzazu Chamorro Jorganes** para optar al grado de Doctor por la Universidad de Alcalá, ha sido realizada bajo la dirección de los Profesores Manuel Rodríguez Puyol y Diego Rodríguez Puyol en el Departamento de Fisiología de la Universidad de Alcalá y que cumple todos los requisitos legales para proceder a su defensa pública.

Y para que conste donde convenga y surta los efectos oportunos, se expide el presente certificado en Alcalá de Henares a 1 de Junio del año 2009.



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UNIVERSIDAD DE ALCALÁ · DEPARTAMENTO DE FISIOLOGÍA · Facultad de Medicina

Fdo.: Dr. D. Manuel Rodríguez Puyol Fdo.: Dr. D. Diego Rodríguez Puyol

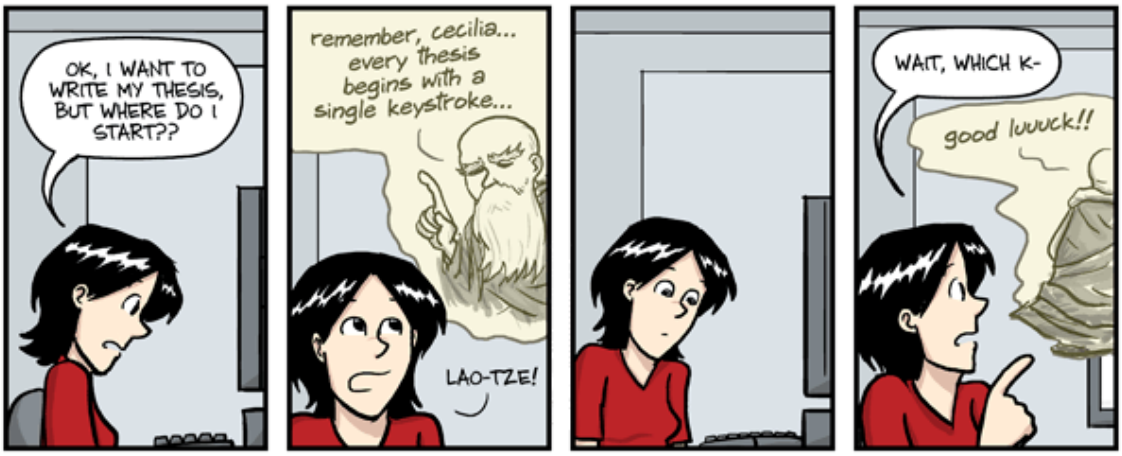
DEPARTAMENTO DE FISIOLOGÍA

MECANISMOS DE CONTRACCIÓN - RELAJACIÓN VASCULAR Y CELULAR: PAPEL CENTRAL DE LA GUANILATO CICLASA SOLUBLE Y DE LA PROTEÍNA KINASA G. ANÁLISIS DE SU MODULACIÓN

TESIS DOCTORAL

ARÁNZAZU CHAMORRO JORGANES

2009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good luuuck!!

A mi familia, Sergio, Toñi e Itziar

AGRADECIMIENTOS

En primer lugar, quiero agradecer al Dr. Manuel Rodríguez Puyol la oportunidad que me ha brindado de formar parte de su grupo de investigación y la confianza que ha depositado en mí desde el primer día que entré en el laboratorio. Después de tantas horas de trabajo compartidas me atrevo a decir que Manuel además de jefe se ha convertido en gran compañero y voy a echar en falta sus consejos y las palmadas por sorpresa en la espalda.

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Cuando pasas más horas en el trabajo que en tu propia casa, que haya personas que estén pendientes de ti y que siempre tengan palabras de apoyo tiene un valor incalculable. Es el caso de la Dra. Luisa Díez-Marqués y Soledad García. A Luisa tengo que agradecerle que me permitiese trabajar en su laboratorio durante dos años y todos sus consejos profesionales y personales. Cuando conocí a Soledad me dijeron que era la secretaría de Manuel pero no es cierto, es la secretaria de todos, prepara powerpoints como nadie y además me pide productos a Sigma fuera de plazo; esto último que no se tenga en cuenta.

Trabajar en laboratorio me ha permitido conocer a personas excelentes que han pasado de ser compañeros de carrera o laboratorio a amigos y un apoyo importantísimo. Me refiero a la Dras. Beatriz Herranz, Gema Pérez y Matilde Alique. Cuando Mati y Bea vuelvan de Nueva York montaremos un laboratorio y contrataremos a Gemita y Merce. ¡Temblad Puyoles!

Me gustaría hablar de mis compañeras de laboratorio, Isabel Serrano y Marta González. Me alegro enormemente de haber compartido estos cuatro años con vosotras. Voy a echar en falta los "momentos confesionario" en el cuarto de cultivos y "la hora golfa" a partir de las 17.15 cuando el fenol se sube a la cabeza y cantamos *Like A Virgin* en alemán. Muchísima fuerza y ánimo para la recta final de tesis, no queda nada campeonas.

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También quiero destacar el cariño de mis amigas Pau, Jose y Cristina que todas renegaban de trabajar en un laboratorio y ahora llevan bata blanca a diario.

Per últim i no per això menys important vull donar les gràcies a Albert, per comprendre la meva feina i no molestar-se perquè pogués arribar tard a algun lloc perquè es complica un experiment al laboratori, per ajudar-me amb l'estadística i ser el meu english assistant, per tots els papers que hem llegit plegats, per ser un apassionat de la ciència i per estar sempre quan el necessito.

SUMMARY

SUMMARY

Recent studies have proven that alterations in the nitric oxide-soluble guanylate cyclase-protein kinase G (NO-sGC-PKG) pathway are related to the genesis of endothelial dysfunction. NO and sGC expression seems to be dependent on the interaction of cells with extracellular matrix through integrins, but no similar effect has been described for PKG. The aim of this study was to evaluate whether the cellular content of PKG could be dependent on the activation of cellular integrins, and to test the possibility that sGC and PKG could be modulated *in vivo* through integrin stimulation. The administration of tirofiban to rats increased the sGC (β1) content in vascular walls. As a consequence of this up-regulation, the pharmacological responses to NO donors increased, the tolerance associated with long-term nitrite treatment improved, and the ability of NO donors to reduce blood pressure in hypertensive rats increased. On the other hand, fibronectin increased the PKG Iα protein content in human mesangial cells (HMC) through interaction with β1 integrin. Akt activation was involved in the fibronectin up-regulation of PKG Iα levels. The changes observed in the PKG Iα protein content were the consequence of increasing levels of its mRNA, probably because of an increased transcriptional activity. The administration of tirofiban to Wistar rats induced an increased PKG Iα protein content in aortic walls, as was expected from the *in vitro* experiments. Tirofiban enhanced the hypotensive response observed in the presence of dibutiryl cGMP (dbcGMP), suggesting the functional relevance of the changes detected in the PKG Iα content in aortas. Elucidation of this novel mechanism provides a rationale for future pharmacotherapy in certain vascular diseases.

Recently, it has been reported that transgenic mice for a constitutively activated form of H-Ras show hypertension and heart hypertrophy, thus suggesting a role for H-Ras in the regulation of blood pressure. We hypothesized that H-Ras$^{-/-}$ mice may show changes in arterial pressure that were opposite to those observed in the mice over-expressing Ras. H-Ras deficient mice showed a decreased blood pressure, and an increased hypotensive response to different agonists of the system NO-sGC-PKG, particularly sodium nitroprusside (SNP) and dbcGMP. That low blood pressure reached values comparable to those of control animals when NOS, sGC or PKG were blocked. The protein content of eNOS, sGC (α) or PKG I was higher in H-Ras$^{-/-}$ mice than in their controls. The metabolic activity of these enzymes was increased, as suggested by

the increased urinary nitrite excretion, the increased SNP-stimulated vascular cGMP synthesis, and the increased content of phosphorylated VASP in aortic tissue. Studies performed in mouse embryonic fibroblasts from these animals suggest that the increased PKG content could be due to an increased expression of its mRNA. Taken together, these results strongly support the up-regulation of the NO-sGC-PKG pathway in H-Ras deficient mice, and the functional relevance of this up-regulation in the genesis of the hypotension observed in these animals. Moreover, they suggest that H-Ras could be considered as a therapeutic target in the field of hypertension treatment.

ÍNDICE

6. RAS GTPASAS EN LA REGULACIÓN DE LA FUNCIÓN VASCULAR.

INTRODUCCIÓN

1. EL ÓXIDO NÍTRICO (NO) COMO MODULADOR DE LA FUNCIÓN VASCULAR.

1. 1. NO: Estructura, composición y regulación.

En la década de los 70 Murad descubrió que la nitroglicerina y otros compuestos vasodilatadores liberaban óxido nítrico (NO, Nitric Oxide) que estimulaba la producción de guanosina monofosfato cíclico (cGMP, Cyclic Guanosine Monophosphate) en diferentes extractos de tejidos que contenían guanilato ciclasa (GC, Guanylyl Cyclase) (*Katsuki y col., 1977*). En 1980, Furchgott y Zawadzki describieron una molécula producida por el endotelio vascular en respuesta a acetilcolina, que denominaron factor relajante derivado del endotelio (EDRF, Endothelium-Derived-Relaxing Factor); esta molécula actuaba en el músculo liso de los vasos produciendo relajación del mismo (*Furchgott y Zawadzki, 1980*). Fue en 1987, cuando separadamente, Palmer e Ignarro demostraron que el EDRF y NO eran la misma molécula (*Palmer y col., 1987; Ignarro col., 1987*).

El NO es un gas soluble altamente reactivo con una vida media de 10 segundos. Es una molécula lipofílica que regula diferentes funciones celulares por difusión, desde la célula donde se sintetiza a las células próximas, sin necesidad de ningún transportador de membrana (*Tsutsui y col., 2006*). Esta molécula está implicada en numerosos procesos fisiológicos como la relajación del músculo liso, neurotransmisión, agregación plaquetaria así como en el sistema inmune. Además juega un papel muy importante en enfermedades inflamatorias como la artritis, miocarditis y nefritis e incluso en condiciones patológicas como cáncer, diabetes y enfermedades neurodegenerativas (*Davis y col., 2001; Hanafy y col., 2001*).

El NO es producido por las óxido nítrico sintasas (NOS, Nitric Oxide Synthases) en respuesta a diferentes estímulos químicos (bradiquinina, acetilcolina, ATP) o fisiológicos. Las NOS son homodímeros compuestos por 3 dominios funcionales: (1) **el dominio oxigenasa amino terminal** que contiene los sitios de unión L-arginina, el grupo hemo y tetrahidrobiopterina (BH_4, Tetrahydrobiopterin) (2) **un sitio de reconocimiento de Calmodulina** (CaM, Calmodulin) y (3) **el dominio reductasa carboxiterminal**, que contiene los sitios de unión para flavín adenín dinucleótido

(FAD, Flavin Adenine Dinucleotide), flavín mononucleótido (FMN, Flavin Mononucleotide) y nicotinamida adenina dinucleótido fosfato (NADPH, Nicotinamide Adenine Dinucleotide Phosphate) (*Alderton y col., 2001*).

Las NOS catalizan la conversión estequiométrica del aminoácido L-arginina en L-citrulina y NO. Esta reacción requiere O_2 y poder reductor en forma de NADPH e implica la transferencia de electrones desde el NADPH pasando por FAD, FMN, BH_4 hasta el grupo hemo. Además precisa de Ca^{2+} y Calmodulina como cofactores.

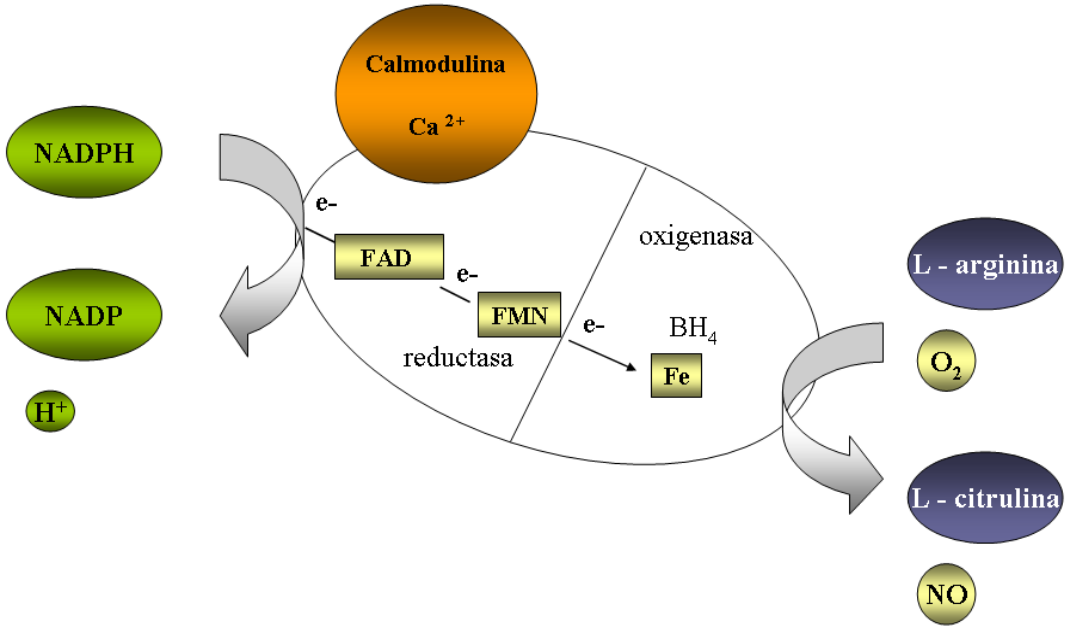

Figura 1. Conversión de L-arginina en L-citrulina por la NOS. Esquema adaptado de *Alderton y col., 2001*.

Se han identificado tres genes distintos localizados en diferentes cromosomas que codifican las proteínas **óxido nítrico sintasa neuronal** (nNOS, NOS 1, Neuronal Nitric Oxide Synthase; localizado en el cromosoma 7), **óxido nítrico sintasa inducible** (iNOS, NOS 2, Inducible Nitric Oxide Synthase; localizado en el cromosoma 17) y **óxido nítrico sintasa endotelial** (eNOS, NOS 2, Endothelial Nitric Oxide Synthase; localizado en el cromosoma 12).

Las isoformas nNOS y eNOS se expresan constitutivamente en el sistema nervioso y el endotelio vascular respectivamente. La isoforma iNOS es una enzima inducible que se expresa en macrófagos y hepatocitos en respuesta a endotoxinas y

citoquinas proinflamatorias. Las isoformas constitutivas son enzimas dependientes de Ca^{2+}. En condiciones basales se encuentran inactivas. Cuando se produce un aumento de Ca^{2+}, éste se asocia con la calmodulina formando el complejo Ca^{2+}/Calmodulina que se une a las NOS activándolas. Por el contrario, iNOS es independiente de Ca^{2+} (*Tsutsui y col., 2006*). La isoforma eNOS se regula principalmente a nivel postraduccional mediante palmitoilación y miristilación que le permite dirigirse al complejo de Golgi y zonas ricas en esfingolípidos y colesterol de la membrana plasmática. Además, la actividad de eNOS es regulada por fosforilación (*Alderton y col., 2001; Shaul, 2002; Mount y col., 2007*).

Para estudiar las funciones de las distintas isoformas de las NOS "*in vivo"* se han generado distintos ratones deficientes para cada una de ellas. **Los ratones deficientes en nNOS (*nNOS* $^{-/-}$)** presentan una hipertrofia del esfínter pilórico (*Huang y col., 1993*), un comportamiento más agresivo (*Nelson y col., 1995*) y una protección frente la isquemia cerebral (*Huang y col., 1994*). **Los ratones deficientes en eNOS (*eNOS* $^{-/-}$)** presentan hipertensión (*Huang y col., 1995*), mayor susceptibilidad a la isquemia cerebral (*Huang y col., 1996*) y una aceleración en la formación de lesiones vasculares (*Yogo y col., 2000*). El fenotipo principal de **los ratones deficientes en iNOS (*iNOS* $^{-/-}$)** es una mayor susceptibilidad a las infecciones por patógenos y resistencia a la respuesta hipotensora cuando son expuestos a lipopolisacáridos bacterianos (LPS, Lipopolysaccharides) (*Wei y col., 1995*).

Tsutsui y colaboradores generaron ratones deficientes para las tres isoformas de NOS. Estos ratones son viables pero presentan una vida media y un número de ratones por camada menor que los ratones control. Además, presentan importantes lesiones arterioscleróticas coronarias que terminan en infarto de miocardio en la mayoría de los casos. También se producen cambios en la estructura renal que derivan en una diabetes insípida (*Tsutsui y col., 2006*).

1. 2. Mecanismos de acción del NO.

En el sistema vascular, el NO producido por la eNOS en las células endoteliales difunde a las células musculares lisas donde se une al grupo hemo de su receptor natural, la guanilato ciclasa soluble (sGC, Soluble Guanylyl Cyclase), dando lugar a la formación de GMPc (*Murad y col., 1990*). Dos moléculas de GMPc son necesarias para la activación de su efector, la quinasa dependiente de GMPc (PKG, Protein Kinase G) que fosforila distintos sustratos produciendo la relajación de las células musculares lisas (*Fukao y col., 1999*). A través de la vía del GMPc-PKG el NO ejerce su actividad vasodilatadora manteniendo el tono vascular, que es esencial para la correcta regulación de la presión arterial (*Moncada y col., 1989*).

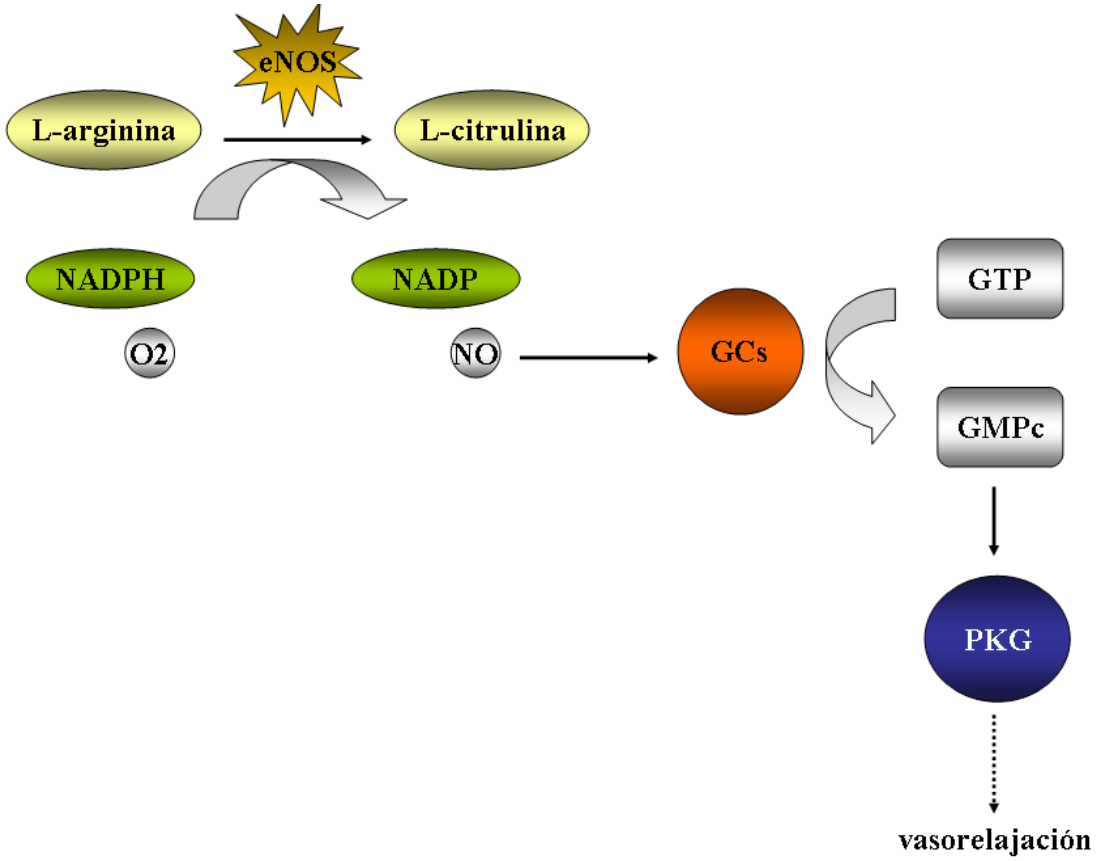

Figura 2. Vía de señalización clásica del NO-GCs-PKG.

El aumento del GMPc inhibe la adhesión plaquetaria y leucocitaria y/o agregación, así como la proliferación y migración de las células musculares lisas. En el sistema nervioso central, el NO actúa como neurotransmisor participando en la coordinación entre la actividad neuronal y el flujo sanguíneo y en procesos de aprendizaje y memoria. En el sistema nervioso periférico regula funciones

gastrointestinales, respiratorias y genitourinarias. En el sistema inmunitario, los macrófagos sintetizan NO en respuesta a patógenos. En este caso, el NO funciona como un agente bactericida, antiviral y tumoricida (*Moncada, 1999*). El NO tiene un efecto citotóxico debido a su capacidad de inhibir enzimas claves en la síntesis de ADN y citocromo c oxidasa favoreciendo la formación de especies reactivas de oxígeno (*Moncada y Erusalimsky, 2002*). Además el NO puede actuar directamente sobre las proteínas produciendo modificaciones que alteran su estructura y funcionalidad (*Gow y col., 2004*). Entre estas modificaciones encontramos la nitrosilación de los grupos tioles de los residuos cisteína (s-nitrosilación) o la nitración de residuos tirosina (*Saura y col., 1999; Lizarbe y col., 2008*). Además el NO puede reaccionar con el oxígeno dando lugar a nitritos y nitratos (*Beckman y col., 1990*).

1. 3. Importancia del NO en situación fisiopatológica.

Como se ha explicado anteriormente, el NO es un agente vasodilatador que además de inhibir la agregación plaquetaria y la proliferación de las células musculares lisas, regula las interacciones entre los leucocitos y la pared de los vasos. Todas estas características establecen al NO como un regulador homeostático en el sistema vascular, y su déficit juega un papel muy importante en distintas situaciones patológicas como la hipertensión (*Moncada y Higgs, 2006*).

La disfunción endotelial es un fenómeno que se observa en los inicios de las enfermedades cardiovasculares y se caracteriza por una disminución de la sensibilidad del NO, o una disminución de su producción y disponibilidad, de forma que la capacidad vasodilatadora del NO está reducida. Aunque el mecanismo que desencadena la disfunción endotelial depende de muchos factores, como un descenso en la expresión de eNOS o cambios en las concentraciones de sustrato y/o cofactores, el incremento en la producción de especies reactivas de oxígeno (ROS, Reactive Oxygen Species) contribuye considerablemente en este fenómeno.

El NO interacciona con especies reactivas del oxígeno, específicamente con el anión superóxido (O_2^-) formando peroxinitritos ($ONOO^-$). Diversos estudios demuestran la implicación de los peroxinitritos en la hipercolesterolemia, diabetes y enfermedad de las arterias coronarias (*Greenacre y Ischiropoulos, 2001*). Las principales fuentes de

producción de O_2^- en el sistema vascular son las enzimas NADPH oxidasa y xantina oxidasa. Las enzimas del citocromo P450 también generan O_2^- y se ha descrito que su inhibición favorece la vasodilatación mediada por el NO dependiente de endotelio en pacientes que sufren enfermedad de las arterias coronarias. Otra fuente potencial de O_2^- es el desacoplamiento de la eNOS que se produce cuando hay un déficit en los niveles de L-arginina o BH_4. Se ha observado este fenómeno en distintas condiciones patológicas como la diabetes, hipertensión e hipercolesterolemia (*Cai y Harrison, 2000; Moncada, 2006*).

Por otro lado, un exceso en la producción de NO también puede producir situaciones patológicas. La inhibición de la respiración mitocondrial es un componente importante en el daño inducido por el NO en tejido. Esta inhibición que es dependiente de NO y reversible, se convierte en persistente con el tiempo como resultado del estrés oxidativo (*Moncada y Erusalimsky, 2002*).

Los estímulos inflamatorios como endotoxinas y citoquinas inducen a la enzima iNOS. El NO producido por la iNOS en el sistema vascular está implicado en la vasodilatación profunda en el shock séptico. Además, la isoforma iNOS está inducida en los macrófagos y células musculares lisas de vasos ateroscleróticos. En placas ateroescleróticas avanzadas, iNOS colocaliza con residuos de nitrotirosina, un marcador de daño inducido por peroxinitrito. La oxidación de las proteínas de baja densidad es uno de los factores claves en la formación de la placa de ateroma y puede ser llevado a cabo por los peroxinitritos (*Moncada y Higgs, 2006*).

Por consiguiente, mientras que bajas concentraciones de NO generado por eNOS tienen una acción protectora contra la ateroesclerosis promoviendo la vasodilatación, inhibiendo la adhesión plaquetaria y leucocitaria y/o agregación, y la proliferación de las células musculares lisas, altas concentraciones de NO generado por iNOS promueven ateroesclerosis.

2. LA GUANILATO CICLASA (GCs) SOLUBLE COMO RECEPTOR INTRACELULAR DEL NO.

2. 1. GCs: Estructura, composición y regulación.

La enzima GCs fue purificada por primera vez en tejido de pulmón en 1980. GCs es un heterodímero formado por dos subunidades α y β de ≈ 82 y ≈ 70 KDa respectivamente. Existen dos isoformas de cada subunidad denominadas α1 (localizado en el cromosoma 4), α2 (localizado en el cromosoma 11), β1 (localizado en el cromosoma 4) y β2 (localizado en el cromosoma 13). El heterodímero más abundante es α1 β1 y se ha clonado en oveja, ratón, rata y humano (*Koesling y col., 1990; Zabel y col., 1998*). El dímero α1β1 se distribuye ubicuamente en mamíferos y se expresa en altas concentraciones en cerebro, pulmón, corazón, riñón, bazo y músculo (*Nakane y col., 1990; Budworth y col., 1999*). La isoforma α2 está presente en placenta, páncreas, bazo y cerebro formando heterodímeros con β1 (*Harteneck y col., 1991; Mergia y col., 2003*). La isoforma β2 se clonó en riñón de rata y contiene 86 aminoácidos adicionales respecto a la isoforma β1. Esta región contiene una secuencia de isoprenilación que le permite unirse a membrana. Se expresa en hígado y riñón. Esta isoforma no forma heterodímeros funcionales "*in vivo*" (*Koesling y col., 2004; Pyriochou y Papapetropoulos, 2005*) y los experimentos llevados a cabo expresando su actividad catalítica en rata no han obtenido resultados (*Koesling, 1999*).

Las subunidades de los heterodímeros están compuestas por 3 dominios funcionales: (1) **el dominio amino terminal**, también denominado dominio regulador, donde se localiza el grupo hemo, que es el centro de unión a ligando (2) **el dominio central** que media la dimerización y (3) **el dominio carboxilo terminal** que contiene la región catalítica y es responsable del reconocimiento del sustrato. El extremo N terminal presenta una menor homología entre las isoformas que el dominio central y catalítico. A diferencia de otras hemoproteínas que emplean su grupo hemo en reacciones redox para el almacenaje y transporte de oxígeno, la GCs utiliza su grupo prostético como receptor de ligando para el NO y CO. Este grupo prostético está formado por un anillo de 5 nitrógenos coordinados centralmente por un ión ferroso que está asociado a la histidina 105 de la subunidad β1. Cuando el NO se une al grupo hemo se disocia el ión ferroso y la histidina 105 desplazándose el hierro del plano porfirínico.

Esta disociación produce un cambio conformacional que favorece la síntesis de GMPc a partir de una molécula de guanosina trifosfato (GTP, Guanosin Tri-Phosphate), por parte del único sitio de unión al sustrato. La GCs une una única molécula de sustrato por dímero (*Russwurm y Koesling, 2004*).

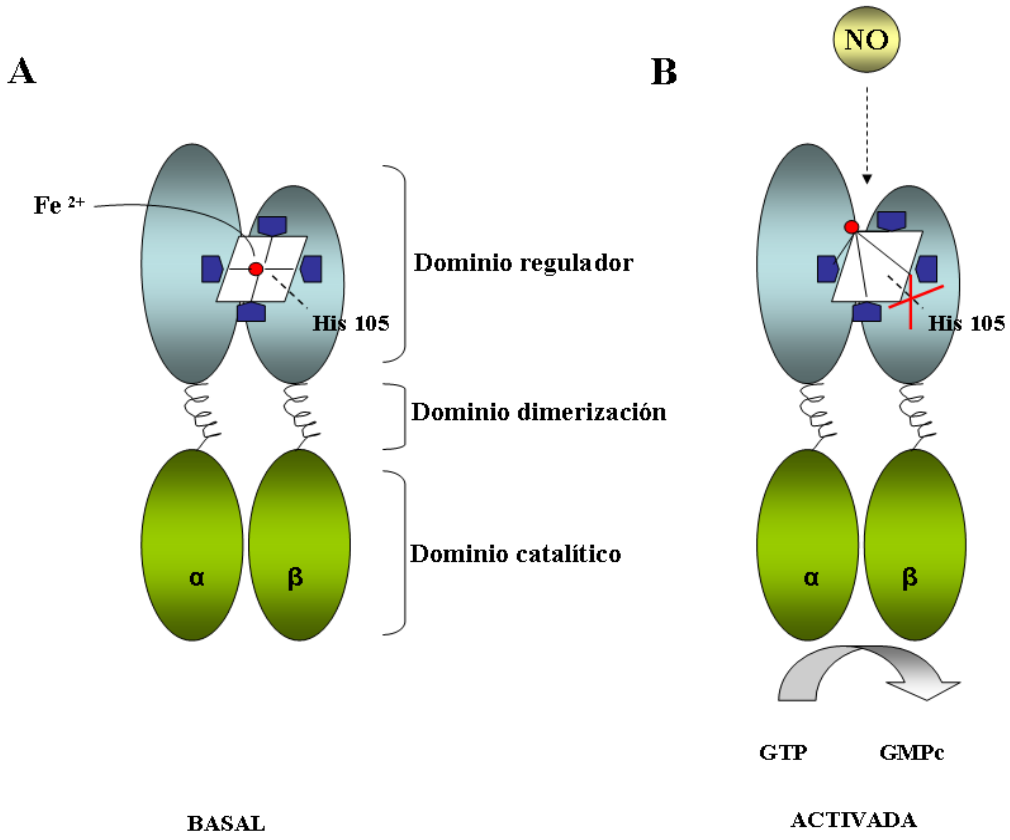

Figura 3. Esquema de la estructura de GCs (A) y su activación (B).

Inicialmente se pensaba que la GCs era una enzima que se localizaba exclusivamente en el citosol pero existen estudios que demuestran su asociación a membrana. En tejido cardiaco de rata, aproximadamente una quinta parte de la GCs está asociada a membrana (*Zabel y col., 2002*). Otros tejidos como la corteza cerebral, glándula adrenal, músculo esquelético y colon presentan una fracción de GCs asociado a membrana. Además, en las células endoteliales la mayor parte de la GCs se encuentra asociado a membrana mientras que en músculo liso su localización subcelular es citosólica (*Venema y col., 2003*).

Con el fin de estudiar el papel fisiológico de esta enzima "*in vivo"* se han generado distintos ratones deficientes en GCs. **Los ratones deficientes en la isoforma β1 (*GCs β1* $^{-/-}$)** tienen una esperanza de vida inferior a un mes, muriendo el 60% los dos primeros días después del nacimiento. Estos animales presentan un incremento en la presión arterial, una disminución en la frecuencia cardiaca, una pérdida en la respuesta de las aortas a la relajación inducida por NO así como una pérdida en la inhibición plaquetaria inducida por el NO. Presentan alteraciones en el tracto gastrointestinal muy importantes como el alargamiento del esófago, estómago dilatado, hinchazón de la vesícula y alargamiento del colón. El peso corporal se reduce en un 40% respecto a los ratones control debido a la malnutrición. La disfunción peristáltica produce la muerte en estos ratones (*Friebe y col., 2007*).

La heterodímero α1β1 es el mediador más importante en la relajación nitrérgica del intestino delgado. En **los animales deficientes en la isoforma α1 (*GCs α1*$^{-/-}$)** no se observan alteraciones de la motilidad intestinal "*in vivo"*. Este resultado puede deberse a que la relajación nitrérgica también puede ocurrir vía activación del heterodímero α2β1 (*Dhaese y col., 2009*). Por otro lado, se han realizado estudios "*in vitro"* en anillos de aorta y arteria femoral de ratones deficientes en α1 que demuestran la implicación de esta isoforma en los mecanismos de vasorelajación inducida por el NO y activadores de GCs independientes del NO pero no se descarta la participación en menor medida de la isoforma α2β1 o algún mecanismo independiente de GCs (*Nimmegeers y col., 2007*).

2. 2. Mecanismos de acción de la GCs.

La GCs a través de la producción de GMPc es capaz de realizar una gran variedad de efectos biológicos como son la regulación del tono vascular, motilidad, fototransducción, flujo intestinal y la homeostasis electrolítica. Para llevar a cabo estos procesos el GMPc actúa directamente sobre tres efectores transmitiendo la señal que se traducirá en el efecto biológico específico. Estos efectores son la proteína kinasa G (PKG, Protein Kinase G), los canales iónicos dependientes de nucleótidos monofosfato cíclicos (CNG, Cyclic Nucleotide-Gated channel) y las fosfodiesterasas (PDE, PhosphoDiEsterase) (*Krumenacker y col., 2004*).

- **PKG:** Es el principal mediador intracelular del GMPc. Hablaremos de esta proteína posteriormente.
- **Canales CNG:** Estos canales regulan la entrada de Na^{+} y Ca^{2+} en la célula y su apertura es mediada por la unión de GMPc. La activación de estos canales es muy importante en la regulación de la fototransducción y neurotransmisión en la retina (*Biel y col., 1999*).
- **PDE:** El papel de estas enzimas es crucial en la hidrólisis y modulación de la concentración de nucleótidos cíclicos intracelular. Debido a su capacidad de regular los niveles tanto de GMPc como adenosina monofosfato cíclico (cAMP, Cyclic Adenosine Monophosphate), van a tener una importante función reguladora de la especificidad de las respuestas mediadas por estos segundos mensajeros (*Krumenacker y col., 2004*).

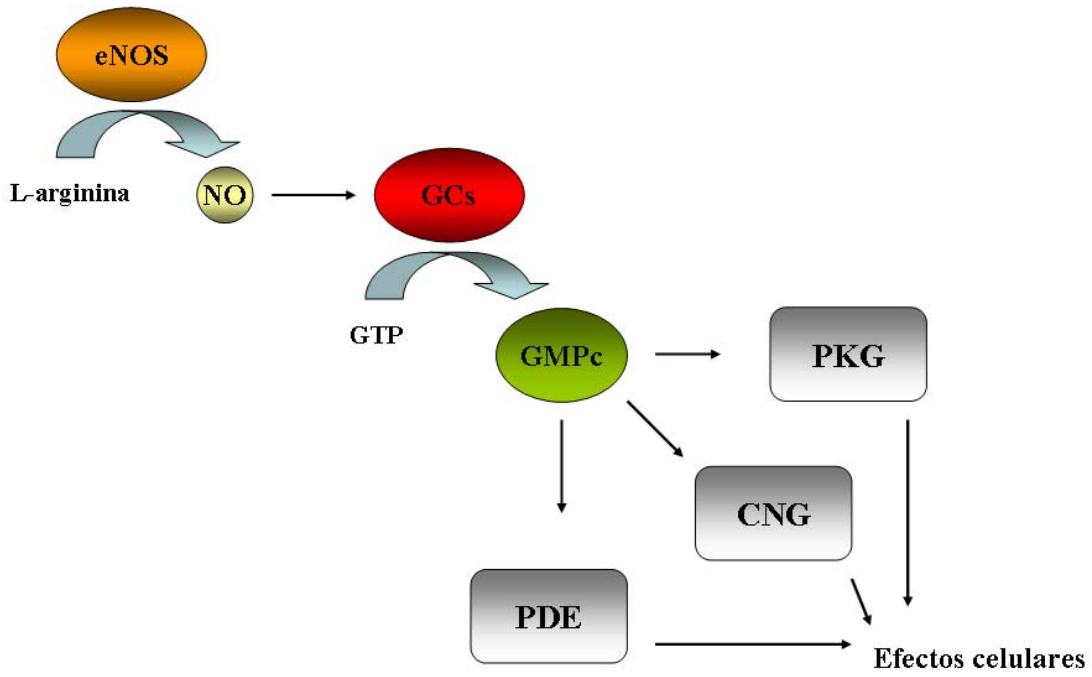

Figura 4. Principales efectores del GMPc.

El GMPc regula la expresión de numerosos genes implicados en la regulación del tono vascular, asociados con la hipertrofia cardiaca, diferenciación de músculo liso, proliferación y apoptosis entre otros. Estos efectos los realiza mediando procesos transcripcionales y postranscripcionales. Además, el GMPc regula la actividad de factores de transcripción como la proteína de unión al elemento de respuesta al AMPc

(CREB, cAMP Response Element Binding) y el factor de respuesta al suero (SRF, Serum Response Factor) (*Pilz y Casteel, 2003*).

2. 3. Importancia de la GCs en situación fisiopatológica.

Es un hecho ampliamente descrito que en algunas enfermedades cardiovasculares como la hipertensión, aterosclerosis y diabetes se producen cambios en los niveles de GCs (*Ruetten y col., 1999; Melichar y col., 2004*). Se ha observado que la exposición al plomo produce hipertensión (*Sharp y col., 1987*). Aunque son pocos los estudios que relacionan la producción de ROS y la regulación de la GCs, Marques y colaboradores demostraron que en la hipertensión inducida por plomo, disminuía de manera significativa la expresión de GCs β1 y que la administración de vitamina C, potente antioxidante, prevenía esa patología (*Marques y col., 2001*). Posteriormente se llevó a cabo un estudio "*in vitro"* que demostró que la disminución de GCs β1 inducida por el plomo estaba mediada por los ROS vía ciclooxigenasa-2 (COX-2) (*Courtois y col., 2003*). Además, se ha descrito que la exposición a estrógenos, altas concentraciones de AMPc, el factor de crecimiento nervioso (NGF, Nerve Growth Factor) e interleuquina-1β (IL-1β, Interleukin-1β) reducen significativamente los niveles de ARNm de GCs α1 y β1 (*Pyriochou y Papapetropoulos, 2005*).

Existen evidencias en la bibliografía que demuestran la participación de la GCs en procesos de inflamación. En un modelo de daño pulmonar inducido por LPS en ratón se observó una disminución en la expresión proteica y ARNm de GCs. Además, cuando se atenuaba la actividad de GCs con inhibidores selectivos de la enzima, se producía un incremento pronunciado del proceso inflamatorio y del daño en el tejido pulmonar (*Glynos y col., 2007*).

En determinadas situaciones se produce una alteración en la respuesta de la GCs al NO, este fenómeno recibe el nombre de sensibilización/desensibilización. Los primeros trabajos sobre el incremento de la sensibilidad de la respuesta de la GCs al NO se llevaron a cabo en vasos sanguíneos aislados sin endotelio o tratados con inhibidores de eNOS (*Shirasaki y col., 1985; Busse y col., 1989*). En todos los casos se observó un incremento en la potencia de los vasodilatadores empleados en la vasorelajación. Estos resultados *"in vitro"* fueron corroborados por Moncada y colaboradores, que

encontraron un aumento en la respuesta antihipertensiva a nitroglicerina en ratas que habían sido tratadas previamente con el inhibidor de eNOS, nitro-L-arginina metil éster (L-NAME, NG-Nitro-L-Arginine Methyl Ester) (*Moncada y col., 1991*). De este modo, la reducción de la concentración de NO produce un incremento en la sensibilidad de la vía de señalización.

Por otro lado, se ha observado que un exceso de ligando, en este caso NO, produce una inactivación de la enzima GCs. Normalmente este término se aplica a la inhibición de la expresión de la GCs tras la administración crónica de donadores de NO, como la nitroglicerina o el dinitrato de isosorbide**,** con la consiguiente pérdida de respuesta. (*Filippov y col., 1997*).

3. LA PROTEÍNA KINASA DEPENDIENTE DE GMPc (PKG) COMO EFECTOR FINAL DEL GMPc.

3. 1. PKG: Estructura, composición y regulación.

La PKG pertenece a la familia de las proteínas serin-treonin kinasa y está presente en diversidad de células eucariotas, desde paramecios hasta humanos (*Lohmann y col., 1997; Francis y col., 1999*). En mamíferos se han identificado dos genes: *Prkg1* (localizado en el cromosoma 10) y *Prkg2* (localizado en el cromosoma 4) que codifican las proteínas PKG I y PKG II respectivamente. A su vez, PKG I presenta dos isoformas PKG Iα y PKG Iβ que difieren en los primeros 90–100 residuos del extremo NH_2 terminal como resultado de un splicing diferencial del ARNm de PKG I (*Feil y col., 2003*). PKG se regula principalmente a nivel transcripcional; existen varios trabajos que demuestran que el NO juega un papel fundamental en su expresión activando o inhibiendo distintos factores de transcripción (*Sellak y col., 2002*).

PKG I y PKG II son homodímeros formados por dos subunidades de ≈ 75 y ≈ 85 KDa respectivamente y comparten características estructurales. Cada subunidad está compuesta por tres dominios funcionales: (1) **dominio amino terminal** responsable de la dimerización de las subunidades mediante una región de cremallera de leucina. Además contiene sitios de autofosforilación lo cual le confiere la capacidad de autorregular la actividad enzimática y también posee secuencias para modificaciones postranscripcionales que determinan las diferentes localizaciones subcelulares (2)

dominio regulador que contiene dos sitios de unión al GMPc y (3) **dominio kinasa** que cataliza la transferencia del γ fosfato del ATP al grupo hidroxilo del residuo serina o treonina de la proteína diana (*Feil y col., 2003*).

El agonista principal de PKG es el GMPc. La unión de GMPc al dominio regulador produce un cambio conformacional que libera al dominio catalítico de la inhibición del extremo N terminal, permitiendo así la fosforilación de las proteínas sustrato (*Wall y col., 2003*). Recientemente se ha descrito un mecanismo de activación de PKG independiente de la vía del NO-GMPc. El H_2O_2, potente agente oxidante, induce la formación de enlaces disulfuro entre los residuos Cys 34 que se localizan en la región N terminal de la isoforma PKG Iα activando la enzima (*Burgoyne y col., 2007*).

Figura 5. Esquema de la estructura de PKG.

PKG Iα y Iβ son enzimas solubles que se localizan en el citosol mientras que PKG II se encuentra anclada en la membrana plasmática por modificaciones postransduccionales como la miristilación en el extremo N terminal. PKG I está presente en músculo liso y plaquetas. También se encuentra en niveles inferiores en el endotelio vascular, cardiomiocitos, fibroblastos, en células renales, leucocitos y en regiones específicas del sistema nervioso, como el hipocampo, células de Purkinje y en el ganglio de la raíz dorsal (*Feil y col., 2005; Keilbach y col., 1992*). La isoforma PKG Iα se expresa en pulmón, corazón, ganglio de la raíz dorsal y cerebelo. Junto con la isoforma Iα, la isoforma Iβ se expresa en músculo liso, concretamente en útero, vaso, intestino y tráquea. En las plaquetas, neuronas del hipocampo y olfatorias se expresa principalmente la isoforma Iβ (*Geiselhöringer y col., 2004*). PKG II se encuentra en cerebro, la mucosa intestinal, riñón, corteza adrenal, condrocitos y pulmón (*De Vente y col., 2001; el-Husseini y col., 1995*, *Lohmann y col., 1997*).

Con el fin de estudiar el papel de esta enzima se han generado distintos ratones deficientes en PKG. **Los ratones deficientes en PKG I (*PKG I* $^{-/-}$)** tienen una esperanza de vida inferior a una semana, muriendo el 50% entre el quinto o sexto día después del nacimiento. Además presentan defectos en la relajación vascular y visceral (*Pfeifer y col., 1998*), disfunción eréctil (*Hedlund y col., 2000*), alteración en la adhesión y activación plaquetaria (*Massberg y col., 1999; Li y col., 2003*) y fallo en la orientación de los axones sensoriales durante la embriogénesis (*Schmidt y col., 2002*).

Por otro lado, **los ratones deficientes en PKG II (*PKG II* $^{-/-}$)** presentan una vida media normal (*Pfeifer y col., 1996*), una disminución en el crecimiento longitudinal del hueso (*Miyazawa y col., 2002*), menor secreción intestinal de cloruro (*Pfeifer y col., 1996; Vaandrager y col., 2000*), una disminución en la inhibición de la reabsorción del sodio mediada por GMPc (*Vaandrager y col., 2000*), también disminuye la inhibición de la secreción de renina inducida por GMPc (*Wagner y col., 1998*), y presentan un ligero defecto en la ritmicidad circadiana (*Oster y col., 2003*).

3. 2. Mecanismos de acción de PKG.

Se han identificado más de diez sustratos que son fosforilados por PKG I "*in vivo"* y uno que es modificado por PKG II. El resto de proteínas descritas como sustratos lo son en sistemas de expresión "*in vitro"*. En la tabla 1 se resumen los principales sustratos de esta enzima. La especificidad de PKG por el sustrato depende de los distintos extremos amino terminal de cada isoenzima.

Sustrato	***Isoforma de PKG***	***Tejido/ tipo celular***	***Función de la fosforilación***
BKca	*PKG I*	*Músculo liso*	*Hiperpolarización de membrana*
Receptor IP3 tipo I	*PKG I*	*Cerebelo*	*Estimula la liberación de Ca^{2+} de los almacenes sensibles a IP3*
IRAG	*PKG Iβ*	*Músculo liso y plaquetas*	*Reduce el Ca^{2+} liberado de los almacenes sensibles a IP3*
MYPT1	*PKG Iα*	*Músculo liso*	*Inhibición de la miosina fosfatasa*
PDE5	*PKG I*	*Músculo liso y plaquetas*	*Degradación de GMPc*
Fosfolamban	*PKG I*	*Músculo liso*	*Favorece la actividad de Ca^{2+} de retículo*
RGS2	*PKG I α*	*Músculo liso*	*Inhibición de generación de IP3*
Telokina	*PKG I*	*Músculo liso*	*Inhibición actividad MLCK*
VASP	*PKG I*	*Músculo liso, plaquetas e hipocampo*	*Regulación citoesqueleto de actina y tráfico vesicular*
RhoA	*PKG I*	*Músculo liso e hipocampo*	*Reduce la fosforilación MLC y tráfico vesicular"in vitro"*
HSP20 *HSP27*	*PKG I*	*Músculo liso y plaquetas*	*Disminuye la polimerización de actina " in vitro"*

Tabla 1. Principales sustratos de PKG. Esquema adaptado de *Hofmann y col., 2006.*

PKG tiene un papel principal en la regulación del tono en las células musculares lisas. Las dianas potenciales de PKG I son los canales de K^+ activados por Ca^{2+} (BKca) (*Sausbier y col., 2000*) e IRAG (*Schlossmann y col., 2000*), que son proteínas implicadas en la modulación de la entrada de Ca^{2+} extracelular y liberación de Ca^{2+} intracelular respectivamente. La fosforilación de BKca induce la hiperpolarización de la membrana y disminuye la entrada de Ca^{2+} a través de los canales de Ca^{2+} dependientes de voltaje (canales de Ca^{2+} tipo L). Otro sustrato alternativo de PKG I es fosfolamban, el cual modula la actividad de la Ca^{2+}-ATPasa del retículo endoplásmico (*Lincoln y col., 2001; Koller y col., 2003*). Esta Ca^{2+}-ATPasa se activa directamente con la fosforilación dependiente de GMPc que produciría una disminución del nivel de Ca^{2+} citosólico (*Lincoln y col., 2001*).

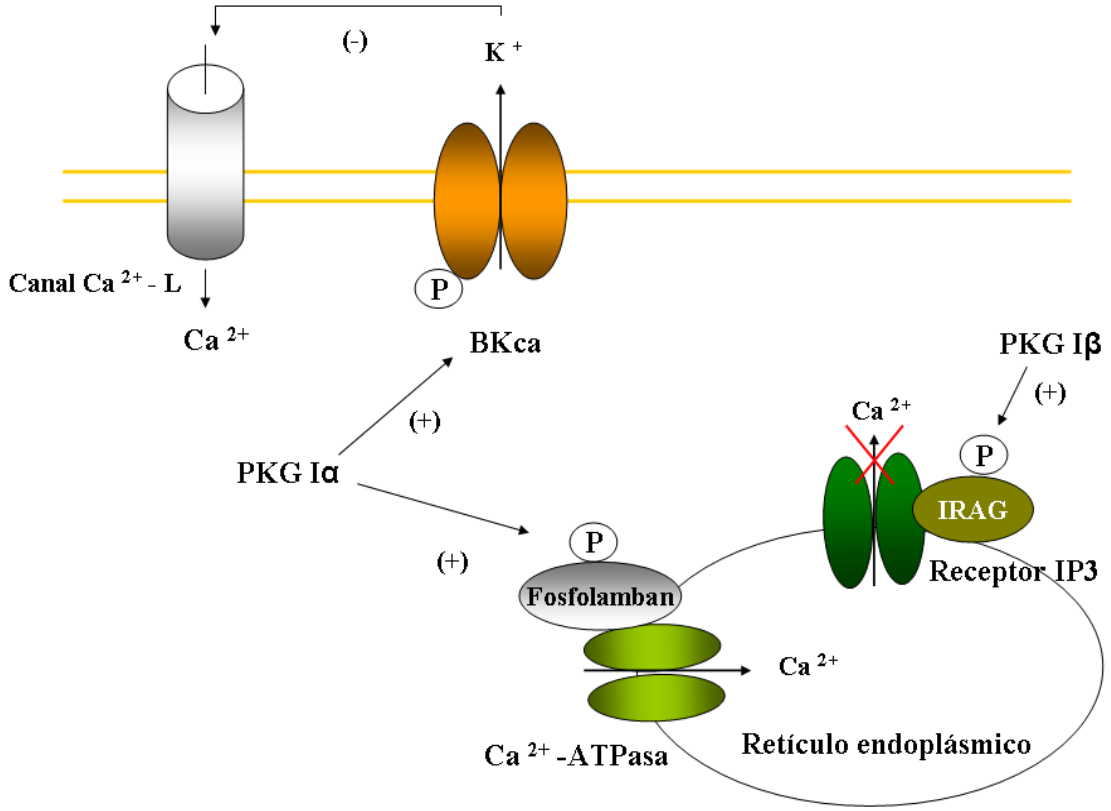

Figura 6. Mecanismos de relajación dependientes de PKG y Ca^{2+}. Esquema adaptado de *Lincoln y col., 2001.*

PKG I puede disminuir el tono vascular por mecanismos adicionales como la desfosforilación de la cadena ligera de la miosina sin afectar a los niveles intracelulares de Ca^{2+} (*Somlyo y Somlyo, 2000*). Estos mecanismos están relacionados con la activación de la fosfatasa de miosina (*Surks y col., 1999*), la inhibición de la

señalización de RhoA (*Sauzeau y col., 2000*) o la fosforilación de la proteína de unión a miosina, telokina (*Walker y col., 2001*). Otras dianas potenciales son la fosfoproteina estimulada por vasodilatadores (VASP, Vasodilator-Stimulated Phosphoprotein) y la proteína de choque térmico 20 (HSP20, Heat Shock Protein), que se unen a los filamentos de actina y regulan la contracción en las células musculares. La fosforilación de VASP y HSP20 disminuye la unión de estas proteínas con los filamentos de actina y reduce el número de adhesiones focales (*Lincoln y col., 2001*). La activación de PKG produce un incremento en la relajación del músculo liso y consecuentemente una disminución de la presión arterial.

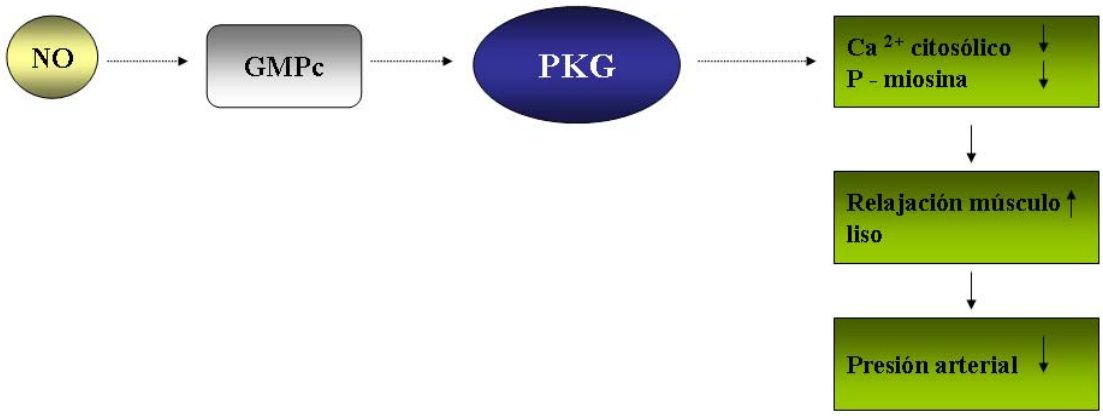

Figura 7. Esquema de la vía de señalización de PKG en la pared vascular.

La vía de señalización del NO juega un papel fundamental en muchas funciones de las células musculares como la regulación de la proliferación, migración, apoptosis celular, así como la formación de matriz extracelular. Parte de estas funciones estarían mediadas por el GMPc y PKG aunque hay autores que proponen que el efecto del NO en las funciones vasculares de las células musculares parece ser independiente de PKG (*Lincoln y col., 1994; Cornwell y col., 1994*). Existen numerosos trabajos que indican que PKG I modula la expresión génica y proliferación de las células musculares lisas activando a las proteínas kinasas dependientes de mitógenos (MAPK, Mitogen-Activated Protein Kinases) y/o fosfoinositol 3-kinasa/Akt kinasa (PI3K/Akt, Phosphoinositol-3-Kinase/Akt Kinase) (*Wolfsgruber y col., 2003; Komalavilas y col., 1999; Kook y col., 2003*). Por el contrario, otros autores han demostrado el efecto antiproliferativo de PKG disminuyendo la vía de señalización de MAPK (*Suhasini y col., 1998; Chiche y col., 1998; Saha y col., 2008*).

Se ha observado que bajo determinadas condiciones, PKG Iα puede translocarse al núcleo y actuar como factor de transcripción activando el promotor de c-fos (*Gudi y col., 1996; Gudi y col., 1997*). Sin embargo, existen muchos trabajos que no observan esta translocación en distintos tipos celulares, incluyendo células musculares lisas (*Feil y col., 2002; Collins y Uhler, 1999; Casteel y col., 2002*) llevando a especular que PKG I podría ser retenida en el citoplasma por proteínas de anclaje específicas de cada célula (*Pilz y Casteel, 2003*).

Una de las funciones mejor descritas de PKG es la modulación del fenotipo de las células musculares. En condiciones fisológicas, éstas células expresan marcadores de fenotipo contráctil tales como α-actina y la cadena pesada de miosina. Variaciones en el contenido intracelular de PKG conlleva el cambio de fenotipo contráctil a sintético en estas células (*Lincoln y col., 2006*).

El NO-GMPc también modula la liberación de renina en riñón. La activación del sistema renina-angiotensina-aldosterona produce un aumento de la presión arterial que estimula la vasoconstricción e inhibe la natriuresis y diuresis. La sobreeexpresión de PKG II en células juxtaglomerulares suprime la liberación de renina y además, en ratones deficientes en PKG II se suprime la inhibición de de la liberación de renina (*Gambaryan y col., 1998*).

3. 3. Importancia de la PKG en situación fisiopatológica.

En determinadas enfermedades como la hipertensión, diabetes o envejecimiento se ha descrito un decremento en el contenido proteico de PKG (*Failli y col., 2005*) o en la actividad de la enzima (*Lin y col., 2001; Russo y col., 2008*).

Desde hace décadas se sabe que en situaciones patológicas como la ateroesclerosis y restenosis se produce un incremento en la proliferación y migración de las células musculares lisas desde la media a la íntima acompañado con un incremento en la producción de proteínas de matriz. Este cambio en el fenotipo de las células musculares se debe a una disminución en la expresión de PKG (*Lincoln y col., 2001; Browner y col., 2004).* Se ha demostrado que las células musculares lisas deficientes en PKG restauraban parcialmente su fenotipo contráctil cuando eran transfectadas con ADNc de PKG (*Boerth y col., 1997; Dey y col., 1998*). Otros compuestos que regulan la

expresión de PKG son el factor de crecimiento derivado de plaquetas (PDGF, Platelet-Derived Growth Factor) y el GMPc (*Lincoln y col., 2001*). Estos datos sugieren que PKG tiene un papel importante en la fisiopatología vascular.

La exposición continuada de donadores de NO o análogos de AMPc reduce significativamente los niveles proteicos y ARNm de PKG en células musculares lisas por una disminución de la transcripción sin afectar a la estabilidad del mensajero (*Soff y col., 1997*). Además, se ha observado que la sobreexpresión de eNOS en endotelio de aorta de ratón produce una disminución de la expresión de PKG (*Yamashita y col., 2000*). Estos resultados podrían proporcionar un mecanismo alternativo de tolerancia a los nitrovasodilatadores.

Otros estudios demuestran la implicación de PKG I en angiogénesis. En concreto, ratones que sobreexpresan PKG Iα presentan un incremento de la angiogénesis inducida por isquemia mientras que se atenúa en ratones deficientes en PKG I, indicando que PKG I es crítico para la neovascularización "*in vivo"* (*Yamahara y col., 2003*). Todos estos resultados sugieren que las vías dependientes de PKG I promueven una variedad de procesos vasculoproliferativos en condiciones patológicas.

4. IMPORTANCIA DE LA MATRIZ EXTRACELULAR (MEC) EN LA REGULACIÓN DEL FENOTIPO DE LA CÉLULA VASCULAR.

4. 1. Estructura de la MEC.

La matriz extracelular (ECM, Extracellular Matrix) está compuesta por un conjunto de macromoléculas que constituyen el entorno donde la célula se multiplica y desarrolla sus funciones. Estas macromoléculas se ensamblan dando lugar a un armazón que determinará la estructura del órgano o tejido, servirá de soporte en la adhesión y migración celular y además, proporcionará a las células información sobre su entorno regulando también procesos de supervivencia, proliferación y diferenciación (*Boudreau y Jones, 1999*).

La MEC está compuesta por una serie de componentes fibrilares, principalmente el colágeno y elastina que se encuentran embebidos en una sustancia amorfa de

componentes no fibrilares, formados predominantemente por proteoglicanos y glicoproteínas. Otro componente importante de la MEC son las glicoproteínas de adhesión, como la fibronectina, laminina y entactina, que son abundantes en las membranas basales e interaccionan con integrinas. La proporción de componentes fibrilares y no fibrilares determinará las propiedades físicas de la MEC. Los componentes de la matriz son sintetizados por los distintos elementos celulares de la pared vascular (*Tanzer, 2006*; *Rhodes y Simons, 2007*).

En condiciones normales, la MEC se encuentra en todos los tejidos en un constante recambio de sus componentes, debido a un estricto balance entre la síntesis y la degradación de matriz. Entre las proteínas que regulan este balance se encuentran las metaloproteasas de matriz (MMP, Matrix Metaloproteases) y el factor de crecimiento transformante-β (TGF-β, Transforming Growth Factor beta). Las MMPs son endopeptidasas que tienen como función degradar los componentes de la MEC (*Mott y Werb, 2004*). Por otro lado, el TGF-β es una de las proteínas reguladoras más importantes de este proceso ya que estimula la síntesis de nuevos componentes de la MEC, la degradación de las proteínas existentes mediante la activación de las MMPs y la inhibición de los inhibidores tisulares de MMPs (TIMPs, Tissue Inhibitors of Metalloproteinases) (*Gauldie y col., 2007*).

4. 2. Las integrinas como receptores celulares de las proteínas de matriz.

Las células interaccionan con la matriz extracelular subyacente a través de receptores expresados en la superfice celular llamados integrinas (*Giancotti y col., 1999*). Consideradas inicialmente como proteínas de anclaje, se ha ido reconociendo progresivamente su papel regulador de la propia función celular. De hecho, hay numerosos estudios que demuestran la estrecha relación existente entre las integrinas y proteínas del citoesqueleto (*Brakebusch y Fässler, 2003*) y, sobre todo, es un hecho ampliamente aceptado que las señales celulares transmitidas por integrinas son claves en la viabilidad celular, ya que tanto ciclo celular (*Aszodi y col., 2003*) como la apoptosis (*Reginato y col., 2003*) son regulados por estas proteínas.

Las integrinas son una familia de receptores transmembrana heterodiméricos, formados por dos tipos de subunidades, α y β, asociadas no covalentemente. Cada

subunidad está compuesta por tres dominios: (1) **el dominio extracelular** por el cual se unen a la MEC, (2) **dominio transmembrana** y (3) **un dominio intracelular o citoplasmático**, a través del cual contactan con el citoesqueleto de actina. Se han identificado en el genoma humano 24 subunidades α y 9 subunidades β que se combinan dando lugar a 24 integrinas funcionales distintas, esto permite que sean muy específicas a la hora de reconocer su ligando indicando que las integrinas pueden tener funciones de señalización especializadas (*Danen y Yamada, 2001*).

Los ligandos de las integrinas incluyen diferentes proteínas de MEC como la fibronectina, laminina, colágeno y vitronectina. La integrina α5β1 es el receptor principal de la fibronectina que reconoce la secuencia RGD (Arg-Gly-Asp) (*Pytela y col., 1985*), aunque se ha descrito otro receptor tipo integrina de FN, α4β1, que no requiere esta secuencia de reconocimiento para activarla (*Sechler y col., 2000*).

La señalización mediada por integrinas se transmite a través de la membrana de forma bidireccional, es decir, transmite señales desde la MEC al citoplasma y también al revés en un proceso conocido como señalización *"inside out"* (*Hynes, 2002*). Las integrinas compensan su menor afinidad por sus ligandos aumentando su presencia en la membrana. Las integrinas que interaccionan con las proteínas de matriz se agrupan formando grupos en regiones especializadas de la membrana plasmática que reciben el nombre de adhesiones focales. Es en estas regiones donde tiene lugar la interacción de las integrinas (subunidad α) con las proteínas de citoesqueleto (talina, parvina, actinina, filaminina, vinculina y paxilina) (*Rhodes y Simons, 2007*).

Las integrinas carecen de actividad enzimática pero pueden interaccionar, a través de su dominio citoplasmático y en respuesta a la unión del ligando, con diversas proteínas con actividad quinasa entre las que destacan la quinasa de adhesión focal (FAK, Focal Adhesion Kinase) y la quinasa ligada a integrinas (ILK, Integrin-Linked-Kinase). Estas quinasas se encargan de reclutar y fosforilar proteínas señalizadoras y adaptadoras que conducen sus señales al citoesqueleto (*Mitra y col., 2005; Rhodes y Simons, 2007*). Las integrinas también intervienen en otras vías de señalización mediadas por Akt/PKB, PI3K, Rap 1, Rho GTPasas y MAPK (*Serini y col., 2006*).

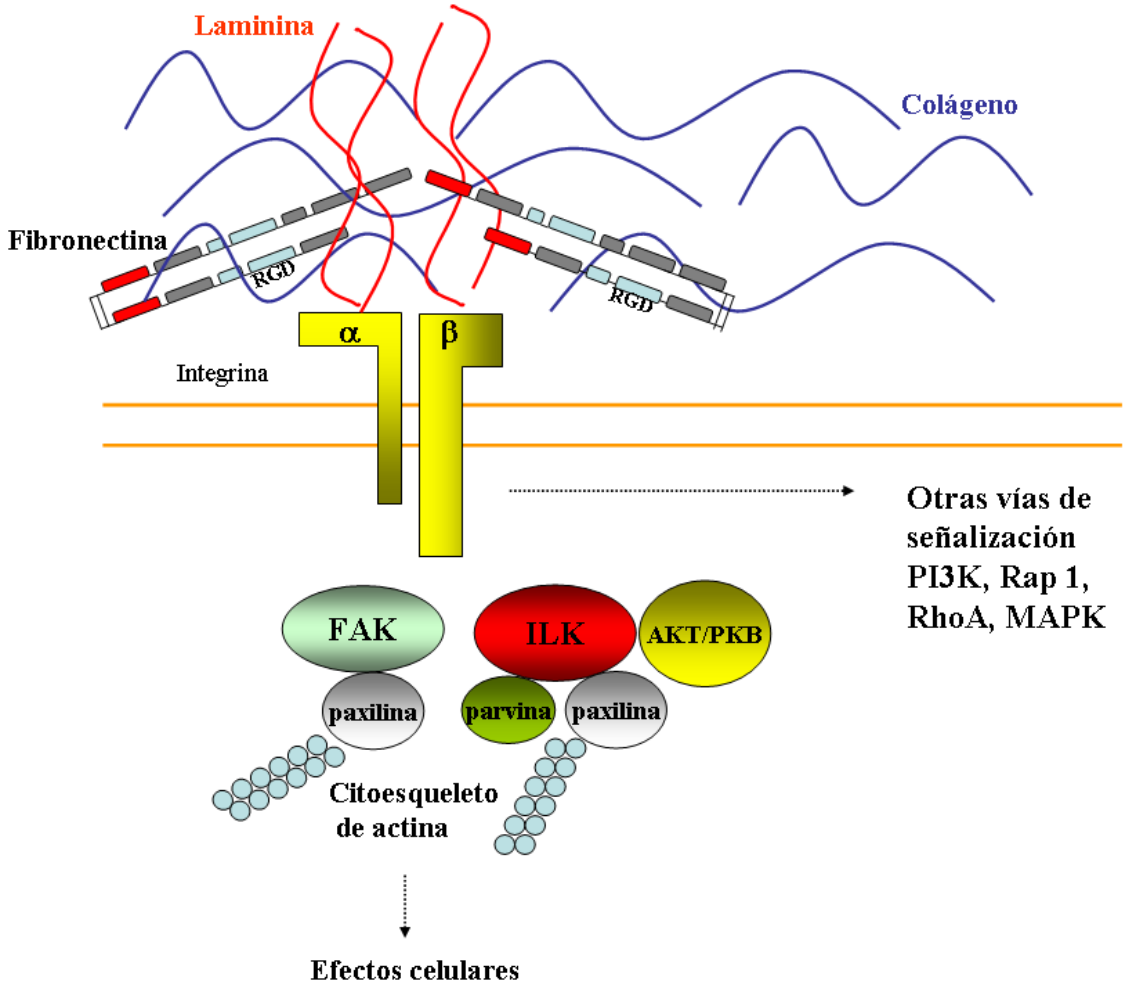

Figura 8. Interacción de las proteínas de matriz extracelular con integrinas.

4. 3. Otros receptores celulares de proteínas de matriz: importancia de las discoidinas.

Los receptores con dominio discoidina (DDRs, Discoidin Domain Receptors) son proteínas transmembrana que pertenecen a la familia de receptores tirosina kinasa (RTK, Receptor Tyrosine Kinase). En 1981 se identificó la primera proteína discoidina (DS, Discoidin Protein) en *Dictyostelium discoideum*; se describió como una lectina con alta afinidad por la galactosa y residuos modificados de galactosa (*Poole y col., 1981*). Posteriormente se han detectado dominios similares en muchas proteínas transmembrana y extracelulares, incluyendo factores de coagulación sanguíneos, enzimas, receptores y proteínas implicadas en el desarrollo neuronal (*Wu y Molday, 2003; Baumgartner y col., 1998; Vogel, 1999; Franco-Pons y col., 2006*).

Se han identificado dos genes distintos *Ddr1* y *Ddr2* que codifican las distintas proteínas discoidinas. DDR1 presenta 6 isoformas generadas por splicing alternativo (*Alves y col., 2001; Mullenbach y col., 2006*). DDR1 y DDR2 son proteínas de ≈ 105 y ≈ 130 KDa respectivamente y comparten cuatro dominios funcionales: (1) **dominio amino terminal** que contiene ≈ 160 aminoácidos que componen el dominio discoidina, (2) **dominio transmembrana,** (3) **un extenso dominio yuxtamembrana** y (4) **dominio catalítico** tirosina kinasa.

El papel biológico de los dominios discoidina se basa en sus interacciones con una gran variedad de moléculas como factores de crecimiento, fosfolípidos y lípidos neutros, galactosa y colágenos (*Franco-Pons y col., 2006; Curat y col., 2001*). Muchas de estas proteínas con dominios discoidinas están implicadas en adhesión celular, migración y organogénesis (*Hvarregaard y col., 1996; Arakawa y col., 2007*).
DDR1 se expresa en células epiteliales, glándula mamaria, cerebro, riñón, pulmón, y en la mucosa colónica. DDR2 se encuentra en músculo esquelético y cardiaco, riñón y piel (*Abdulhussein y col., 2004*). DDR1 se emplea como marcador tumoral ya que se encuentra sobreexpresado en cáncer de mama, colon, pulmón y esófago entre otros. También está implicado en distintas enfermedades como fibrosis pulmonar, insuficiencia cardiaca y ateroesclerosis (*Agarwal y col., 2007*).

DDR1 se activa por colágenos (tipo I a VI y VIII) mientras que DDR2 se estimula selectivamente por colágenos fibrilares (tipo I a III, V y también X) (*Vogel y col., 1997; Leitinger y Kwan, 2006*). La unión del colágeno al DDR induce una fosforilación de las tirosinas del dominio kinasa (*Vogel y col., 1997*; *Shrivastava y col., 1997*). El colágeno desnaturalizado o gelatina no induce la actividad kinasa de los DDRs. Cuando se produce una activación prolongada de los dominios kinasa se incrementan los niveles de MMPs tipo 1, 2, 9 y 13 encargados de la ruptura y degradación de las fibras de colágeno de la MEC (*Vogel y col., 1997*).

Las células musculares lisas procedentes de **ratones deficientes en DDR1 (*Ddr1*$^{-/-}$)** presentan una reducción en la proliferación, migración y actividad de las MMPs "*in vitro"* y la sobreexpresión de DRR1 revierte esos déficits (*Hou y col., 2002*). En consonancia con estos resultados, se ha observado que la sobreexpresión de DDR1 y DDR2 en células musculares lisas se produce además de un incremento de la expresión

del ARNm de MMP-1, una disminución de procolágeno α1 (*Ferri y col., 2004*). Por otro lado, se ha descrito que DDR2 inhibe fibrinogénesis de colágeno tipo 1 (*Mihai y col., 2006*). Con estos resultados podemos decir que los DDRs juegan un papel fundamental en el remodelado y recambio del colágeno.

5. CONSECUENCIA DE LA ACTIVACIÓN DE LAS INTEGRINAS EN EL FENOTIPO CELULAR.

5. 1. Células endoteliales y MEC.

La MEC proporciona un soporte crítico al endotelio vascular. Fundamentalmente, a través de sus interacciones adhesivas con las integrinas de la célula endotelial, la MEC actúa como sostén manteniendo la organización vascular y también está implicada en la regulación de otros procesos como la proliferación, migración, morfogénesis y supervivencia de las células endoteliales (*Davis y Senger, 2005*). Aunque la importancia de la MEC en todos estos procesos está ampliamente descrita en la bibliografía, no se conoce con exactitud la importancia de los componentes específicos de la matriz y con frecuencia resulta difícil establecerlo debido a un solapamiento funcional. Perruzzi y colaboradores demostraron que la proliferación y activación de MAPK de las células endoteliales eran similares cuando se crecían en colágeno I o vitronectina (*Perruzzi y col., 2003*). Por otro lado, existen estudios realizados en células endoteliales en cultivo, que demuestran que éstas exhiben un comportamiento diferente según se cultiven sobre una matriz similar a la matriz existente en el vaso sano o sobre colágeno tipo I. Gónzalez-Santiago y colaboradores vieron que la expresión de eNOS, la producción de nitritos y la actividad de ILK de las células endoteliales cultivadas en colágeno tipo I eran significativamente menores a las que presentaban las células cultivadas en colágeno tipo IV (*Gónzalez-Santiago y col., 2002*). Del mismo modo células endoteliales de vena de cordón umbilical humano (HUVEC, Human Umbilical Vascular Endotelial Cell) cultivadas sobre colágenos tipo I y IV mostraron un incremento en la expresión de endotelina 1 y una disminución en la actividad de ILK en las células crecidas en colágeno I respecto al colágeno IV. Esta inhibición era revertida cuando se trataba las células endoteliales con un anticuerpo β1 estimulante lo que sugería la implicación de esa integrina en el desarrollo de los cambios observados (*Ruiz-Torres y col., 2005*).

La composición de la MEC es muy importante en el proceso de migración celular. Así, Murasawa y colaboradores demostraron que el colágeno tipo V fibrilar inducía motilidad en células endoteliales glomerulares a través de la interacción con la integrina β1 activando la fosforilación de las proteínas paxilina y FAK (*Murasawa y col., 2008*). Van Horssen y colaboradores realizaron ensayos de migración de células endoteliales en diferentes tipos de matrices y demostraron que la fibronectina inducía migración y que este efecto era dependiente de concentración (*Van Horssen y col., 2006*).

La MEC en contacto con las células endoteliales juega un papel clave en el proceso de angiogénesis. Deroanne y colaboradores emplearon un modelo de tubulogénesis "*in vitro"* en el que cultivaron HUVECs en matrigel o matrigel suplementado con gelatina, y observaron una disminución de la expresión de la subunidad α2 de la integrina β1α2 (receptor principal de laminina y colágeno) y de las proteínas vinculina, talina, actina y actinina. La remodelación de los componentes de la placa de adhesión focal y citoesqueleto produjo cambios en el fenotipo de las células, que se organizaron formando túbulos. Cuando las células fueron cultivadas en matrigel suplementado con colágeno nativo (sin desnaturalizar) no se observaron cambios (*Deroanne y col., 2001*).

5. 2. Células musculares lisas y MEC.

En condiciones fisiológicas, las células musculares lisas están rodeadas por una MEC altamente estructurada compuesta principalmente por colágenos I y III, elastina y proteoglicanos. Estas proteínas de matriz son importantes en el mantenimiento de la estructura del tejido y tienen un papel clave en la función celular. Las células se unen a la matriz mediante receptores específicos tipo integrina y esta interacción puede afectar directamente en la función celular. Las señales pueden ser de tipo bioquímico como los iones o factores de crecimiento, o de tipo mecánico como la tensión, fuerzas de compresión o de cizallamiento, que son transmitidas por la MEC a la célula vía integrinas.

Durante la formación de los vasos el fenotipo de las células musculares lisas en la túnica media sufre una serie de modificaciones entre las que cabe destacar una

disminución en la producción de proteínas de matriz y un aumento en la formación de miofilamentos intracelulares. Esta transición, desde un estado "sintético" a "contráctil" es necesaria para que las células musculares lleven a cabo su función principal, que es la contracción y dilatación de la pared de los vasos para regular la presión y flujo sanguíneo. En condiciones fisiopatológicas, las células musculares pueden revertir su fenotipo contráctil a proliferativo; en este estado sintético las células pueden migrar de la túnica media a la región íntima (*Stegemann y col., 2005*).

Heidin y colaboradores demostraron que la fibronectina promueve la modulación de un fenotipo proliferativo en células musculares lisas (*Hedin y col., 1990*). Existen otros trabajos que han relacionado la MEC con la proliferación de estas células, incluyendo un papel autocrino para la trombospondina (*Majack y col., 1986*), y proliferación en respuesta a componentes de matriz sintetizados por el endotelio (*Herman y Castellot, 1987*). Cuando las células musculares lisas se trataron con alilamina, amina tóxica que se metaboliza produciendo acroleína y peróxido de hidrógeno, se produjo un daño oxidativo que promovió un fenotipo proliferativo (*Ramos y col., 1993*) y esta respuesta era dependiente de la integrina αV (*Parrish y Ramos, 1997*). Wilson y colaboradores demostraron que el fenotipo proliferativo inducido por alilamina en células musculares revertía a un fenotipo sintético cuando eran cultivadas en colágeno tipo I, no así en plástico, fibronectina o pronectina (*Wilson y col., 2002*).

La estructura de la MEC también es muy importante en la regulación de la función de estas células. Estudios llevados a cabo en matrices bidimensionales demostraron que los sustratos de fibronectina y colágeno tipo I inducían cambios hacia el estado sintético (*Hedin y Thyberg, 1987; Yamamoto y col., 1993*), mientras que laminina producía el efecto contrario (*Qin y col., 2000*). La vía de señalización de ERK juega un papel principal en la modulación del fenotipo de las células musculares (*Roy y col., 2001*), regulando los marcadores contráctiles (*Schauwienold y col., 2003*) y de síntesis (*Moses y col., 2001*). Stegemann y colaboradores demostraron que las células musculares crecidas en geles tridimensionales de colágeno tipo I presentaban una disminución significativa de la proliferación y una menor expresión de α-actina respecto a las células cultivadas en matrices bidimensionales (*Stegemann y Nerem, 2003*).

5. 3. Células mesangiales y MEC.

La acumulación de la MEC en los glomérulos renales es un fenómeno característico que se observa en numerosas enfermedades renales. Las células mesangiales son una fuente productora de proteínas de matriz en condiciones normales y patológicas. Esta síntesis está regulada por factores solubles y componentes no difusibles de la MEC (*Gauer y col., 1997*). Los componentes de la MEC pueden ejercer sus efectos sobre las células mesangiales indirectamente, debido a su capacidad de secuestrar y liberar citoquinas solubles y factores de crecimiento, o directamente vía receptores específicos de la MEC como las integrinas y discoidinas. Cuando se cultivaron células mesangiales sobre colágeno tipo IV o laminina, se observó un aumento en la secreción y deposición de otros componentes de la matriz extracelular (*Ishimura y col., 1989*). Por otro lado, las células epiteliales tubulares cultivadas en colágeno tipo IV presentaron un aumento significativo en la proliferación que no se observó en otras matrices como laminina o plástico (*Cybulsky y col., 1990*).

También se ha observado que las células mesangiales cultivadas en colágeno tipo I presentaban una mayor expresión de proteínas de matriz como colágeno tipo I –IV y fibronectina, así como un aumento en la actividad de ILK respecto a las células mesangiales cultivadas en colágeno tipo IV. Estos efectos fueron potenciados por la citoquina TGF-β1 y bloqueados por anticuerpos anti-integrina αVβ1 (*Ruiz-Torres y col., 2005*).

La sobreexpresión de la integrina α1β1, receptor principal de colágeno y vinculina, produjo una inhibición del crecimiento de las células mesangiales, hipertrofia, un aumento en la expresión de α-actina y remodelación de la matriz de colágeno (*Kagami y col., 2000*). La integrina α8 β1 se expresa en glomérulo y arteriolas renales. Es el receptor principal de fibronectina, vitronectina y osteopontina en riñón. Se ha observado que los ratones deficientes en la subunidad α8 presentaban defectos muy importantes en el desarrollo glomerular (*Müller y col., 1997*).

La composición de la MEC tiene un papel importante en la regulación de la GCs. De Frutos y colaboradores observaron que las células mesangiales crecidas en colágeno tipo I presentaban una menor expresión proteica de GCs respecto a las células

cultivadas en colágeno IV (*De Frutos y col., 2005*). Por otro lado, Díez-Marqués y colaboradores demostraron que el RGDS, secuencia que se encuentra en la fibronectina, incrementaba la expresión de la subunidad β1 de la GCs. Este efecto era bloqueado con anticuerpos anti-integrinas αVβ1 (*Díez-Marqués y col., 2006*).

6. RAS GTPASAS EN LA REGULACIÓN DE LA FUNCIÓN VASCULAR.

6. 1. Ras GTPasas: Estructura, composición y regulación.

La superfamilia de las proteínas Ras monoméricas de unión a GTP está compuesta por las subfamilias de proteínas Ras, Rho, Ran, Rab, Rac, Rheb, Arf y Kir/ReM/Ras. A su vez, la subfamilia Ras está compuesto por las proteínas clásicas Ras (p21 Ras: H-K y N-Ras), M-Ras, R-Ras (R-Ras, TC21, R-Ras23), Rap (Rap 1A, Rap 1B, Rap 2A, Rap 2B) y Ral (Ral A, Ral B). En eucariotas, las proteínas Ras están altamente conservadas desde levaduras a humanos. Se han identificado tres protooncogenes, *Hras-1*, *Nras* y *Kras-2* que codifican las proteínas H-Ras (o Ha-Ras), N-Ras y K-Ras (o Ki-Ras) respectivamente. A su vez, K-Ras presenta dos isoformas Ki(A)-Ras o (K-Ras4A) y Ki(B)-Ras o (K-Ras4B). Las proteínas Ras se expresan ubicuamente y tienen un peso molecular ≈ 21 KDa (*Takai y col., 2001*). Funcionan como relojes intracelulares en las vías de transducción de señales controlando numerosas acciones biológicas como el crecimiento y diferenciación celular, o la proliferación y apoptosis en condiciones fisiológicas (*Rojas y Santos, 2002*).

Las proteínas Ras presentan actividad reguladora GTP-hidrolasa y alterna dos conformaciones estructurales, bien unidas a GTP (activas) o GDP (inactivas). En su estado inactivo, el factor intercambiador de nucleótido de guanina (GEF, Guanine Nucleotide Exchange Factor) aumenta la reacción de intercambio GDP/GTP promoviendo el estado activo Ras-GTP. Debido a su actividad GTPasa intrínseca, la proteína Ras vuelve a su estado inactivo Ras-GDP finalizando la vía de señalización RasGTPasa. La proteína activadora de GTPasa (GAP, GTPase-Activating Protein) favorece la hidrólisis de Ras-GTP (*Martínez-Salgado y col., 2008*).

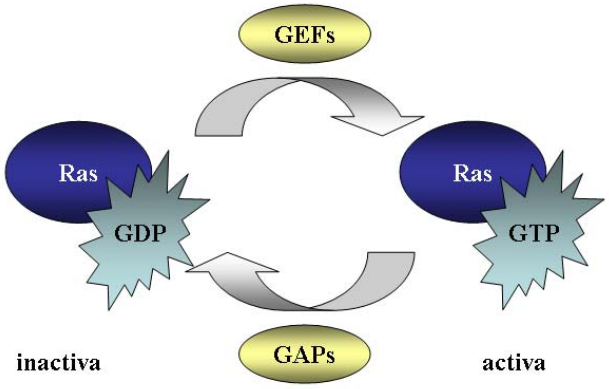

Figura 9. Posibles conformaciones de las Ras GTPasa monoméricas.

Las proteínas Ras sufren modificaciones postransduccionales, generalmente la adición de un grupo prenilo en el extremo carboxitermal, ya sea farnesil o geranilgeranil, que le permite anclarse a la membrana plasmática donde se asocian a receptores transmembrana tipo integrinas, receptores tirosina kinasa (RTKs, Receptor Tyrosine Kinases) y receptores acoplados a proteínas G (GPCRs, G Protein-Coupled Receptors) (*Philips y Cox, 2007*). Cuando están en su conformación activa actúan sobre numerosos efectores, los más destacados son las vías de señalización RAF-MEK-ERK1/2 y PI3K-Akt (*Rodríguez-Peña y col., 2005*).

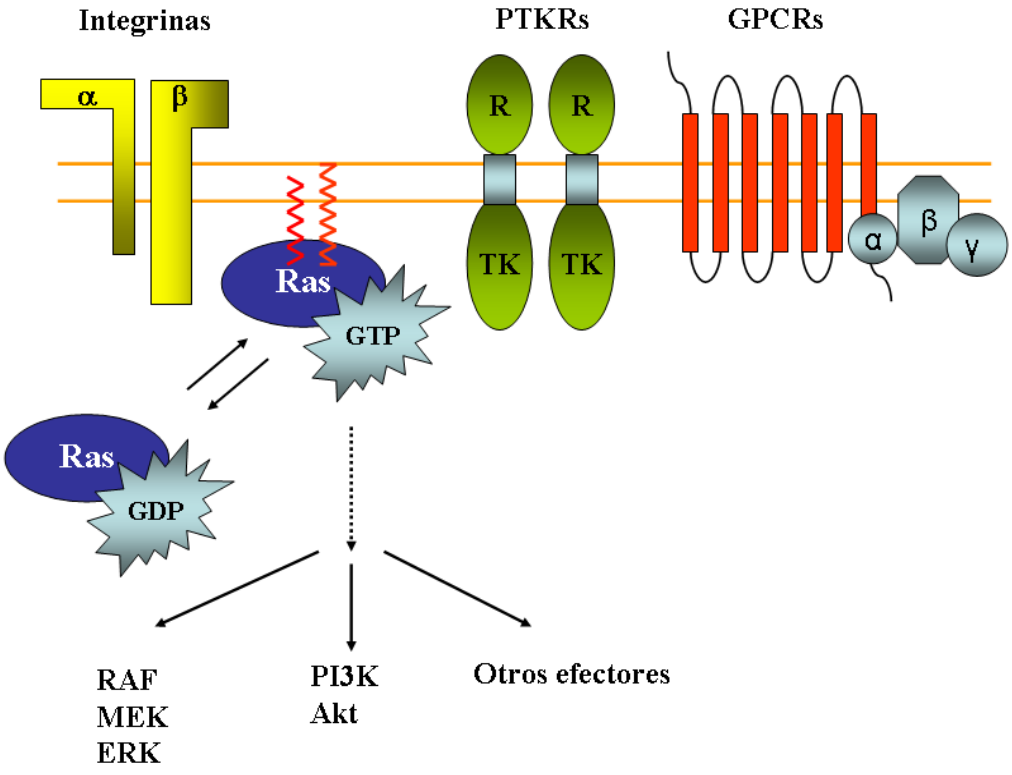

Figura 10. Localización subcelular y efectores principales de las Ras GTPasas monoméricas.

En determinadas ocasiones se producen mutaciones puntuales en el protooncogen Ras produciendo una proteína GTPasa constitutivamente activa, unida a GTP, que estimula proliferación celular, este proceso conlleva la formación de tumores (*Schubbert y col., 2007*). Las mutaciones en las isformas H-Ras y N-Ras están asociadas a cáncer de vejiga y leucemia, mientras que en el 90% de los casos de cáncer de páncreas y el 50% de colon la isoforma K-Ras está mutada (*Bos, 1989; Mo y col., 2007*).

Los ratones deficientes en H-Ras, N-Ras o K(A)-Ras son viables y la mutación no afecta en su esperanza de vida (*Koera y col., 1997; Esteban y col., 2001*); por el contrario **los ratones deficientes en la isoforma K(B)-Ras** mueren durante la embriogénesis entre los días doce y catorce. K(B)-Ras es esencial en el desarrollo embrionario (*Johnson y col., 1997; Plowman y col., 2003*).

6. 2. Ras GTPasas y regulación de la síntesis de la MEC.

Desde hace años se sabe que pacientes que sufren distintas patologías como hipertensión y nefropatía diabética sufren fibrosis renal que se caracteriza por una excesiva deposición de ECM en el instersticio y/o mesangio glomerular (*Johnson y col., 1992*). En estos procesos el TGF-β juega un papel muy importante, existiendo una estrecha relación entre las vías de señalización del TGF-β y Ras. TGF-β es un activador de Ras y está aumentado tanto en la fibrosis renal humana como en distintos modelos experimentales de fibrosis renal donde juega un papel principal en la génesis de daño renal (*Rodríguez-Peña y col., 2005*). Experimentos llevados a cabo por Martínez-Salgado y colaboradores, con fibroblastos procedentes de ratones deficientes en H- y N-Ras, mostraron una mayor acumulación de proteínas de matriz y activación de Akt que los fibroblastos control (*Martínez-Salgado y col., 2006*). La activación de Akt está relacionada con la síntesis de MEC porque la inhibición de PI3K disminuye la expresión de fibronectina y colágeno tipo 1 (*Wisdom y col., 2005*). Por otro lado la pérdida de las isoformas H y N-Ras estaba acompañada por una menor proliferación en respuesta a TGF-β, junto con una disminución en la fosforilación de ERK. Estos resultados sugieren que H y N-Ras disminuyen la síntesis de ECM y median proliferación, en parte, a través de la activación de MEK/ERK (*Martínez-Salgado y col., 2006*).

6. 3. Posible papel de las Ras GTPasas en la regulación de la contracción – relajación vascular.

En el campo de la regulación del tono vascular sabemos que las proteínas Ras aumentan la sensibilidad al calcio en la contracción del músculo liso (*Satoh y col., 1993*). La angiotensina II, potente vasoconstrictor, actúa activando la vía Ras GTPasa/Raf1/MEK1 produciendo la contracción de los vasos (*Roffe y col., 1996; Benter y col., 2005*). Algunos autores proponen que H-Ras podría regular la expresión de eNOS vía PI3K-Akt (*Jeon y col., 2004*).

Thorburn y colaboradores estudiaron el papel del protooncogen H-Ras en el crecimiento celular cardiaco y la hipertrofia en un modelo "*in vitro"* de miocitos ventriculares de rata. H-Ras no indujo proliferación celular aunque si se observó un aumento de la expresión de los genes c-fos y del factor natriurético atrial (ANF, Atrial Natruretic Factor). La expresión de ambos genes está asociado con la respuesta hipertrófica en los miocitos ventriculares sugiriendo que Ras está implicado en la vía de señalización hipertrófica (*Thorburn y col., 1993*). Siguiendo esta línea, Hunter y colaboradores sobreexpresaron el oncogen H-Ras en ventrículos de ratones observando una inducción de la hipertrofia y una disfunción diastólica (*Hunter y col., 1995*).

En ratones genéticamente modificados en los que se reemplazó K-Ras por H-Ras se observó que estos animales eran viables pero sufrían cardiopatías asociadas con hipertensión arterial, lo cual sugiere que las proteínas Ras podrían tener un papel importante en la homeostasis cardiovascular (*Potenza y col., 2005*). Existen evidencias recientes del papel de las proteínas Ras en el proceso de contracción–relajación en un estudio realizado en ratas espontáneamente hipertensas sometidas a un tratamiento crónico de L-NAME. Cuando estos animales se trataron posteriormente con el inhibidor de la proteína farnesil transferasa III (FPTIII, Farnesyl Protein Transferase Inhibitor III), se observó una disminución de la presión arterial y daño renal (*Benter y col., 2005*). Por último, Schuhmacher y colaboradores generaron ratones que sobreexpresaban H-Ras constitutivamente. Estos animales presentaron alteraciones en la homeostasis del sistema cardiovascular, incluyendo el desarrollo de una hipertensión sistémica, remodelación vascular extensiva y fibrosis en corazón y riñón (*Schuhmacher y col., 2008*).

HIPÓTESIS

HIPÓTESIS

El sistema de NO-GCs-PKG es uno de los principales reguladores del grado de contracción-relajación vascular, y la modulación de cada uno de sus componentes puede condicionar el nivel de activación del sistema. Existen evidencias que demuestran que la expresión y la actividad del NO y de la GCs dependen de la interacción entre células y matriz extracelular, y que las integrinas juegan un papel crítico en esta interacción.

Existe la posibilidad del que el contenido celular y el grado de activación de la PKG dependa también de la interacción células-matriz, a través de la modulación de integrinas, y de que sea posible modular el nivel de expresión tanto de GCs como de PKG, mediante la activación de estas integrinas, condicionando así un mayor grado de vasodilatación. Por otra parte, otros sistemas de señalización celulares ligados a la activación de proteínas transmembrana, en concreto *Ras*, podrían jugar también un papel crítico en la regulación del sistema NO-GCs-PKG.

OBJETIVOS

OBJETIVOS

Objetivo general

Analizar en profundidad los mecanismos reguladores de los sistemas intracelulares efectores del NO, en concreto la GCs y PKG, y su importancia fisiopatológica.

Objetivos específicos

1. **Estudiar el efecto de la modulación de integrinas en la expresión de GCs, en condiciones basales y fisiopatológicas.**

- Analizar el efecto del tirofiban en la expresión de GCs "*in vitro"* en distintos tipos celulares.

- Estudiar el efecto del tirofiban en la expresión de GCs "*in vivo"* y sus consecuencias funcionales.

- Estudiar el papel del tirofiban en la regulación de GCs en un modelo genético de hipertensión arterial sistémica.

2. **Estudiar el efecto de la modulación de integrinas en la regulación de PKG.**

- Analizar las consecuencias de la activación de integrinas en la expresión de PKG y sus vías de señalización.

- Evaluar, "*in vivo",* las consecuencias funcionales de la modulación de PKG tras la activación de integrinas.

3. Analizar la importancia de H-Ras en la regulación de la presión arterial.

- Estudiar los cambios en la presión arterial en ratones deficientes en H-Ras.

- Analizar los mecanismos moleculares implicados en los cambios en la presión arterial, en particular la importancia del sistema NO-GCs-PKG.

RESULTADOS

Tirofiban increases soluble guanylate cyclase in rat vascular walls: pharmacological and pathophysiological consequences.

Ruiz-Torres MP, Griera M, Chamorro A, Díez-Marqués ML, Rodríguez-Puyol D and Rodríguez-Puyol M.

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La guanilato ciclasa soluble (GCs) actúa como receptor intracelular del óxido nítrico. Los cambios en el contenido de esta proteína se han relacionado con la disfunción endotelial en determinadas enfermedades cardiovasculares como la hipertensión, aterosclerosis y diabetes. Nuestro grupo publicó recientemente que el péptido RGDS (Arg-Gly-Asp-Ser), a través de su interacción con integrinas, incrementa el contenido de la subunidad β1 de la GCs, favoreciendo la respuesta a los donadores de óxido nítrico en células musculares lisas de rata. Por ello, se quiso evaluar la capacidad de análogos del RGD, como el tirofiban, para modular el contenido vascular de GCs en animales de experimentación, en condiciones basales y en situaciones fisiopatológicas.

En primer lugar, se confirmó que el tirofiban incrementa los niveles proteicos y RNAm de la GCs β1 en distintas células contráctiles y en distintas especies: células musculares lisas de rata y humanas así como en células mesangiales humanas. Este efecto dependía de integrinas, ya que desaparecía cuando las células se incubaron con un anticuerpo bloqueante de la isoforma β1. El efecto "*in vivo"* del tirofiban fue estudiado en ratas Wistar observándose un incremento de la GCs β1 en forma tiempo y dosis-dependiente. Para analizar las consecuencias funcionales de esta sobreexpresión proteica dependiente del tirofiban, se analizaron los cambios en la presión arterial de ratas Wistar en respuesta a la administración ip de un donador de óxido nítrico, nitroprusiato sódico, con o sin pre-tratamiento de tirofiban. Las ratas pre-tratadas con tirofiban mostraron una mayor respuesta hipotensora así como un incremento en la producción de GMPc en anillos procedentes de aortas. El uso de un antagonista específico de la GCs, el ODQ, revertió el efecto. Para confirmar estos resultados, se realizó un estudio de contracción vascular en tiras de aorta desendotelizadas, incubadas con tirofiban o vehículo. Se observó una mayor respuesta vasorelajante, al incubar los anillos con nitroprusiato sódico, tras el pretratamiento con tirofiban. El efecto fue bloqueado con el ODQ, demostrando así su dependencia de la GCs.

La importancia fisiopatológica de esta modulación se analizó en dos contextos. En primer lugar, en relación con un fenómeno bien conocido que se observa tras la administración de nitritos, la taquifilaxia al tratamiento. Se confirmó que el tratamiento crónico con dinitrato de isosorbide redujo los niveles proteicos de la guanilato ciclasa en aorta, con la subsiguiente pérdida de respuesta a la administración de nitroprusiato.

Estos resultados se revirtieron con la administración de tirofiban, restaurando la respuesta hipotensora al donador de óxido nítrico.

En segundo lugar, se estudió el efecto del tirofiban en un modelo genético de hipertensión sistémica, ratas espontáneamente hipertensas (SHR). El tirofiban incrementó el contenido proteico de GCs en aortas de ratas SHR y favoreció la respuesta hipotensora al nitroprusiato sódico alcanzando niveles en la presión arterial sistólica similares a las ratas control. Estos resultados apoyan la utilidad del tirofiban y por extensión la modulación de integrinas, en el tratamiento de enfermedades cardiovasculares.

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Tirofiban increases soluble guanylate cyclase in rat vascular walls: pharmacological and pathophysiological consequences

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Arterial pressure;
Integrin

Aims Our aim was to evaluate whether tirofiban, which mimics the structure of arginine-glycine-aspartic acid (RGD) peptides, up-regulates soluble guanylate cyclase β1 subunit (sGC-β1) expression in vascular smooth muscle cells (VSMCs) and in aorta from rats, and to investigate the pharmacological and pathophysiological consequences of this up-regulation.

Methods and results Wistar, Wistar Kyoto, and spontaneously hypertensive rats (SHRs) were used. sGC-β1 content was assessed by immunoblotting. Arterial pressure was recorded using a tail-cuff sphygmomanometer. Sodium nitroprusside (SNP) and isosorbide dinitrate (IDN) were used as nitric oxide (NO) donors. Tirofiban increased the sGC-β1 content in VSMCs and in aortic walls from rats after 6 h of treatment. Rats treated with tirofiban experienced a more pronounced decrease in their arterial pressure after acute SNP treatment than vehicle-treated rats. Isolated rat aortic rings incubated with tirofiban showed a higher relaxing response to SNP than control rings as well as an increased sGC-β1 content and SNP-induced cyclic guanosine monophosphate synthesis. Animals receiving IDN for 1 week showed decreased sGC-β1 in aortic walls and did not respond to SNP treatment with changes in arterial pressure. Tirofiban restored the decreased sGC-β1 content in IDN-treated rats and promoted a decreased arterial pressure in response to SNP administration. SHRs showed reduced sGC-β1 levels, and tirofiban increased these levels and led to a higher response to SNP.

Conclusion Tirofiban increased the sGC-β1 content in contractile cells and aortic walls of rats, enhancing the response to SNP and reversing the NO donor tachyphylaxis.

1. Introduction

Soluble guanylate cyclase (sGC) acts as the nitric oxide (NO) receptor,[1] and changes in sGC levels have been related to endothelial dysfunction in some cardiovascular diseases such as hypertension, atherosclerosis, and diabetes.[2,3] The mechanisms responsible for the down-regulation of sGC in these pathological conditions have not been extensively explored, although in the case of lead-induced hypertension, it has been attributed to reactive oxygen species.[4]

An sGC deficiency has been described in the presence of NO. In fact, the continuous exposure of cells to NO leads to the increased degradation of the enzyme.[5] NO donors are currently used for the treatment of ischaemic cardiomyopathy,[6] and they have also been proposed for the treatment of other diseases such as other arteriopathies,[7] acute and chronic inflammatory conditions,[8] and degenerative diseases.[9] However, the fact that tolerance builds up after chronic administration has precluded their therapeutic usefulness.

Taking these data into account, it could be proposed that the up-regulation of sGC in vascular walls may be a useful strategy for the prevention or treatment of different pathological conditions, particularly cardiovascular diseases. However, no previous studies have analysed this possibility. We have recently published that the peptide RGDS (Arg-Gly-Asp-Ser) promotes an increased sGC-β1 subunit content in rat vascular smooth muscle cells (RVSMCs), enhancing the response to NO donors.[10] These *in vitro* findings would suggest that the systemic administration of RGD analogues could modify the vascular content of sGC

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in vivo, but a direct evaluation of this hypothesis has not been performed.

Tirofiban is an inhibitor of the platelet receptor glycoprotein IIb-IIIa, also known as integrin α_{IIb} β_3, and is used as a potent anti-thrombotic drug.[11] Tirofiban was developed based on the structure of the disintegrin echistatin, optimizing the tyrosine analogue that structurally mimicked the RGD-loop.[12] Disintegrins are low-molecular-weight proteins derived from snake venom which contain RGD or KGD motifs in their structure and interact with specific integrins to modulate their function.[13] RGD motifs are present in many extracellular matrix proteins, such as fibronectin, and are the binding sites to integrins for many of them. RGD-related peptides have been used to analyse integrin function for a long time. The role of RGD motifs has been described in the regulation of angiogenesis, cell adhesion and survival, and TGF-β1 expression.[14–16]

In this paper, we demonstrate that the administration of tirofiban to rats increases the sGC content in vascular walls. As a consequence of this up-regulation, the pharmacological responses to NO donors increase, the tolerance associated with long-term nitrite treatment improves, and the ability of NO donors to reduce blood pressure in hypertensive rats increases.

2. Methods

2.1 Studies in animals (*in vivo* and *ex vivo*)

Twelve-week-old male Wistar, Wistar-Kyoto (WKY) rats, and spontaneously hypertensive rats (SHRs) were obtained from Harlan Charles River (Germany). Animals were housed in a pathogen-free, temperature-controlled room (22 ± 2°C). Food and water were available *ad libitum*. Arterial pressure (MAP) was measured in conscious animals using a tail-cuff sphygmomanometer (LE 5001 Pressure Meter, Letica Scientific Instruments, Hospitalet, Spain) as described previously.[17] The preparation of the aortic rings was essentially similar to that described.[18] Aortas were treated with 0.3 mg/mL saponin in Krebs buffer for 10 min, to remove endothelium, rinsed, and cut into segments. Segments were incubated for 3 h with tirofiban or vehicle, and then contracted with noradrenaline (100 μmol/L, NA). After that, the bath was replaced, aortic rings were treated with different doses of sodium nitroprusside (SNP), and tension was recorded. Some experiments were performed in the presence of 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (1 μmol/L, ODQ). The investigation conforms with the Guide for the care and use of laboratory animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996) and was supervised and approved by the veterinary authority of the animal facilities from the Universidad de Alcalá.

2.2 Cell cultures

Rat vascular smooth muscle cells were obtained from thoracic aortas of Wistar rats using previously described methods.[10] Human aortic smooth muscle cells (HASMCs) were a gift from Drs Peiro and Sánchez-Ferrer.[19] Human mesangial cells (HMCs) were obtained from nephrectomy specimens histologically free of lesions and cultured according to previously described procedures.[20] Approval was granted by the Hospital Universitario Principe de Asturias Ethics Committee. The use of human cells conforms with the Declaration of Helsinki.

2.3 Analytical procedures

Cyclic guanosine monophosphate (cGMP) was determined by radioimmunoassay in endothelium-denuded aortic rings.[21] For western blot analysis, cell or thoracic aorta was washed in PBS and lysed for 30 min at 4°C. For immunoblot analysis, a rabbit polyclonal antibody against sGC-β1 was used.[10] The blots were reblotted with an antibody against α-actin to guarantee that an equal amount of protein was loaded in each case. For northern blot analysis, total RNA was extracted from cells as described[22] and transferred to a nitrocellulose membrane. Hybridization was performed as described previously.

2.4 Statistical analysis

The data are presented as the mean ± SEM of a variable number of experiments (see each figure legend). The differences between groups were analysed by one-way ANOVA. Two-way ANOVA was used to analyse the results shown in *Figures 4A* and *6*. *Post hoc* pair comparisons were performed using the least-significant difference (LSD) test. For the analysis of the measure of mean arterial pressure during 30 min, the linear slope of change for each animal was computed and compared between different treatment groups using an ANOVA *F*-test. *Post hoc* pair comparisons were performed by means of an LSD test. The effect of the length of treatment was analysed for each treatment using an ANOVA *F*-test and the LSD test. A value of $P < 0.05$ was considered statistically significant. Each experiment was repeated at least five times.

A more extensive description of the methods can be found in Supplementary material online.

3. Results

3.1 Tirofiban increases soluble guanylate cyclase β1 content in rat and human contractile cells

Rat vascular smooth muscle cells were treated with tirofiban or vehicle for different periods of time (*Figure 1A*) or for 6 h with vehicle or different doses of tirofiban (*Figure 1B*). The results show that tirofiban increased the sGC-β1 protein content in a time- and dose-dependent manner, reaching its maximum effect at 50 μmol/L after 6 h of treatment. At this same time and concentration, tirofiban also increased the steady-state sGC-β1 mRNA expression (*Figure 1C*). The stimulatory effect of tirofiban (50 μmol/L after 6 h) on sGC-β1 content was blunted by preincubation of the cells with specific anti-integrin-blocking antibodies (*Figure 1D*). Similar changes in the sGC-β1 content were observed in human contractile cells, HASMCs and HMCs, incubated with tirofiban (see Supplementary material online, *Figure S1*).

3.2 Tirofiban increases soluble guanylate cyclase β1 content in the aortic walls of rats and enhances the vascular effects of the nitric oxide donor sodium nitroprusside

To analyse the *in vivo* effect of tirofiban, rats were treated with three consecutive doses of intraperitoneal (ip) tirofiban (50 μg/kg b.w., times 0, 2, and 4 h), and the aortic sGC-β1 content was measured at different times after the first injection (*Figure 2A*). In a similar way, variable doses of tirofiban were given to rats at times 0, 2, and 4 h, and sGC-β1 content was analysed at 6 h (*Figure 2B*). The sGC-β1 increased in aortic walls from tirofiban-treated rats, in a time- and dose-dependent way.

To test the functional consequences of tirofiban treatment, rats were treated with three consecutive doses of ip tirofiban for 6 h (2 h between doses) or vehicle, and then SNP was administered, also ip. Arterial pressure was

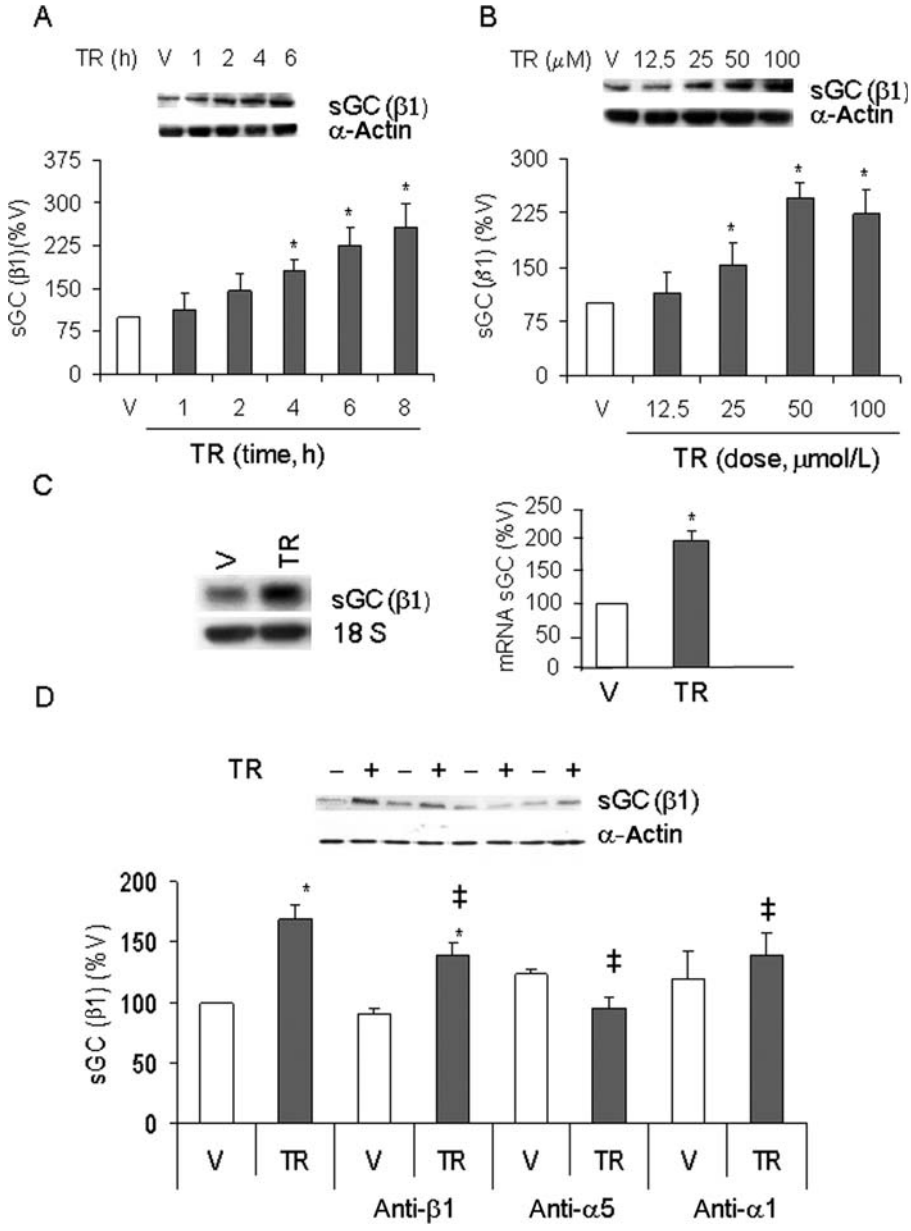

Figure 1 Tirofiban (TR) increases the soluble guanylate cyclase β1 (sGC-β1) protein and mRNA in rat vascular smooth muscle cells (RVSMCs) through integrin-dependent mechanisms. RVSMCs were treated with tirofiban (50 μmol/L, grey bars) for different periods of time or vehicle V (white bar) (*A*) or for 6 h with different doses of TR or V (*B*), and the sGC-β1 protein was evaluated by western blot. The steady-state sGC-β1 mRNA expression in the presence of TR was assessed by northern blot (*C*). RVSMCs were also treated with TR (50 μmol/L) or V for 6 h either in the presence or absence of specific integrin-blocking antibodies (10 μg/mL anti-β1, 30 μg/mL anti-α5, and 30 μg/mL anti-α1), and sGC-β1 protein content was measured (*D*). A representative blot is shown in each case. The bar graphs represent the densitometric analysis of the bands and are expressed as the ratio between sGC-β1 and α-actin. The results are the percentage of V and are the mean ± SEM of five different experiments.*$P < 0.05$ vs. V, in the same experimental conditions. ‡$P < 0.05$ vs. tirofiban-treated cells without blocking antibodies.

measured through all treatments, and tirofiban treatment alone did not modify arterial pressure (see Supplementary material online, *Figure S2*). SNP administered after vehicle led to a significant decrease in the arterial pressure, an effect that started at 15 min and was maximal (15.5 ± 1.4 mmHg lower than basal values) at 30 min. When both tirofiban and SNP were administered, the drop in the arterial pressure was more pronounced, reaching a difference at 30 min of 31.1 ± 4.5 mmHg, with respect to basal values (*Figure 3A*). Additionally, the SNP-induced cGMP synthesis was measured in rat aortas isolated from rats, after 6 h of treatment with tirofiban (50 μg/kg b.w., times 0, 2, and 4 h) or vehicle, and treated with SNP 30 min in the absence or presence of ODQ. Results showed an increase in the cGMP production in the aortic rings exposed to SNP, which was higher in aortas from tirofiban-treated rats. Incubation of rings with ODQ inhibited the increase in cGMP (*Figure 3B*).

To analyse the direct effect of tirofiban in vascular walls, rat aortic rings were isolated, endothelium was removed, and rings were incubated with vehicle or tirofiban during 3 h. Thereafter, NA was added to the rings to promote maximum vasoconstriction, and the mechanical force was recorded in the presence of different SNP concentrations.

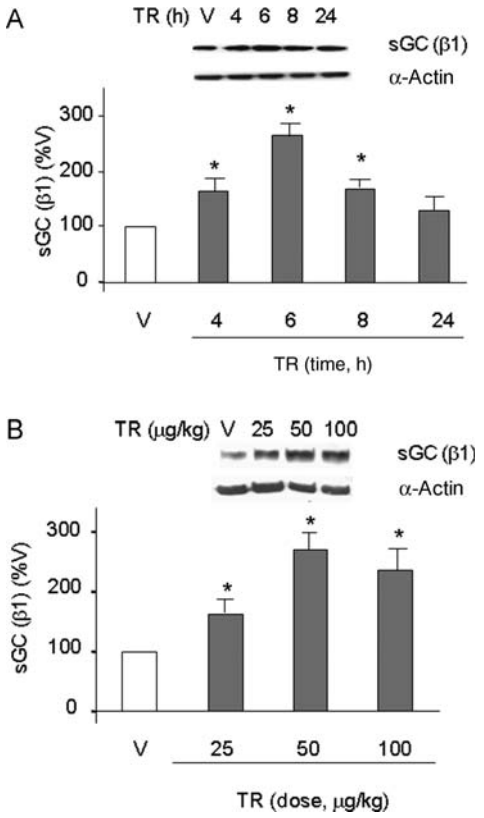

Figure 2 Tirofiban (TR) increases the soluble guanylate cyclase β1 (sGC-β1) content in the aorta of rats in a time- and dose-dependent manner. (*A*) Rats were treated with tirofiban (three consecutive doses of 50 μg/kg b.w., at times 0, 2, and 4 h) (TR, grey bars) or vehicle (V, white bar). Aortas were removed at the times showed, proteins were extracted, and the sGC-β1 protein content was assayed by immunoblotting. (*B*) A similar analysis was performed at 6 h after the first injection, but with different doses of TR. In both panels, a representative blot is shown. The bar graphs represent the densitometric analysis of the bands and are expressed as the ratio between sGC-β1 and α-actin. The results are the percentage of V and are the mean ± SEM of five different animals. $^{*}P < 0.05$ vs. V.

Some experiments were performed with ODQ. No changes were found in response to NA after tirofiban or vehicle administration (VH: 0.8 ± 0.15; TR: 0.92 ± 0.16 absolute units). The results showed that aortic rings preincubated with tirofiban were more responsive to the relaxing effect of SNP, an effect that was inhibited by ODQ (*Figure 4A*). The incubation of the endothelium-denuded aortic rings with tirofiban (50 μmol/L, 3 h) also increased the sGC-β1 content (*Figure 4B*) and the SNP-induced cGMP synthesis, an effect that was inhibited by ODQ (*Figure 4C*).

3.3 Tirofiban reverses the down-regulation of soluble guanylate cyclase content promoted by chronic treatment with nitric oxide donors and nitric oxide donor tachyphylaxis

Rat vascular smooth muscle cells incubated with SNP for 24 h had lower sGC-β1 content than control cells. Adding tirofiban in the last 6 h of SNP treatment reversed this down-regulation (*Figure 5A*). Similar results were observed in rats. Animals treated for 1 week with isosorbide dinitrate (IDN) in their drinking water showed a significant reduction in sGC-β1 content in their aortas (*Figure 5B*). The administration of tirofiban for the last 6 h (three consecutive

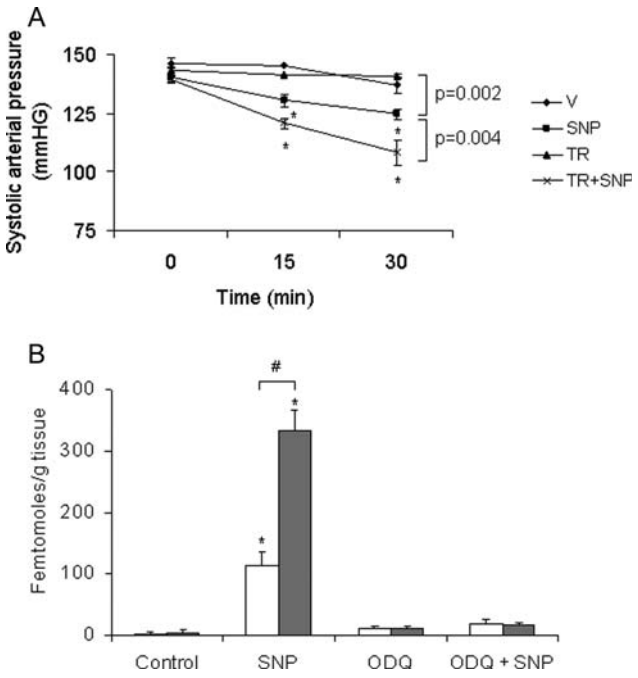

Figure 3 Tirofiban (TR) increased the hypotensive effect of sodium nitroprusside (SNP). (*A*) Rats were treated with three consecutive intraperitoneal doses of TR (50 μg/kg b.w., 2 h between injections) or vehicle (V). Six hours after the first injection, a single dose of SNP (10 μg/kg b.w.) or vehicle was administered intraperitoneally. The arterial pressure was recorded for 30 min afterwards by using a tail sphygmomanometer. $^{*}P < 0.05$ vs. time 0. (*B*) Rats were treated as in (*A*) with TR (grey bars) or V (white bars) and aortas were isolated. Cyclic guanosine monophosphate synthesis was measured after SNP addition (1 μmol/L, 30 min) in the presence or absence of ODQ (1 μmol/L). Results are mean ± SEM from six different experiments. $^{*}P < 0.05$ vs. control. $^{\#}P < 0.05$ vs. vehicle.

doses of 50 μg/kg b.w., every 2 h) of the seventh day normalized the aortic sGC-β1 content (*Figure 5B*).

The functional effect of treating rats with tirofiban after IDN administration was analysed by recording the arterial pressure after a single ip dose of SNP (*Figure 5C*). Rats were treated for 1 week with or without IDN, then administered vehicle or tirofiban for the last 6 h of this time frame, and then given SNP. Arterial pressure was recorded for 30 min. SNP induced a moderate but significant decrease (15.2 ± 0.8 mmHg) in the arterial pressure after 30 min of treatment. However, no changes in the blood pressure were observed in rats treated successively with IDN and SNP (*Figure 5C*). This lack of response was reversed by tirofiban treatment. In fact, in animals that were administered IDN and tirofiban, the SNP reduction in the arterial pressure was ∼13.8 ± 1.8 mmHg (*Figure 5C*).

3.4 Tirofiban increases the antihypertensive effect of nitric oxide donors in a model of genetic hypertension with decreased soluble guanylate cyclase vascular content

The aortic sGC-β1 content was lower in SHRs than in WKY rats (*Figure 6A*). The treatment with tirofiban significantly increased this protein in both groups of animals (*Figure 6A*). Administering SNP significantly decreased the arterial pressure of WKY rats and SHRs, an effect that was enhanced by tirofiban in both strains (*Figure 6B*). Only when the SHRs were treated with tirofiban plus SNP were

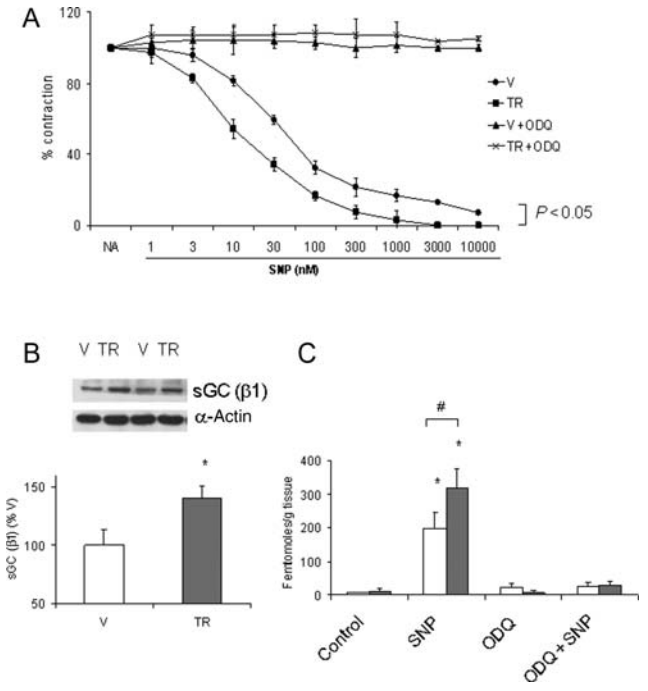

Figure 4 Tirofiban (TR) increased the vascular reactivity to sodium nitroprusside (SNP) in endothelium-deprived aortic rings, an effect that was blunted by the soluble guanylate cyclase (sGC) inhibitor ODQ. (*A*) Aortic rings of rats were isolated and suspended in an organ bath. The rings were treated with TR (50 μmol/L) or vehicle (V) for 3 h in the presence or absence of ODQ (1 μmol/L) and then contracted with noradrenaline (NA) (100 μmol/L). Later, different doses of SNP (ranging from 0.1 nmol/L to 1 μmol/L) were added to each ring and the changes in isometric force were recorded. The results are the mean ± SEM of 10 different experiments. $^{*}P < 0.05$ vs. aortic rings treated with vehicle at the same SNP dose. (*B*) Aortic rings were incubated with TR (50 mmol/L, grey bars) or V (white bars) for 3 h and the sGC-β1 content was evaluated by immunoblotting. A representative blot is shown. The bar graph represents the densitometric analysis of the bands and is expressed as the ratio between sGC-β1 and α-actin. The results are the percentage of V and are the mean ± SEM of five different animals. $^{*}P < 0.05$ vs. V. (*C*) Aortic rings were incubated with TR or V in the presence or absence of ODQ (1 μmol/L) and cyclic guanosine monophosphate synthesis was evaluated after addition of SNP (1 μmol/L, 30 min). Results are mean ± SEM from six different experiments. $^{*}P < 0.05$ vs. control. $^{\#}P < 0.05$ vs. V.

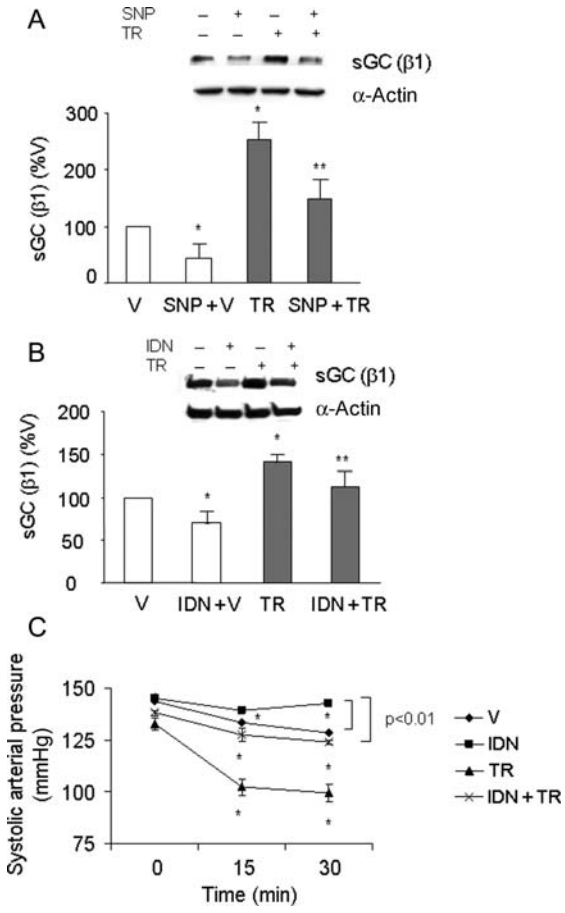

Figure 5 Tirofiban (TR) reverts the decrease in soluble guanylate cyclase β1 (sGC-β1) content promoted by chronic treatment with nitric oxide donors. (*A*) Rat vascular smooth muscle cells were either treated or not with sodium nitroprusside (SNP, 10 μmol/L) for 24 h and incubated either in the presence of TR (50 μmol/L, grey bars) or vehicle (V, white bars) for another 6 h. sGC-β1 content was assayed by immunoblotting. A representative blot is shown. The bar graph represents the densitometric analysis of the bands and is expressed as the ratio between sGC-β1 and α-actin. The results are the percentage of the V and are the mean ± SEM of five different experiments. $^{*}P < 0.05$ vs. V. $^{**}P < 0.05$ vs. SNP + V. (*B*) Rats were treated for 1 week with isosorbide dinitrate (IDN, 300 mg/kg/day) in their drinking water. At the end of this period, TR (50 μg/kg b.w.) or V was administered intraperitoneally in three consecutive doses. Six hours later, the rats were treated with a single intraperitoneal dose of SNP (10 μg/kg b.w.). sGC-β1 protein content in their aortic walls was assayed by immunoblotting. A representative blot is shown. The bar graph represents the densitometric analysis of the bands and is expressed as the ratio between sGC-β1 and α-actin. The results are the percentage of the V and are the mean ± SEM of five different experiments. $^{*}P < 0.05$ vs. V. $^{**}P < 0.05$ vs. IDN + V. (*C*) After the treatment described in (B), the arterial pressure was recorded for 30 min by using a tail sphygmomanometer. The results are the mean ± SEM of five animals per group. $^{*}P < 0.05$ vs. time 0 in the same treatment.

the arterial pressure values reached similar to those observed in WKY animals (*Figure 6B*).

4. Discussion

Arg-Gly-Asp motifs interact with cell integrins and are able to promote changes in cellular function.[23] Our group demonstrated that RGDS, an RGD-containing tetrapeptide, increased the sGC-β1 content in contractile cells.[10] As a consequence of this increase, smooth muscle cells responded better to NO donors. These *in vitro* findings led us to hypothesize that RGD-derived analogues, through sGC-β1 up-regulation, could have a significant therapeutic effect on those conditions characterized by decreased guanylate cyclase content in vascular walls. Since no significant information could be found regarding the pharmacological properties of RGDS administered parenterally, and since it was considered to be highly unstable due to its structure, tirofiban, an anti-thrombotic agent that mimics the RGD sequence[12] was chosen. Tirofiban presented two additional advantages. Its therapeutic use in human beings is widely known[13] and it has been used in rats to inhibit the platelet–neuthrophil interactions,[24] although platelet from rats is less responsive to peptidomimetics containing RGD motif than human platelet.[25]

First, we confirmed that tirofiban and RGDS had the same effects on sGC-β1 in RVSMCs and in human aortic and renal contractile cells.[13] In addition, we also demonstrated that tirofiban's cellular mechanism of action was similar to that of RGDS since (i) the sGC-β1 mRNA expression also increased in the presence of tirofiban and (ii) the observed effect depended on the interaction with integrins.[13] Moreover, the sGC content increased as soon as 2 h after tirofiban

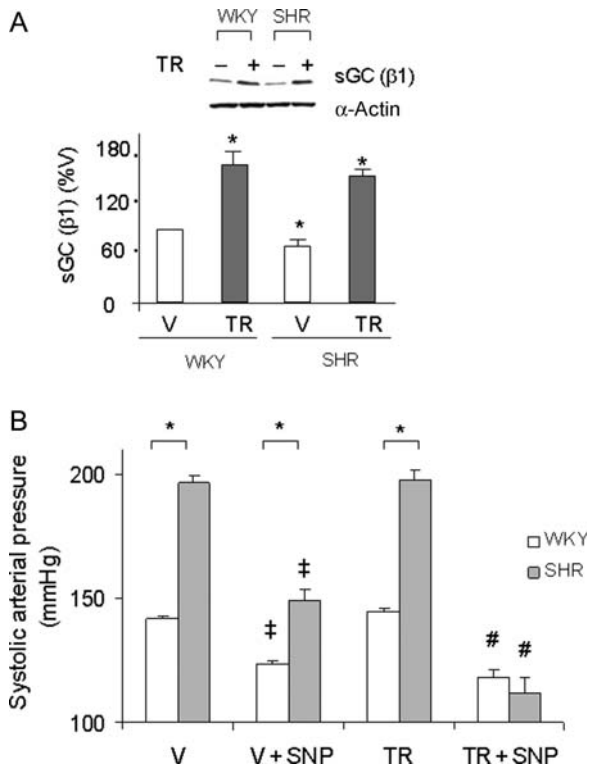

Figure 6 Tirofiban (TR) increases sGC-β1 content in spontaneously hypertensive rats (SHRs). (*A*) SHR and normotensive Wistar-Kyoto (WKY) rats were treated with TR (three consecutive doses of 50 μg/kg b.w., at times 0. 2 and 4 h) (TR, grey bars) or vehicle (V, white bars). sGC-β1 protein content in their aortic walls after 6 h was assayed by immunoblotting. A representative blot is shown. The bar graph represents the densitometric analysis of the bands and is expressed as the ratio between sGC-β1 and α-actin. The results are the percentage of V and are the mean ± SEM of five different experiments. $^{*}P < 0.05$ vs. V. (*B*) After TR or V treatment, as described in (A), a single dose of SNP (10 μg/kg b.w.) was administered to both WKY rats (white bars) and SHRs (grey bars). Arterial pressure was recorded after 30 min by using a tail sphygmomanometer. The results are the mean ± SEM of six animals per group. $^{*}P < 0.05$ vs. WKY rats. $^{\ddagger}P < 0.05$ vs. rats treated with V in the same rat genotype. $^{\#}P < 0.05$ vs. rats treated with V + SNP in the same rat genotype.

treatment in rats and human smooth muscle cells, the maximum effect being after 6 h of treatment in a similar way to RGDS.[10] The RGDS effect was dependent on AP-1 stimulation of sGC-β1 promoter. Although we have not analysed the signalling pathways involved in the tirofiban effect, it could be similar to those promoted by RGDS, but more experiments would be necessary to assess this point.

Soluble guanylate cyclase expression and activity is regulated by multiple factors. Whereas there is not much information about the stimuli which are able to up-regulate its expression. It is reduced in freshly isolated vessels exposed to ROS-generating agents,[26] hypoxia,[27] high salt intake,[28] and some cytokines.[29] The sGC expression is also regulated by integrins in other contexts; in HMCs, the sGC expression was down-regulated in the presence of collagen I, through the β1 integrin activation.[21]

The *in vivo* effect of tirofiban was tested in Wistar rats. Increased sGC-β1 content was found in the aortas of treated rats in a time- and dose-dependent manner. sGC-β1 is just one of the sGC isoforms of smooth muscle cells. The α isoform could have been tested as well, but previous studies, including our demonstration that RGDS increased sGC-β1 in RVSMCs, point to the relevance of the β1 isoform in the regulation of enzyme activity.[1] It could be argued that aortas are not resistance vessels, and they are not necessarily involved in the regulation of systemic haemodynamics. Since the main biological function of the sGC is to act as the NO intracellular receptor, we decided to test the functional consequences of the tirofiban-dependent sGC-β1 increase by studying the vascular response to NO donor administration.

We first analysed the changes in blood pressure in response to ip SNP, with or without tirofiban pretreatment. Administering tirofiban to rats enhanced the hypotensive response observed in the presence of SNP, as well as the SNP-induced cGMP synthesis by isolated rings, suggesting the functional relevance of the changes detected in the sGC-β1 content in aortas. The incubation of endothelium-denuded aortic rings with tirofiban increased the vasorelaxing properties of an NO donor such as SNP and the ability of this donor to stimulate cGMP synthesis in these vascular structures. The increased sGC-β1 content detected in aortic rings incubated with tirofiban, as well as the fact that the observed effect was completely blocked by ODQ, strongly support that the changes in the sGC isoform levels are critical in the increased vascular response to NO donors induced by tirofiban. A previous work described that tirofiban improves endothelium-dependent NO bioavailability in patients with coronary artery disease by inhibiting platelet aggregation.[30] Our experiments have been specifically directed to analyse the endothelium-independent vasorelaxing effect of tirofiban, but they do not exclude a possible endothelium-dependent effect. At this point, we considered the possibility that administering tirofiban could be useful to treat those pathophysiological conditions characterized by decreased sGC vascular content.

Organic nitrates exert their vasodilatory effects acting as NO donors within blood vessels. However, with repeated administration, the haemodynamic actions of nitrate therapy are frequently lost as tolerance is built up. Tolerance has limited the utility of nitrates as monotherapy. Different mechanisms accounting for nitrate tolerance have been postulated. Several studies suggest that oxidative stress induced by increased nicotinamide adenine dinucleotide phosphate oxidase activity contributes to the development of nitrate tolerance because of the NO scavenging properties of the superoxide anion.[26] However, one of the better known mechanisms is that NO decreases sGC mRNA stability via a transcription- and translation-dependent mechanism.[5] Recently, NO desensitization was described as being due to the *S*-nitrosylation of sGC.[31] We confirmed that chronic treatment with an organic nitrate such as IDN[32] reduced aortic sGC-β1 content and promoted a lack of response to the acute administration of SNP. Administering tirofiban for 6 h reversed the IDN-dependent down-regulation of sGC-β1 content, restoring the hypotensive response to SNP.

Reduced sGC activity has been described in certain physiopathological conditions such as systemic and pulmonary hypertension,[33] and ageing.[34] Tirofiban could increase sGC-β1 content in some of these situations, thus increasing the hypotensive effect of NO donors. To analyse this question, experiments on a genetic model of systemic hypertension, SHRs, were performed. The sGC-β1 content in the aortas of SHRs was lower than in age-matched normotensive WKY rats, as reported previously.[35] Treatment with tirofiban

increased the sGC-β1 content in the aortas of both SHRs and WKY rats in similar ways but it did not modify the basal arterial pressure in any of them. Both strains of rats showed a decrease in blood pressure after SNP treatment, but spontaneously hypertensive rats only reached arterial pressure values comparable to those of control animals when treated with tirofiban prior to the administration of SNP.

The relevance of this result is based on the idea that stimulating sGC could be a useful therapeutic tool in the treatment of cardiovascular diseases. In addition to the well-studied organic nitrites, a new class of NO-independent sGC stimulators, such as BAY 41-2272, is being tested in the treatment of cardiovascular diseases.[36] It has antihypertensive properties, attenuates remodelling in models of systemic arterial hypertension,[37] improves the heart function in experimental congestive heart failure,[38] reduces pulmonary vascular resistance in acute and chronic experimental pulmonary hypertension,[33] and slows the progression of anti-Thy-1-induced glomerulosclerosis.[39] However, BAY 41-2272 needs normal amounts of vascular sGC to be effective, which may be too low in many diseases. Thus, strategies devoted to modifying the vascular content of sGC could also be very relevant from a therapeutic point of view. Our studies point to RGDS analogues and integrin modulation as potential tools and targets, respectively, in the management of cardiovascular diseases.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Acknowledgements

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Regulation of Protein Kinase G type Iα by Extracellular Matrix Proteins.

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La disfunción endotelial es un fenómeno que se observa en los inicios de la enfermedad cardiovascular. El fallo en la respuesta vasodilatadora después de la administración de acetilcolina que caracteriza la disfunción endotelial no parece ser el único mecanismo implicado en la respuesta anómala observada en estas enfermedades.
Se han relacionado cambios en el contenido de GCs con la disfunción endotelial en determinadas enfermedades como la hipertensión, aterosclerosis y diabetes. Nuestro grupo propuso recientemente que la disfunción endotelial podría deberse a una acumulación anormal de proteínas de matriz en la pared vascular. Además el contenido de GCs puede ser modulado por proteínas de matriz extracelular en células renales y vasculares.

La vía del NO-GCs ejerce la mayoría de sus acciones celulares a través de la activación de PKG. En determinadas situaciones patológicas, se ha observado una disminución en el contenido o actividad de esta enzima lo que sugiere que PKG podría estar involucrado en la disfunción vascular de las enfermedades cardiovasculares. Por ello se quiso evaluar la capacidad de la fibronectina, proteína de matriz extracelular que contiene la secuencia RGD, para modular el contenido de PKG en células contráctiles y en animales de experimentación.

En primer lugar, se confirmó que la fibronectina incrementa los niveles proteicos y la actividad de PKG Iα en células mesangiales humanas. Un efecto similar a la fibronectina se observó cuando las células fueron tratadas con RGDS. Por el contrario, el tratamiento con RGES no modificó el contenido de PKG Iα. Este efecto dependía de integrinas, ya que desaparecía cuando las células se incubaron con un anticuerpo bloqueante de la isoforma β1. En el segundo bloque de resultados se observó que Akt era uno de los mediadores intracelulares implicados en el aumento del contenido proteico de PKG Iα. Cuando las células fueron tratadas con distintos inhibidores de kinasas solamente desapareció la estimulación del sistema de PKG bloqueando Akt. En tercer lugar, se estudió el efecto de la fibronectina en el contenido de ARNm y en la actividad transcripcional de la región del promotor humano de PKG de 2 Kb. En ambos casos se produjo un incremento de los niveles de ARNm de PKG Iα y de la actividad del promotor. Este efecto desapareció cuando las células mesangiales transfectadas fueron posteriormente tratadas con el inhibidor de Akt.

Por último, se testó que la fibronectina y el tirofiban, análogo del RGD, tuviese el mismo efecto sobre PKG en células musculares lisas de rata que el observado en mesangio. Ambos tratamientos incrementaron el contenido proteico de PKG Iα de manera dosis dependiente. La administración de tirofiban en ratas Wistar produjo un incremento en el contenido proteico de PKG Iα en la pared vascular. Finalmente, se estudió el efecto biológico de estos resultados midiendo la presión arterial en respuesta a la administración intraperitoneal (ip) de dibutiril GMPc en ratas pretratadas o no con tirofiban. Se observó una mayor respuesta vasorelajante en las ratas pretratadas con tirofiban. Estos resultados muestran un nuevo mecanismo capaz de incrementar el contenido proteico y actividad de PKG Iα en células contráctiles.

TITLE PAGE:

Title:

Regulation of Protein Kinase G type Iα by Extracellular Matrix Proteins.

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Running title:

Extracellular Matrix modulates PKG Iα

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ABSTRACT

Recent studies have proven that alterations in the nitric oxide-soluble guanylate cyclase (NO-sGC) pathway are related to the genesis of endothelial dysfunction. This pathway exerts most of its cellular actions through the activation of the protein kinase G (PKG). The aim of this study was to evaluate whether extracellular matrix proteins, particularly fibronectin, modulate PKG expression and activity in human mesangial cells (HMC), rat vascular smooth muscle cells (RVSMC) and rat aorta. Fibronectin increased the PKG Iα protein content in HMC and RVSMC, and the effect on human cells depended on β1 integrin interaction. The fibronectin up-regulation of PKG Iα seemed to depend on Akt activation, as this protein was phosphorylated after cell incubation with fibronectin, and the increased PKG Iα protein content elicited by fibronectin was completely abolished by treating the cells with an Akt inhibitor. Tirofiban, a pharmacological analogue of fibronectin, increased PKG Iα content in RVSMC and aortic walls of rats. As a consequence of these changes, tirofiban magnified the dbcGMP induced drop in arterial pressure in rats. In conclusion, the present results provide evidence of a mechanism able to increase the PKG Iα protein content linked to increased activity in contractile cells. Elucidation of this novel mechanism provides a rationale for future pharmacotherapy in certain vascular diseases.

INTRODUCTION

Endothelial dysfunction has been proposed as being responsible for the abnormal hemodynamic status of patients with hypertension, diabetes or atherosclerosis (Perticone *et al.* 2001; Giles, 2003; Davignon and Ganz, 2004). However, the impaired vasodilatory response after acetylcholine administration which characterizes endothelial dysfunction does not seem to be the only mechanism involved in the abnormal vascular responses observed in these diseases (Drexler and Hornig, 1999). Some studies have demonstrated a decreased soluble guanylate cyclase (sGC) content in the vascular walls of animals with experimental hypertension or atherosclerosis, and an attenuated cGMP production and renal vasodilation in diabetic rats (Wang *et al.* 1993; Ruetten *et al.* 1999; Marques *et al.* 2001; Kagota *et al.* 2001; Melichar *et al.* 2004), pointing to the importance of this intracellular receptor for nitric oxide (NO) in the genesis of vascular dysfunction. Different mechanisms have been proposed to explain the NO and sGC down-regulation in pathophysiological conditions. Recently, our group has proposed that endothelial dysfunction could be the consequence of the accumulation of abnormal extracellular matrix (ECM) proteins in vascular walls. In fact, cultured human umbilical vein endothelial cells exhibited a decreased NO synthase expression when cultured in collagen type I, when compared with the same cells grown in collagen type IV (González-Santiago *et al.* 2002). Moreover, sGC cellular content may be modulated by extracellular matrix proteins, in renal and vascular cells (De Frutos *et al.* 2005; Díez-Marqués *et al.* 2006). These effects seem to depend on integrin activation.

The NO-sGC pathway exerts most of its cellular actions through the activation of the protein kinase G (PKG). Two types of PKG are found in mammalian cells, type I and type II, and they are products of two distinct genes (Francis *et al.* 1988; Wernet *et al.* 1989; Orstavik *et al.* 1997). Type I is expressed as two isoforms, PKG Iα and PKG Iβ, generated by alternate mRNA splicing. Although PKG I is widely distributed in mammalian cells, it is most abundant in smooth muscle cells (SMC), platelets and Purkinje cells (Lincoln and Cornwell, 1993; Tamura *et al.* 1996; Walter, 1996). Activation of PKG I mediates smooth muscle relaxation through lowering of intracellular Ca^{2+} levels in vascular smooth muscle (Raeymaekers *et al.* 1988; Robertson *et al.* 1993; Archer *et al.* 1994) or inhibition of myosin light chain phosphorylation (Somlyo and Somlyo, 1994; Surks *et al.* 1999).

In some pathological situations, such as hypertension, diabetes or aging, a decreased PKG content (Failli *et al.* 2005) or decreased activity of the enzyme (Lin *et al.* 2001; Russo *et al.* 2008) have been described, suggesting that changes in PKG could also be involved in the vascular dysfunction of cardiovascular diseases.

Considering the previous information concerning the ability of extracellular matrix proteins to modulate the elements of the NO-sGC pathway, a similar effect could be hypothesized for PKG. However, no previous studies have analyzed this possibility. Thus, in the present study, we tested the possibility that extracellular matrix proteins, through integrin activation, could also modulate PKG content or activity.

MATERIALS AND METHODS

Drugs and other reagents

Arg–Gly–Asp–Ser (RGDS), Arg-Gly-Glu-Ser (RGES), fibronectin, N^2,2′-O-dibutyrylguanosine 3′,5′-cyclic monophosphate sodium salt hydrate (dibutyryl cGMP), leupeptin, pepstatin A, aprotinin, phenylmethylsulphonyl fluoride (PMSF), ammonium persulphate, collagenase IV, and anti-Actin antibodies were purchased from Sigma Chemical (St. Louis, MO, USA). Tirofiban and acrylamide-bisacrylamide were purchased from MERCK (Darmstadt, Germany). Akt inhibitor, anti-pVASP (Ser 239) and anti-VASP antibodies were purchased from Calbiochem (La Jolla, CA, USA). Anti-PKG antibody was purchased from Stressgen Biotechnologies (Victoria, BC, Canada). Anti-pGSK-3β (Ser 9), anti-GSK-3β, anti-p44/42 MAPK (Thr 202/Tyr 204), anti- 44/42 MAPK, anti-pAkt (Ser 473) and Akt antibodies were purchased from Cell Signalling (Danvers, MA, USA). Phosphotyrosine antibody was purchased from BD Transduction Laboratories (Franklin Lakes, NJ, USA). Peroxide-conjugated goat antirabbit Ig and anti–integrin β_1 antibody were purchased from Chemicon (Temecula, CA, USA). Lipofectamine and Opti-MEM were purchased from Invitrogen (Carlsbad, CA, USA). Dual-luciferase reporter assay system was purchased from Promega (Madison, WI, USA). RPMI 1640, DMEM/Ham's F-12 medium, fetal calf serum, trypsin-EDTA (0.02%), L-glutamine and penicillin-streptomycin were purchased from Biowhittaker (Walkersville, MD, USA). Culture plates were from Nunc (Kamstrup, Denmark). X-OMAT films were from Kodak (Rochester, NY, USA). The ECL chemiluminescence system was from Amersham-Pharmacia Biotech (Buckinghamshire, UK). Electrophoresis equipment and protein molecular weight standard were from Bio-Rad

(Richmond, CA, USA). The polyvinylidene difludide membrane was from Perkin Elmer (Boston, MA, USA). The bicinchoninic acid (BCA) assay kit was from Pierce (Rockford, IL, USA). The human PKG I promoter constructs were a gift from Drs. M. Lincoln and H. Sellak. All the reagents employed were of the highest commercially available grade.

Cell culture

Human mesangial cells (HMC) were cultured according to previously described procedures (Díez- Marqués *et al.* 1995). Portions of macroscopically normal, cortical tissue were quickly obtained from human kidneys immediately after nephrectomy for renal cell carcinoma. Isolated glomeruli were treated with collagenase, plated in plastic culture dishes, and maintained in RPMI 1640, supplemented with 10% FBS, in a 5% CO_2 atmosphere. The identity of the cells was confirmed by morphological and functional criteria, as previously described (Díez- Marqués *et al.* 1995). All procedures were performed in accordance with the Declaration of Helsinki.

Vascular smooth muscle cells (VSMC) were obtained from thoracic aortas of Wistar rats by methods described previously (Chamley-Campbell *et al.* 1979). Wistar rats (125-200 g) were quickly sacrificed and exsanguinated. Thoracic aortas were removed, cleaned of surrounding tissues, dissected into small strips, and incubated in DMEM/Ham's F-12 medium with collagenase type IV at 37°C for 45 min. The digested strips were seeded onto 100-mm diameter dishes and maintained in 10 ml of DMEM/Ham's F-12 medium with 10% fetal calf serum, at 37°C, in a humidified atmosphere of 5% CO_2. Confluent cultures were serially passaged by trypsinization (trypsin-EDTA). The cells were used between the second and fourth passages. Cells exhibited characteristics of VSMC (Chamley-Campbell *et al.* 1979).

Animals

The experiments were carried out in accordance with the European Union legislation and were approved and supervised by the University Animal Care facility. All efforts were made to minimize animal suffering and to reduce the number of animals used. The experiments were performed in male Wistar rats (Harlan Charles River, Germany) weighing 150 g housed in a pathogen-free, temperature and light-controlled room (22 $\pm$ 2ºC; 12/12 hours light/ dark cycle). Food and water were available *ad libitum*.

Blood pressure measurement

Arterial pressure (AP) 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 30ºC with a heater (LE5660/6, Letica Scientific Instruments).

Protein extraction and Western blot analysis

Following treatment, cells or tissues were washed in PBS and solubilised (10mmol/L Tris-HCl pH 7,4, 1 mmol/L EDTA, 1% Triton X-100, 0,1% sodium deoxycholate, 500 nmol/L sodium orthovanadate, 50 nmol/L NaF, 1 mmol/L pepstatin/leupeptin/aprotinin, 1 mmol/L PMSF) for 30 min at 4ºC. To perform Western blot analysis, total cell extracts (20µg/lane) were size-fractionated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were blocked for 1 h and then incubated with the corresponding antibodies. After washing, blots were incubated with a secondary antibody and a specifically bound antibody was detected with the ECL method. Films were scanned and analyzed with appropriate software (NIH Imagine 1.55; National Institutes of Health, Bethesda, MD).

RNA extraction and analysis of mRNA expression by Northern blot

Cells were homogenized using guanidinium isothiocyanate, and total RNA was isolated by repeated phenol-chloroform extractions and isopropanol precipitation as described (Chomczynski and Sacchi, 1987). Total RNA (10 µg per lane) was denatured and electrophoresed through a 1% agarose gel containing 0.66 mol/L formaldehyde. RNA was transferred to a nitrocellulose membrane and UV-cross-linked. The membranes were hybridized at 60°C in hybridization solution (10% dextran sulphate, 1% SDS, 1 mol/L NaCl, 0,1 mg/ml denatured salmon sperm DNA) with an α-^{32}[P]-dCTP (10^6 cpm/ml) radiolabeled 1000-bp fragment of PKG I cDNA spanning the catalytic region (*Browning et al. 2001*) and an 18S RNA probe. Densitometric analysis of the exposed films was performed with a scanner and analyzed using appropriate software (NIH Image 1.55 from the National Institutes of Health, Bethesda, MD, USA).

Transient transfection and Luciferase Assays

A total of 3.5 x 10^5 cells/well were plated in six-well plates 24h before transfection. The cells in every well were then incubated 4 h at 37ºC with 2 ml Opti-MEM medium containing complexes of 2 µl lipofectamine, 0.1 µg human PKG reporter (Sellak *et al.* 2005) and 0.01 µg renilla luciferase reporter as an internal control. Transfected cells were next incubated with complete growth medium for 16 h and then they were treated as indicated in the legend of Figure 7. Finally, firefly luciferase activity of the PKG reporter was measured with a luminometer (FB12 Berthold luminometer) and normalized against the renilla luciferase activity by using the dual-luciferase reporter assay system. Protein concentration was determined by BCA.

Statistical analysis

The number of experiments performed is reflected in the "Figure legends". As this number was under 10, non-parametric statistics were used for comparisons (Friedman's and Wilcoxon's tests, for paired data, and Kruskal-Wallis' or Mann-Whitney' tests, for non-paired data). The data are expressed as the mean ± S.E.M. A $P<0.05$ was considered statistically significant.

RESULTS

Fibronectin increases the PKG Iα protein content in human mensangial cells (HMC) through interaction with β1 integrin.

The effect of fibronectin on PKG Iα protein content in HMC is shown in Fig. 1. Fibronectin induced a significant increase in the PKG Iα level, which was time- (Fig. 1A) and dose-dependent (Fig. 1B). The minimal fibronectin concentration that elicited a significant change in PKG was 5 µg/ml, and the effect was detected after 4 h of incubation. The functional consequences of this change in PKG Iα cellular content were evaluated by examining the Ser 239 phosphorylation of one of the main PKG I substrates in contractile cells, VASP. Dibutyryl cGMP induced a significantly increased VASP phosphorylation in HMC, which was stimulated by cell preincubation with fibronectin (Fig. 2). A similar effect to that of fibronectin on PKG Iα protein content was observed in HMC treated with 50 µM of RGDS for 6 h. By contrast, RGES, a tetrapeptide similar to RGDS in which glutamic substitutes aspartic acid, did not modify the PKG Iα protein content (Fig. 3A).The blockade of the β1 subunit of integrins with a

blocking antibody blunted the stimulatory effect of fibronectin on PKG Iα overexpression (Fig. 3B).

Akt activation is involved in fibronectin up-regulation of PKG Iα levels.

The effect of fibronectin on Tyrosine, ERK, Akt and GSK- 3β phosphorylation in HMC is shown in Fig. 4. Fibronectin induced a significant increase of Akt (Ser 473) (Fig. 4C) and GSK- 3β (Ser 9) phosphorylation (Fig. 4D), that was not observed with the other proteins (Fig. 4A and 4B). Neither tyrosine kinase inhibitors such as genistein and herbimycin nor the ERK 1/2 blocker PD9805 modified the action of fibronectin (data not shown). A complete blockade of the fibronectin –induced PKG Iα up-regulation was observed after inhibiting Akt with an Akt inhibitor (Fig. 5A).

Fibronectin increases the mRNA expression and the transcriptional activity of human PKG I promoter region in HMC.

The changes in the PKG Iα protein content were paralleled by modifications in mRNA expression. Thus, fibronectin induced an increase in the PKG Iα mRNA expression with a similar time- and dose- dependent pattern to that observed for the protein content (Fig. 6). Moreover, fibronectin induced a significant increase on the transcriptional activity of the human PKG I 2 Kb promoter region, which was time-dependent: the promoter activity increased 2 fold with respect to basal activity after 2-4 hours of fibronectin treatment (Figure 7A). This effect was blocked when HMC were pre-incubated with the Akt inhibitor (Fig. 5B). When HMC were transiently transfected with serial deletions of human PKG I promoter, it was observed that the fibronectin-dependent stimulated promoter activity slightly, but significantly, decreased after 1.5 kb deletion and completely disappeared with the 1 kb fragment (Fig. 7B).

Fibronectin and tirofiban increases PKG Iα content in rat vascular smooth muscle cells (RVSMC) and aortic walls of rats.

The effect of fibronectin on PKG Iα protein content in RVSMC is shown in Fig. 8A. Fibronectin induced a significant increase in the PKG Iα level, which was dose-dependent. The minimal fibronectin concentration that elicited a significant change in PKG Iα was 5 μg/ml, for 6 h. A similar increase was observed on PKG Iα protein content in RVSMC treated with tirofiban, a pharmacological analogue of fibronectin (Fig. 8B).To analyse these effects *in vivo*, rats were treated with three consecutive doses

of intraperitoneal tirofiban (50 µg/kg b.w., times 0, 2, 4 h), and the aortic PKG Iα content was measured at different times after the first injection. As shown in figure 9A, PKG Iα increased in vascular walls with the tirofiban treatment. To test the functional consequences of these changes, dibutiryl cGMP (5 mg/kg b.w) was administrated to these rats, and the arterial pressure was measured. The dibutiryl cGMP induced drop in arterial pressure was magnified by tirofiban pretreatment (Fig. 9B).

DISCUSSION

PKG plays a central role in the regulation of vascular function, since it acts as a cGMP downstream effector (Hofmann *et al.* 2000; Lohmann and Walter, 2005). Defective NO/cGMP signaling is associated with many forms of vascular disease (Feil *et al.* 2003; Münzel *et al.* 2003). However, the regulation of downstream components of this signalling cascade has not been extensively studied. The data presented here provide a clear demonstration of the role of ECM composition in the regulation of PKG expression and activity. Our results show that fibronectin increases PKG Iα in HMC. As a consequence of this increased protein content, cGMP-dependent VASP phosphorylation increased after cell incubation with fibronectin. To our knowledge, this study is the first to demonstrate such a modulation of PKG Iα activity by an ECM component.

We furthermore have provided some insights into the mechanisms underlying this process. First, the fact that RGDS mimics and anti-ß1 antibody blocks fibronectin effects on PKG Iα, strongly support a role for integrin-containing ß1 subunit in the genesis of the fibronectin effects. It has been previously demonstrated that RGD motifs of fibronectin interact with ß1-containing integrins (Hynes, 2002), and these integrins are present in HMC (Pröls *et al.* 1999; Kagami and Kondo, 2004). Second, our results point to Akt, and exclude tyrosine kinases and ERK 1/2, as one of the intracellular mediators responsible for the changes observed in the PKG Iα protein content. Although fibronectin increased the phosphorylation of various intracellular proteins, only the Akt blockade prevented the stimulation of the PKG system. Akt phosphorylation has been proposed as a critical pathway following integrin activation (Gibson *et al.* 2005; Ruiz-Torres *et al.* 2007; Kotha *et al.* 2008). Third, the changes observed in the PKG Iα protein content were the consequence of increasing levels of its mRNA, probably

because of an increased transcriptional activity. Although a more detailed analysis is needed to better understand the fibronectin-dependent regulation of PKG transcriptional activity, present results stress the relevance of the region -1000, -2000 of the 5'-flanking region of the PKG gene in response to fibronectin. To analyze the relevance of these *in vitro* findings, some experiments were performed in Wistar rats. However, before starting the *in vivo* studies, the ability of fibronectin to increase the PKG Iα protein content in rat vascular cells was tested, as our previous experiments were performed in human cells of renal origin. HMC and RVSMC share different properties, as both are mesenchimal, contractile cells (Johnson *et al.* 1992), but it was necessary to demonstrate that rat vascular cells showed a similar response, concerning the PKG Iα content after integrin activation, to human renal cells. In fact, fibronectin induced a dose-response stimulation of PKG Iα protein content in RVSMC. Moreover, as *in vivo* experiments were going to be performed with tirofiban, an anti-thrombotic agent that mimics the RGD sequence and activates integrins (Deckelbaum *et al.* 1997; Proimos, 2001; Ruiz-Torres *et al.* 2009), it was also necessary to demonstrate that this agent increased PKG Iα protein content in RVSMC, which it did.

Administering tirofiban to Wistar rats induced an increased PKG Iα protein content in aortic walls, as was expected from the *in vitro* experiments. To test the biological relevance of this finding, the changes in blood pressure in response to ip dibutiryl cGMP were analyzed, with and without tirofiban pretreatment. Tirofiban enhanced the hypotensive response observed in the presence of dibutiryl cGMP, suggesting the functional relevance of the changes detected in the PKG Iα content in aortas.

These findings highlight the importance of the ECM-integrin interactions in the regulation of the NO-sGC-PKG pathway. Previous studies from our group demonstrated an abnormal regulation of endothelial nitric oxide synthase (González-Santiago *et al.* 2002) and sGC (De Frutos *et al.* 2005) in cells cultured in collagen type I. Moreover, they demonstrated the possibility of up-regulating sGC vascular content through integrin activation (Díez-Marqués *et al.* 2006). Now, evidence is provided concerning the dependence of PKG on integrins, and the possibility of regulating its vascular content.

The pathophysiological importance of PKG down-regulation has been demonstrated in several pathological cardiovascular diseases such as hypertension and late atherosclerosis (Pfeifer *et al.* 1998; Sausbier *et al.* 2000; Melichar *et al.* 2004). Decreased enzyme levels in vascular walls would determine a defective relaxation of

these structures. Moreover, nitrovasodilator resistance or nitrate tolerance has also been attributed to PKG, as continuous exposure to NO-releasing agents, cGMP, or cAMP analogues suppresses PKG mRNA and protein levels in VSMCs by decreasing transcription without affecting mRNA stability (Soff *et al.*1997).

In consequence, an impairment of PKG function and/or expression could be viewed as a mechanism involved in vascular dysfunction, and PKG could constitute a therapeutic target for the reversal of these alterations.

Results of the present study provide evidence of a novel mechanism that is able to increase the PKG Iα protein content linked to increased activity in contractile cells. As a decreased PKG may be a relevant feature of vascular dysfunction, at least in some pathophysiological conditions, these data provide the basis for future interventions in the reversal of functional abnormalities of certain vascular diseases.

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Conflict of interest

The authors state no conflict of interest.

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FIGURE LEGENDS

Fig 1. PKG Iα protein content in human mesangial cells (HMC) incubated with fibronectin. HMC were treated with 10 μg/ml fibronectin for different periods of time (Panel A) or with different fibronectin concentrations for 6 h (Panel B). The PKG Iα protein content was analyzed by immunoblotting. A representative blot is shown in each case. Bar graphs represent the densitometric analysis of the bands (ratio between the densitometric signals of PKG Iα and α-Actin) of six independent experiments. The results are expressed as a percentage of control (C) and are the mean ± SEM. **P* < 0.05 vs. C.

Fig 2. Changes in vasodilator-stimulated phosphoprotein (VASP) phosphorylation in human mensangial cells (HMC) treated with fibronectin. HMC were incubated with 10 μg/ml fibronectin for 6 h, and then treated with 100 μM dibutyryl cGMP (dbGMP) for 15 min. VASP phosphorylation (P-VASP) was evaluated by immunoblotting. A representative blot is shown. Bar graphs present the densitometric analysis of the bands (ratio between the densitometric signals of P-VASP and VASP) of three independent experiments. The results are expressed as a percentage of control (C) and are the mean ± SEM. **P* <0.05 vs. C, ** *P* < 0.05 vs. dbGMP.

Fig 3. Role of integrins in the fibronectin-induced changes in the PKG Iα protein content in human mensangial cells (HMC). HMC were incubated with 10 μg/ml fibronectin, 50 μM Arg–Gly–Asp–Ser (RGDS), or 50 μM Arg-Gly-Glu-Ser (RGES) for 6 h (Panel A), or with 30 μg/ml anti β1 blocking antibody for 4h and then 10 μg/ml fibronectin for 6 h (Panel B). The PKG Iα protein content was analyzed by immunoblotting. A representative blot is shown in each case. Bar graphs represent the densitometric analysis of the bands (ratio between the densitometric signals of PKG Iα and α-Actin) of four independent experiments. The results are expressed as a percentage of control (C) and are the mean ± SEM. **P* < 0.05 vs. C.

Fig 4. Changes in intracellular kinase phosphorylation in human mensangial cells (HMC) treated with fibronectin. HMC were treated with 10 μg/ml fibronectin for different periods of time. Phospho-tyrosine (P-tyrosine, Panel A), phospho-ERK1/2 (P-

ERK, Panel B), phospho-Akt (P-AKT, Panel C) and phospho-GSK- 3β (P-GSK, Panel D), and their respective non-phosphorylated proteins or α-Actin, were analyzed by immunoblotting. A representative blot is shown in each case. Bar graphs represent the densitometric analysis of the bands (ratio between the densitometric signals of phosphorylated proteins and α-Actin, or phosphorylated and non-phosphorylated proteins) of four independent experiments. The results are expressed as a percentage of control (C) and are the mean ± SEM. **P* < 0.05 vs. C.

Fig 5. Importance of the Akt activation in the fibronectin-induced changes in the PKG Iα protein content and promoter activity in human mensangial cells (HMC). HMC were treated with 30 μM Akt inhibitor and 30 min later, 10 μg/ml fibronectin was added and maintained for 6 h (Panel A). PKG Iα protein content was evaluated by immunoblotting. A representative blot is shown. Bar graphs represent the densitometric analysis of the bands (ratio between the densitometric signals of PKG I and α-Actin) of four independent experiments. The results are expressed as a percentage of control (C) and are the mean ± SEM. **P* <0.05 vs. C. HMC were transfected with the human PKG I 2 Kb promoter, treated with 30 μM Akt inhibitor and 30 min later, 10 μg/ml fibronectin added and maintained for 3 h (Panel B). The promoter-dependent luciferase activity was normalized to renilla activity. Data are representative of at least five independent experiments. The results are expressed as a percentage of basal transcriptional activity of control (C) and are the mean ± SEM. **P* <0.05 vs. C.

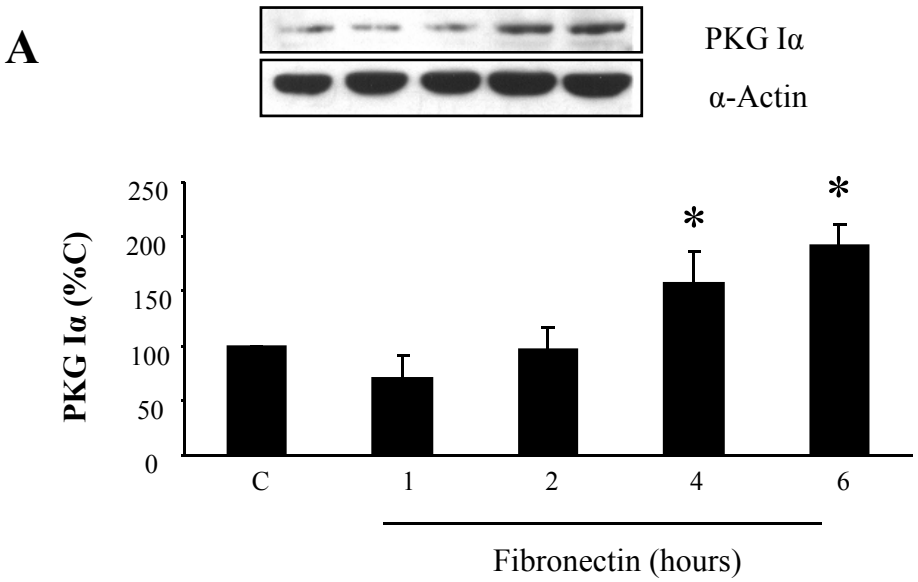
Fig 6. PKG Iα mRNA content in human mesangial cells (HMC) incubated with fibronectin. HMC were treated with 10 μg/ml fibronectin for different periods of time (Panel A) or with different fibronectin concentrations for 6 h (Panel B). PKG Iα mRNA expression was evaluated by northern blotting. A representative blot is shown in each case. Bar graphs represent the densitometric analysis of the bands (ratio between the densitometric signals of PKG Iα and 18S) of three independent experiments. The results are expressed as a percentage of control (C) and are the mean ± SEM. **P* <0.05 vs. C.

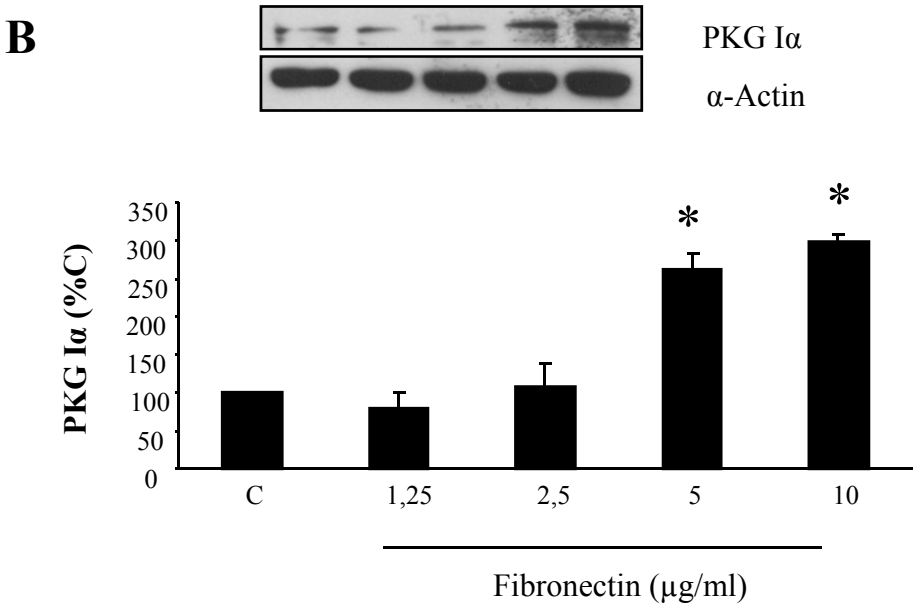
Fig 7. Changes in the transcriptional activity of the human PKG I 2 kb promoter region in human mensangial cells (HMC) treated with fibronectin. HMC were transfected with the human PKG I 2 Kb promoter region for 4 h. Twenty-four hours

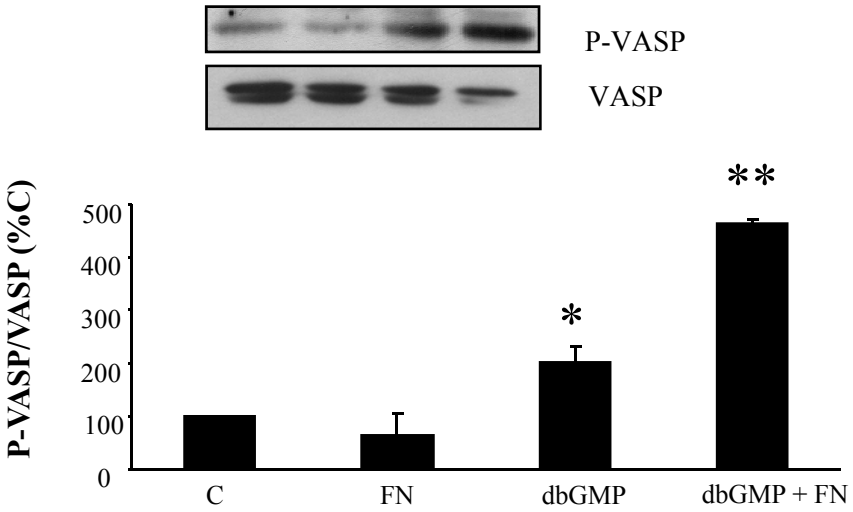
later, cells were treated with 10 µg/ml of fibronectin for different periods of time, and luciferase activity was measured (Panel A). HMC were transfected with different serial deletions of the human PKG I 2 Kb promoter region for 4 h. Twenty-four hours later, cells were treated with 10 µg/ml of fibronectin for 3 h, and luciferase activity was measured (Panel B). In each condition, the promoter-dependent luciferase activity was normalized to renilla activity. In both panels, the results are expressed as a percentage of basal transcriptional activity of control (C) and are the mean ± SEM. **P* <0.05 vs. C ***P* <0.05 vs. fibronectin.

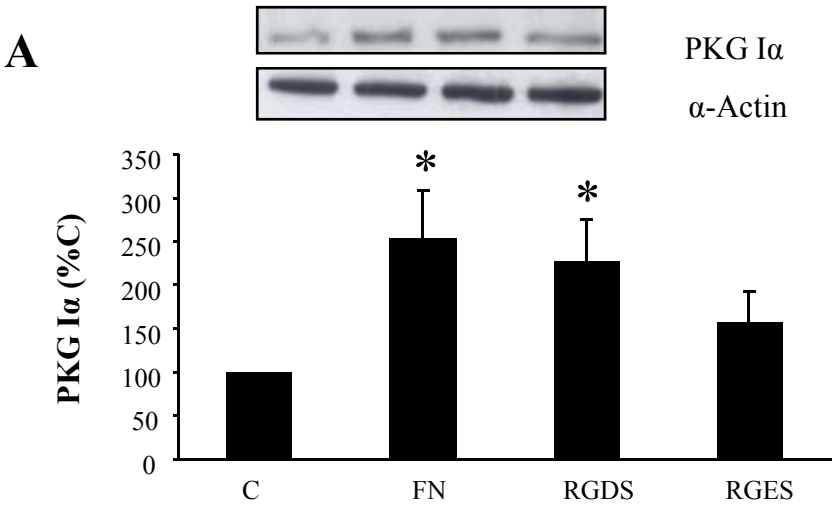
Fig 8. PKG Iα protein content in rat vascular smooth muscle cells (RVSMC) incubated with fibronectin and tirofiban. RVSMC was treated with different fibronectin (Panel A) or tirofiban (Panel B) concentrations for 6 h. The PKG Iα protein content was analyzed by immunoblotting. A representative blot is shown in each case. Bar graphs represent the densitometric analysis of the bands (ratio between the densitometric signals of PKG Iα and α-Actin) of three independent experiments. The results are expressed as a percentage of control (C) and are the mean ± SEM. **P* < 0.05 vs. C.

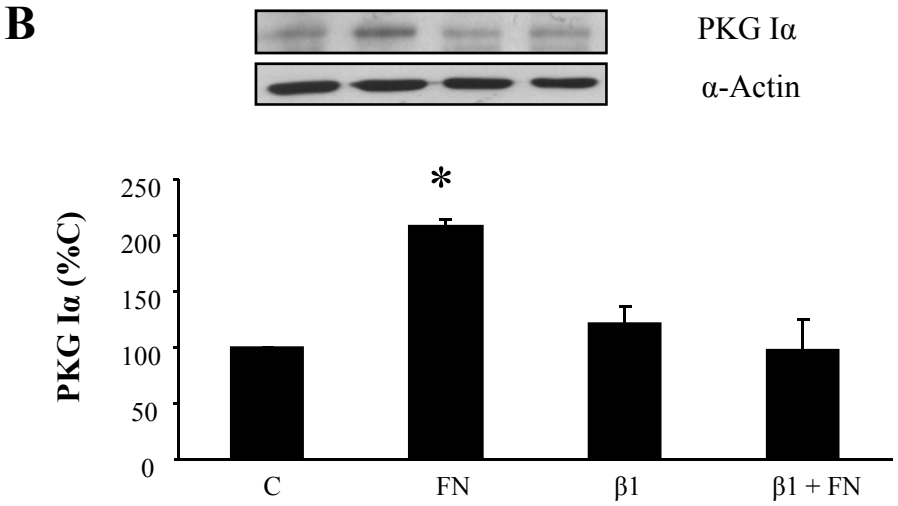
Fig 9. Effects of tirofiban in rats: changes in aorta PKG Iα protein content, and in systolic arterial pressure (SAP) in response to cyclic GMP (cGMP) analogues.
Rats were injected i.p. with 50 µg/Kg tirofiban for different periods of time (Panel A). The PKG Iα protein content in aorta was analyzed by immunoblotting. A representative blot is shown. Bar graphs represent the densitometric analysis of the bands (ratio between the densitometric signals of PKG Iα and α-Actin) of eight independent animals. The results are expressed as a percentage of control (C) and are the mean ± SEM. **P* < 0.05 vs. C. Changes in SAP in response to dibutyryl cGMP (dbcGMP, 5 mg/kg b.w i.p) in control rats (VH) and in rats treated with tirofiban (TIR, 50 µg/kg b.w., times 0, 2, 4 h, i.p.) (Panel B). The results are shown as mean ± SEM of eleven animals. **P* <0.05 vs. basal values (closed bars). ***P* <0.05 vs VH after dbcGMP treatment.

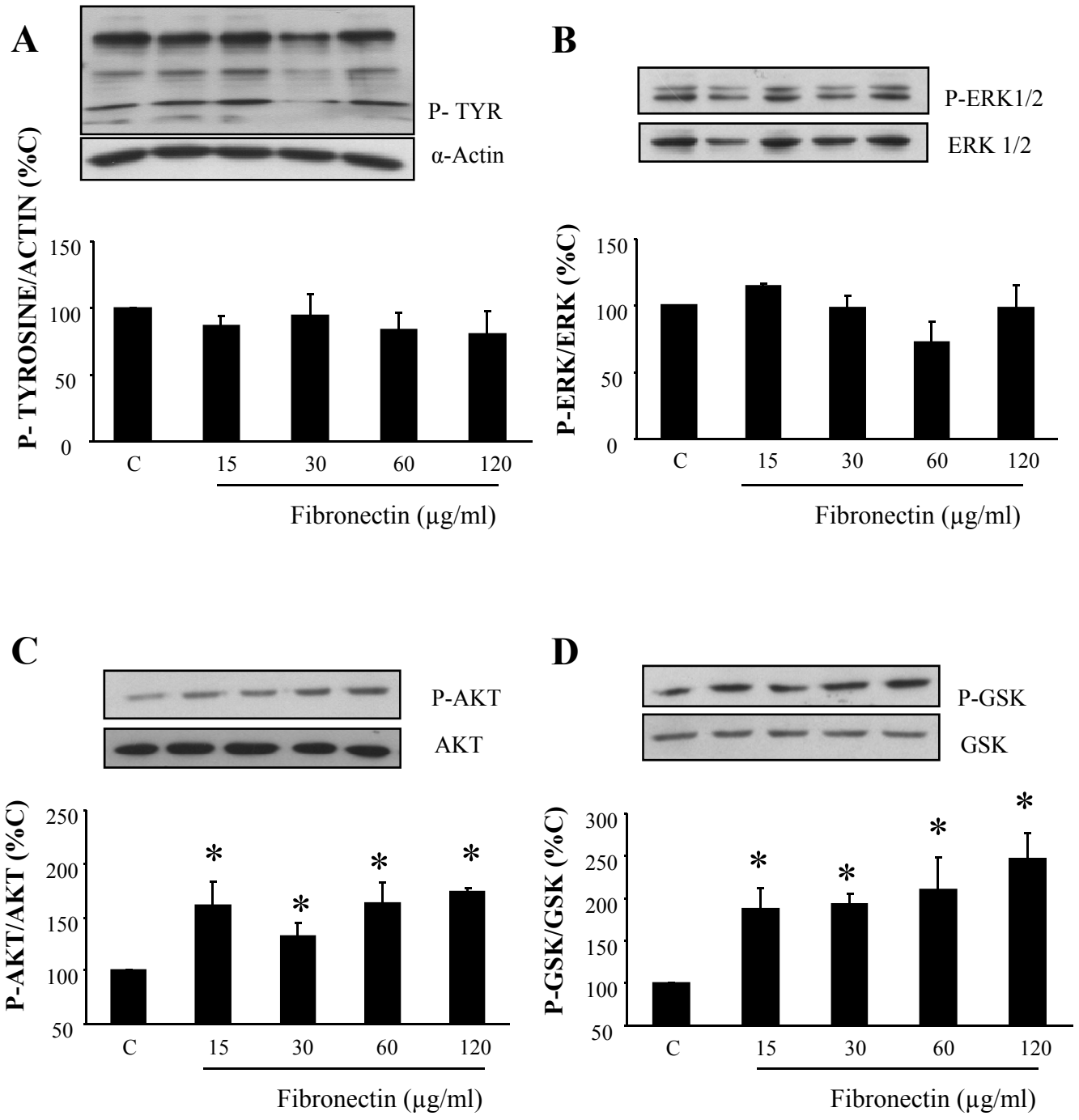

A
PKG Iα
α-Actin
PKG Iα (%C)
250
200
150
100
50
0
C
1
2
4
6
*
*
Fibronectin (hours)


B
PKG Iα
α-Actin
PKG Iα (%C)
350
300
250
200
150
100
50
0
C
1,25
2,5
5
10
*
*
Fibronectin (µg/ml)

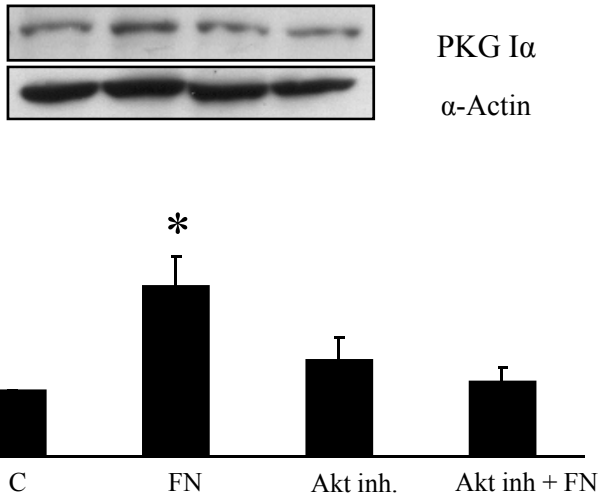

P-VASP
VASP
P-VASP/VASP (%C)
500
400
300
200
100
0
*
**
C
FN
dbGMP
dbGMP + FN


A
PKG Iα
α-Actin
PKG Iα (%C)
350
300
250
200
150
100
50
0
*
*
C
FN
RGDS
RGES

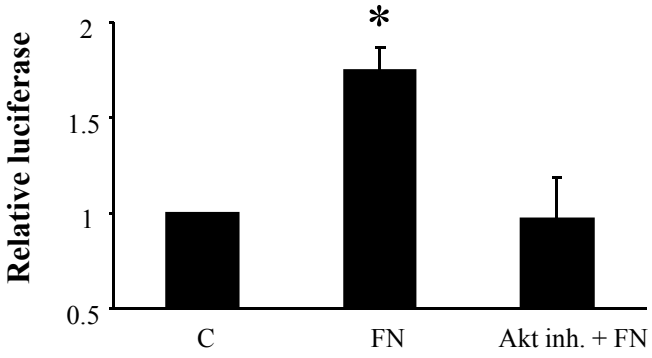

B
PKG Iα
α-Actin
PKG Iα (%C)
250
200
150
100
50
0
*
C
FN
β1
β1 + FN


A
P- TYR
α-Actin
P- TYROSINE/ACTIN (%C)
150
100
50
0
C
15
30
60
120
Fibronectin (μg/ml)
B
P-ERK1/2
ERK 1/2
P-ERK/ERK (%C)
150
100
50
0
C
15
30
60
120
Fibronectin (μg/ml)
C
P-AKT
AKT
P-AKT/AKT (%C)
250
200
150
100
50
C
15
30
60
120
Fibronectin (μg/ml)
D
P-GSK
GSK
P-GSK/GSK (%C)
300
250
200
150
100
50
C
15
30
60
120
Fibronectin (μg/ml)

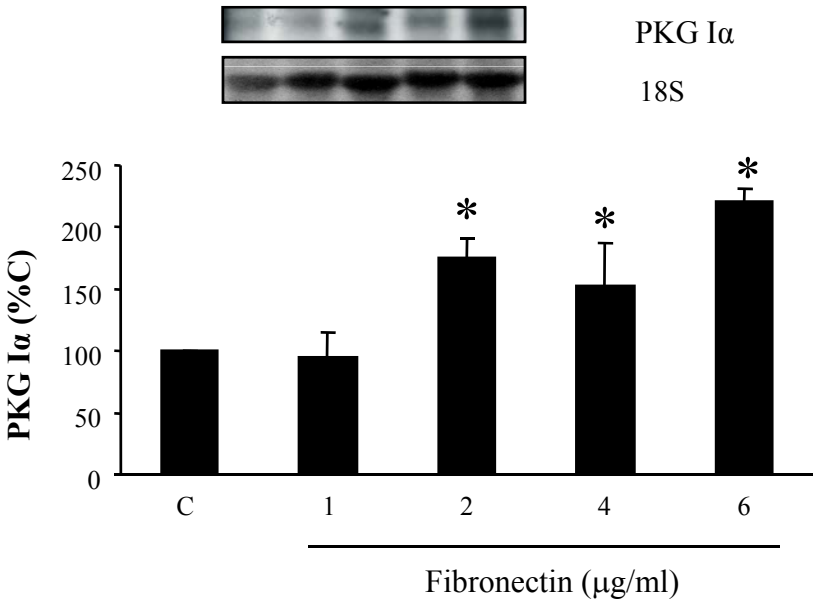
A


PKG Iα
α-Actin
PKG Iα (%C)
250
200
150
100
50
*
C
FN
Akt inh.
Akt inh + FN

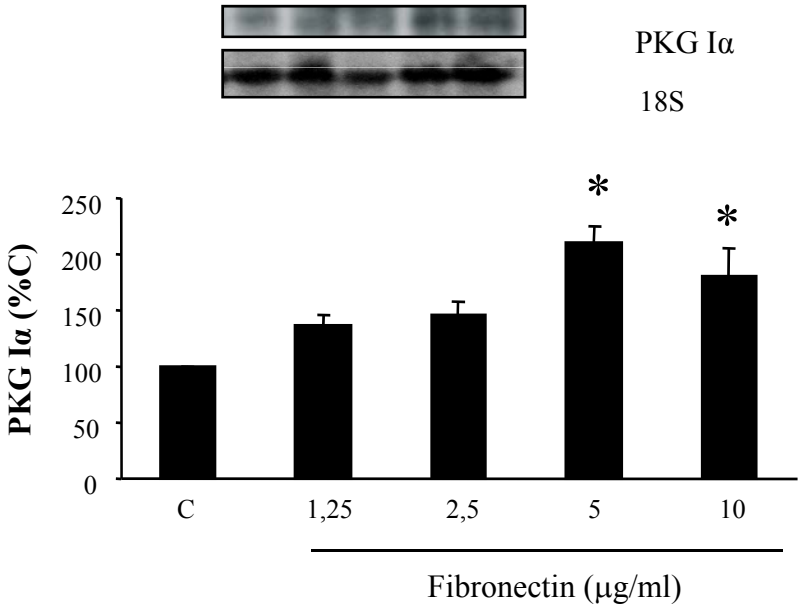
B


Relative luciferase
2
1.5
1
0.5
*
C
FN
Akt inh. + FN

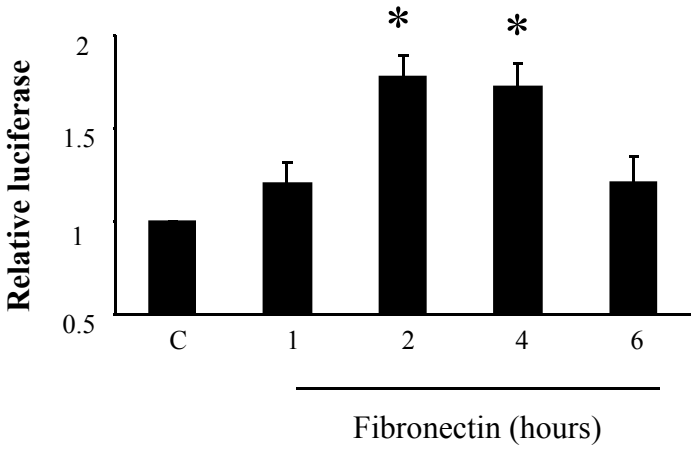
A


PKG Iα
18S
PKG Iα (%C)
250
200
150
100
50
0
*
*
*
C
1
2
4
6
Fibronectin (μg/ml)

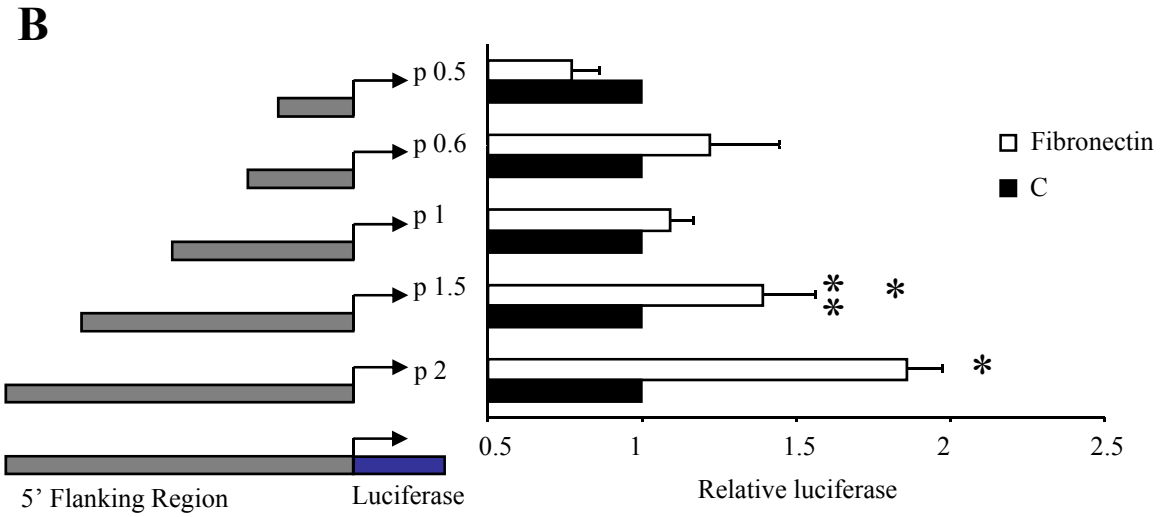
B

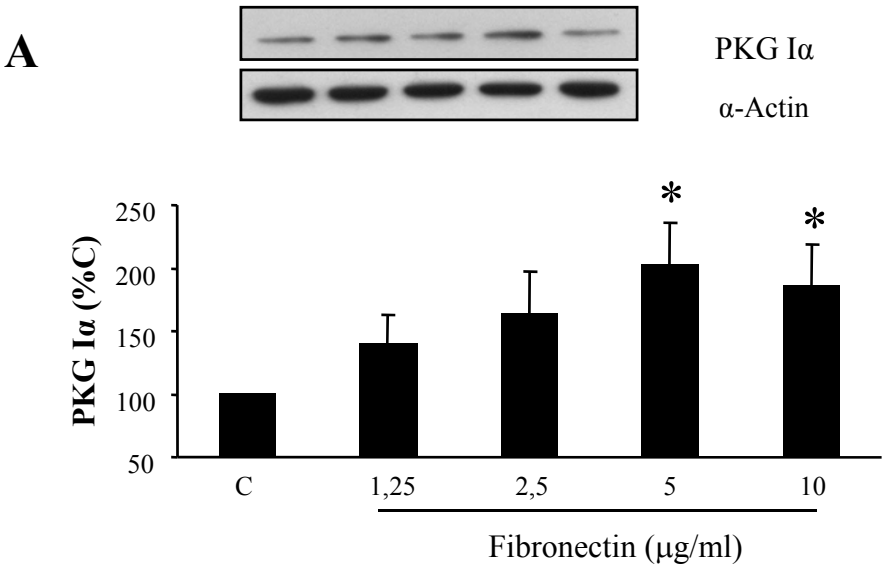

PKG Iα
18S
PKG Iα (%C)
250
200
150
100
50
0
*
*
C
1,25
2,5
5
10
Fibronectin (μg/ml)

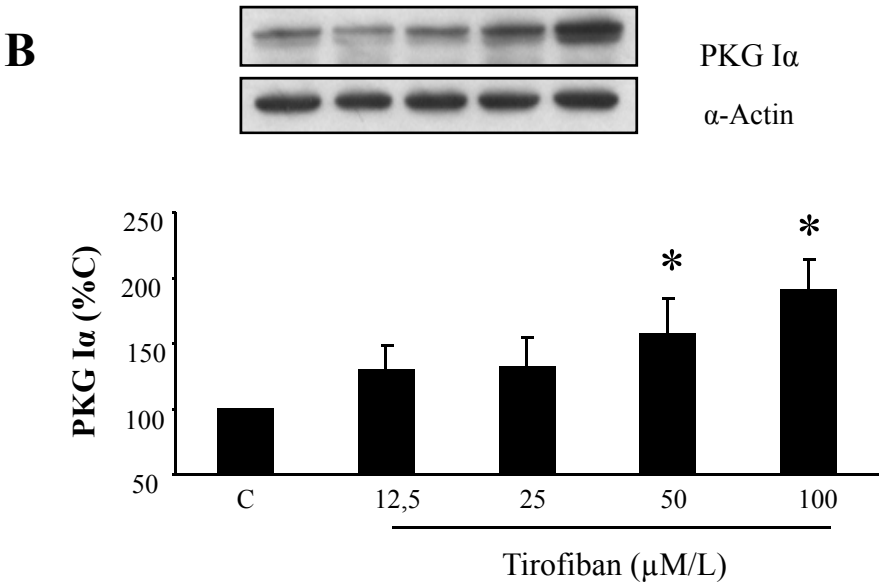
A

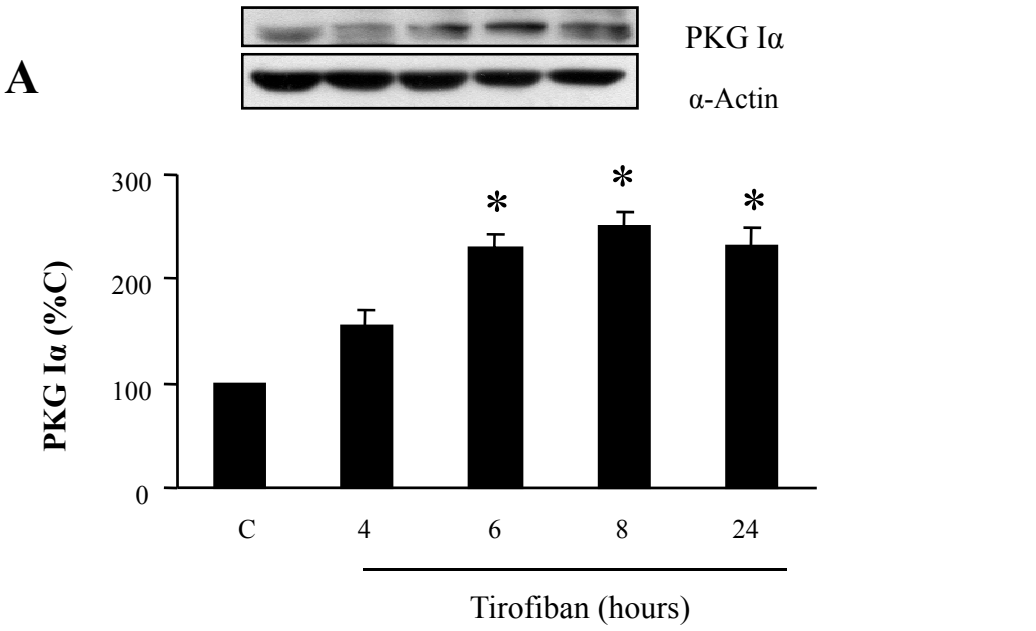

Relative luciferase
2
1.5
1
0.5
C
1
2
4
6
Fibronectin (hours)

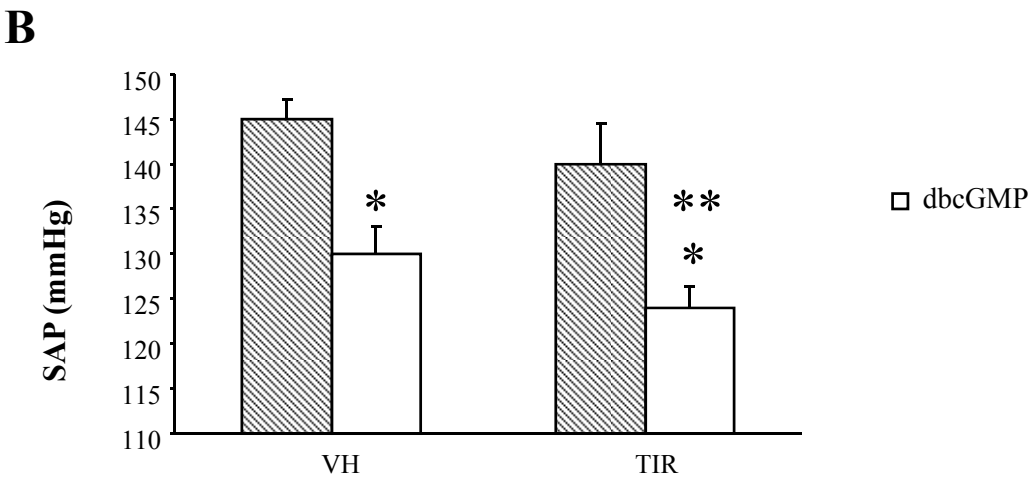
B


p 0.5
p 0.6
p 1
p 1.5
p 2
5' Flanking Region
Luciferase
Fibronectin
C
0.5
1
1.5
2
2.5
Relative luciferase


A
PKG Iα
α-Actin
PKG Iα (%C)
250
200
150
100
50
*
*
C
1,25
2,5
5
10
Fibronectin (μg/ml)


B
PKG Iα
α-Actin
PKG Iα (%C)
250
200
150
100
50
*
*
C
12,5
25
50
100
Tirofiban (μM/L)


A
PKG Iα
α-Actin
PKG Iα (%C)
300
200
100
0
C
4
6
8
24
Tirofiban (hours)


B
SAP (mmHg)
150
145
140
135
130
125
120
115
110
VH
TIR
dbcGMP

Targeted genomic disruption of H-Ras induces hypotension through a mechanism dependent on the activation of the NO-sGC-PKG pathway.

Aranzazu Chamorro-Jorganes, Maria Teresa Grande, Beatriz Herranz, Mercedes Griera, Eugenio Santos, Diego Rodriguez Puyol, Jose M Lopez-Novoa and Manuel Rodriguez Puyol.

(Enviado a Cardiovascular Research, Junio 2009)

La familia de las proteínas Ras monoméricas de unión a GTP está compuesta por las proteínas H-Ras, N-Ras, K-Ras4A y K-Ras4B. Presentan una alta homología entre ellas y tienen un peso molecular de 21 kDa. Las proteínas Ras funcionan como relojes moleculares alternando dos posibles conformaciones, activas cuando están unidas a GTP o inactivas a GDP. Los principales efectores de Ras son las vías de señalización Raf/MAPK y PI3K. Existen trabajos previos que sugieren que Ras tiene un papel importante en la respuesta celular a angiotensina II, pero no se ha demostrado concluyentemente una relación entre esta proteína y la presión arterial. Recientemente se ha generado un ratón que sobreexpresa H-Ras constitutivamente y presenta hipertensión e hipertrofia.

El objetivo de este trabajo fue estudiar el efecto de H-Ras en la presión arterial en un modelo experimental de ratón deficiente para la isoforma H-Ras. Para ello, se midió la presión arterial por 3 procedimientos diferentes, anestesiados y conscientes por telemetría y esfingomanómetro de cola. En todos los casos los animales deficientes en H-Ras presentaron hipotensión respecto a los animales control. La administración de ACh, SNP y dbGMPc produjo un descenso mayor en la presión arterial sistólica de los ratones deficientes en H-Ras. Estas diferencias en la presión arterial desaparecieron en ambos grupos de animales cuando se les administró L-NAME, ODQ y DT-3. El contenido proteico de eNOS, GCs (α) y PKG I en aorta fue mayor en los ratones deficientes en H-Ras así como la actividad enzimática de estas proteínas observando una mayor excreción de nitritos en orina, mayor producción de GMPc y fosforilación de VASP respecto a los ratones control.

Para profundizar en los mecanismos responsables de la hipotensión observada en los ratones deficientes en H-Ras se llevaron a cabo una serie de experimentos en fibroblastos embrionarios procedentes de estos ratones. En primer lugar se observó un incremento en el contenido proteico y actividad de GCs (α) y PKG I en fibroblastos embrionarios de ratones deficientes en H-Ras respecto a las células control. En segundo lugar, se estudió la actividad del promotor de PKG que fue mayor en los fibroblastos embrionarios de ratones deficientes en H-Ras. Todos estos resultados demuestran que los ratones deficientes en H-Ras son hipotensos y presentan un aumento de la vía del NO-GCs-PKG. H-Ras podría ser considerado como una diana terapéutica en el campo del tratamiento de la hipertensión.

TITLE PAGE:

Title:

Targeted genomic disruption of H-Ras induces hypotension through a mechanism dependent on the activation of the NO-sGC-PKG pathway.

Authors:

Aranzazu Chamorro-Jorganes[1*], Maria Teresa Grande[3], Beatriz Herranz[1], Mercedes Griera[1], Eugenio Santos[3], Diego Rodriguez Puyol[1,2], Jose M Lopez-Novoa[3] and Manuel Rodriguez Puyol[1].

Institutional Affiliations:

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Keywords

Arterial pressure, nitric oxide, soluble guanylyl cyclase, protein kinase G.

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E-mail address: aranzazu.chamorro@uah.es

ABSTRACT

Aims. Our aim was to evaluate whether H-Ras lacking mice show changes in arterial pressure and, if this were the case, to analyze the mechanisms involved in the genesis of these changes.

Methods and results. H-Ras lacking mice and mouse embryonic fibroblast (MEF) from these animals were used. Blood pressure was measured using three different methods: direct intra-arterial measurement in anesthetized animals, tail-cuff sphygmomanometer, and radiotelemetry. Acetylcholine (ACh), sodium nitroprusside (SNP), and dibutyryl cGMP (dbcGMP) were used to evaluate the consequences of the activation of the nitric oxide (NO) - soluble guanylyl cyclase (sGC) - protein kinase G (PKG) pathway, while N(G)-nitro-L-arginine methyl ester (L-NAME), 1H-[1,2,4]Oxadiazole[4,3-a]quinoxalin-1-one (ODQ) and DT-3 were used to evaluate the consequences of inhibiting the NO-sGC-PKG pathway. Endothelial oxide nitric synthase (eNOS), sGC (α), and PKG content were assessed by immunoblotting and immunohistochemistry. A functional evaluation of the NO-sGC-PKG pathway in mice lacking H-Ras activity was assessed using the Griess method, cGMP measurements, and immunoblotting. The PKG promoter activity was evaluated by transient transfection. H-Ras$^{-/-}$ showed lower blood pressure than control animals. Moreover, the protein content of eNOS, sGC (α), or PKG I was higher in H-Ras$^{-/-}$ mice than in their controls. The metabolic activity of these enzymes was increased, as the increased urinary nitrite excretion, the increased SNP-stimulated vascular cGMP synthesis, and the increased content of phosphorylated VASP in aortic tissue all suggest. MEFs from H-Ras$^{-/-}$ mice showed higher sGC (α) and PKG I protein content, a greater increase in cGMP synthesis, and greater VASP phosphorylation than control cells. Furthermore, MEFs from H-Ras$^{-/-}$ showed higher PKG promoter activity than control cells.

Conclusion. These results strongly support the up-regulation of the NO-sGC-PKG pathway in H-Ras deficient mice. Moreover, they suggest that H-Ras could be considered as a therapeutic target in the field of hypertension treatment.

INTRODUCTION

Small GTP-binding proteins (G proteins) are monomeric G proteins with a molecular weight of 20 to 40 kDa. A small G protein acts as a molecular switch between inactive GDP-bound and active GTP-bound cycles. Ras proteins are highly conserved from yeast to humans. The small G proteins in this superfamily are structurally classified into 5 families (Ras, Rho, Rab, Sar/Arf, and Ran families), all of them acting as biological switches for diverse cellular processes. Three functional Ras genes are ubiquitously expressed in mammals. These genes are located on different chromosomes and encode four highly homologous 21 kDa proteins: H-Ras, N-Ras, K-Ras4A, and K-Ras4B.[1-3] Multiple downstream effectors of small G proteins, some of which are protein kinases, have been identified. Ras mediates its effect on cell proliferation mainly through the activation of its effector Raf, initiating the mitogen-activated protein kinase cascade.[4] In addition, a variety of Ras effectors have been identified, such as a phosphatidylinositol 3-kinase (PI3K).[5]

Ras isoforms have a very high degree of homology (~80%) at the protein level. H- and K-Ras have a >90% homology at the protein level, excluding the last 25 amino acids. Most of the differences between these proteins are found within the hypervariable region at the carboxyl end of the molecule. However, the different Ras isoforms seem to have different functions.[6] Thus, mice KO for H-Ras, N-Ras, or both isoforms are viable whereas mutations in K-Ras are lethal, indicating that K-Ras is not only essential, but also sufficient for normal mouse development. Targeted genomic disruption of H-Ras and N-Ras, individually or in combination, reveals the dispensability of both loci for mouse growth and development.[7]

Until recently, the relationship between Ras and the regulation of blood pressure have scarcely been studied. Previous reports suggested a role for Ras in the cellular response to angiotensin II,[8,9] but no definite relationship between this protein and blood pressure was established. However, genetic manipulation of the different isoforms of the Ras gene has stressed the relevance of this protein as a hemodynamic regulator. Thus, it has been reported that the knock-in of the H-Ras coding sequence at the K-Ras locus (HRasKI) is viable, though a dilated cardiomyopathy associated with arterial hypertension was shown.[10] More recently, it has been reported that transgenic mice for a

constitutively activated form of H-Ras have hypertension and heart hypertrophy,[11] thus suggesting a role for H-Ras in the cardiovascular system. However, an analysis of the intrinsic cardiovascular mechanisms involved in the genesis of this hypertension has not been extensively performed.

The present experiments were devoted to answer part of these questions, using a different experimental approach. H-Ras lacking mice are viable and apparently normal, but no study has evaluated these animals from a hemodynamic point of view. We hypothesized that H-Ras$^{-/-}$ mice may show changes in arterial pressure that were opposite to those observed in the mice over-expressing Ras and, if correct, that vascular vasodilator mechanisms may be involved in the genesis of these changes.

MATERIALS AND METHODS

Drugs and other reagents

Acetylcholine (ACh), sodium nitroprusside (SNP), N^2,2'-O-dibutyrylguanosine 3',5'-cyclic monophosphate sodium salt hydrate (dibutyryl cGMP), 8-Bromoguanosine-3',5'-cyclomonophosphate sodium salt (8 Br cGMP), N(G)-nitro-L-arginine methyl ester (L-NAME), 3-isobutyl-1-methylxanthine (IBMX), leupeptin, pepstatin A, aprotinin, phenylmethylsulfonyl fluoride (PMSF), ammonium persulphate, anti-actin, and anti-guanylyl cyclase α1 antibodies were purchased from Sigma Chemical (St. Louis, MO, USA). 1H-[1,2,4]Oxadiazole[4,3-a]quinoxalin-1-one (ODQ) and DT-3 were from A.G. Scientific (San Diego, CA, USA) and Biolog (Bremen, Germany), respectively. Acrylamide-bisacrylamide was purchased from Merck (Darmstadt, Germany). Anti-pVASP (ser 239) and anti-VASP antibodies and were purchased from Calbiochem (La Jolla, CA, USA). Anti- eNOS antibody was purchased from BD Biosciences (San Jose, CA, USA). Anti-PKG antibody was purchased from Stressgen Biotechnologies (Victoria, BC, Canada). Peroxide-conjugated goat anti-rabbit IgG was purchased from Chemicon (Temecula, CA, USA). Biotin-labeled goat anti-mouse and anti-rabbit antibodies, avidin-peroxidase complex, and diaminobenzidine were purchased from Dako (Carpinteria, CA, USA). Lipofectamine and Opti-MEM were purchased from Invitrogen (Carlsbad, CA, USA). A dual-Luciferase Reporter Assay System was purchased from Promega (Madison, WI, USA). Dulbecco's Modified Eagle's Medium,

fetal bovine serum, trypsin-EDTA (0.02%), L-glutamine and penicillin-streptomycin were purchased from BioWhittaker (Walkersville, MD, USA).

The culture plates came from Nunc (Kastrup, Denmark). X-OMAT films came from Kodak (Rochester, NY, USA). The ECL chemiluminescence system was purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). The electrophoresis equipment and the protein molecular weight standard were purchased from Bio-Rad (Richmond, CA, USA). The polyvinylidene difluoride membrane came from Perkin Elmer (Boston, MA, USA). The bicinchoninic acid (BCA) assay kit came from Pierce (Rockford, IL, USA). All of the antibodies were used at a 1:1000 dilution. All of the reagents employed were of the highest commercially available grade.

Animals

H-Ras deficient mice (*H-Ras*$^{-/-}$) were obtained as previously reported[7] and maintained under standard conditions. A breeding colony of adult *H-Ras*$^{-/-}$ animals has been maintained in our laboratory for over 7 years. The animals appear healthy and normal with no signs of any apparent associated lesions. The growth rates of these animals were indistinguishable from those of wild-type animals, and mutant mice reproduced normally. Routine genotyping of DNA isolated from mouse tail biopsies was performed by PCR using the previously reported primers.[7] Our research conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Cell culture

Mouse embryonic fibroblasts were isolated using the previously described methods. Studies were performed on confluent monolayers at passages 2-4.[7]

Blood pressure measurements

Blood pressure was measured using three different methods: direct intra-arterial measurement in anesthetized animals,[12-13] tail-cuff sphygmomanometer,[13-14] and radiotelemetry.[15] For the first procedure, mice were anaesthetized with sodium pentobarbital, 40 mg/kg body weight (b. wt.). Body temperature was maintained at 37ºC and a tracheotomy was performed. The right carotid artery was cannulated with PE-10 tubing connected to PE-50 tubing and to a pressure transducer. Arterial pressure (AP) was continuously recorded by a digital data recorder (MacLab/4e, AD Instruments,

Australia) and analyzed using Chart v 3.4. Indirect measurements ofAP were obtained in conscious animals by means of a tail-cuff sphygmomanometer (LE 5001 Pressure Meter, Letica Scientific Instruments, Hospitalet, Spain). The animals were trained for 3 days before starting the measurement to prevent stress and were prewarmed to 30ºC with a heater (LE5660/6, Letica Scientific Instruments).

In addition, direct measurements of AP in conscious animals were performed using radiotelemetry. This method records AP through a catheter implanted into an artery of the mouse. The catheter is attached to a combination of a pressure transducer, a transmitter, and a battery, all encapsulated in an implantable microminiaturized electronic monitor (PA-C20, Data Sciences International, DSI; St. Paul, MN, USA). The implantation of the device in the mice was performed as described.[16] Briefly, after anesthesia by intraperitoneal injection of a mixture containing ketamine 78 mg/Kg, diazepam 6 mg/Kg, and atropine 0.15 mg/Kg, the ventral body wall of the mouse was shaved and wiped clean with topical antiseptic and alcohol and a midline skin incision 2–3 cm long from pelvis to xiphoid process was made. A second incision from chin to manubrium exposed the salivary glands, and a subcutaneous channel from the neck site to the abdominal site was made by blunt dissection. A 16-gauge trocar was then passed from the abdominal cavity through the lateral aspect of the left rectus muscle and under the skin to the neck incision. The implant catheter was introduced into the trocar, and the body of the implant was inserted into the abdominal cavity. The body of the implant was secured to the midline of the abdominal cavity with interrupted nonabsorbable sutures. The skin was closed with staples and a tissue adhesive, and a topical antiseptic was applied. Subsequently, the carotid artery was cannulated using the device's catheter, holding it in place with tissue adhesive and securing it with the middle tie. The skin was closed and approximately 1 ml of normal saline was injected subcutaneously into two or more sites to assure adequate postoperative hydration. The animal was kept in a warmed. Ventilated environment for at least 24 h with continuous blood pressure monitoring. Most animals were fully ambulatory within 30 min, with a return to their drinking and eating habits and bowel function within 1 h. An antibiotic, cefazolin 25 mg/Kg (Normon, Spain), was administered at the time of operation and twice daily during the recovery period. An analgesic, buprenorphine 0.1 mg/Kg IM (Buprex, Schering-Plough, Madrid, Spain), was provided if the animal's behavior suggested the presence of pain, e.g. attention to the wound site, lethargy, or aggression. After instrumentation, each animal was housed individually in a standard polypropylene cage

placed over a radio receiver, and in a quiet environment. After recovery, repeated measurements of basal systolic and diastolic arterial pressure and heart rate were taken for each animal between 10:00 and 12:00 a.m. for at least 3 days. Data was digitally recorded on a computer and calculated using the software provided by Data Sciences.Blood pressure was measured under basal conditions and after the administration of the following treatments: acetylcholine (ACh, 1 μg/Kg b.w. i.p.), sodium nitroprusside (SNP, 2 μg/Kg b.w. i.p.), dibutyryl cyclic GMP (dbcGMP, mg/Kg b.w. i.p.), N(G)-nitro-L-arginine methyl ester hydrochloride (L-NAME, 20 mg/Kg b.w./day, drinking water for 2 weeks), 1H-[1,2,4]Oxadiazole[4,3-a]quinoxalin-1-one (ODQ, 5 mmol/Kg b.w. i.p.), and DT-3 (500 μg/Kg b.w. i.p.).

Protein extraction and Western blot analysis

Tissues were studied under basal conditions. Cells were studied under basal conditions and after treatments with SNP (10^{-4}M, 15 min) or 8-bromo-cyclic GMP (8 Br cGMP, 10^{-4}M, 15 min). In the SNP experiments, to assess the specificity of the observed effects, cells were preincubated with ODQ (10^{-6}M, 30 min). Tissues or cells were washed in PBS and solubilized (10 mmol/L Tris-HCl pH 7.4, 1 mmol/L EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 500 nmol/L sodium orthovanadate, 50 nmol/L NaF, 1 mmol/L pepstatin/leupeptin/aprotinin, 1 mmol/L PMSF) for 30 min at 4ºC. To perform Western blot analysis, total protein extracts (20 μg/lane) were size-fractionated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were blocked for 1 h and then incubated with the corresponding antibodies. After washing, blots were incubated with a secondary antibody and specifically bound antibody was detected using the ECL method. Films were scanned and analyzed using appropriate software (NIH Imagine 1.55; National Institutes of Health, Bethesda, MD).

Immunohistochemistry

Aortas were harvested from *H-Ras*$^{+/+}$ and *H-Ras*$^{-/-}$ mice, fixed with formalin, and subjected to increasing concentrations of ethanol and xylene. Tissues were embedded in paraffin, cut into serial sections 3-5 μm thick, incubated with the anti eNOS (dilution 1:100), sGC (α) (dilution 1:100), and PKG antibodies (dilution 1:200), and then with biotin-labeled goat anti-mouse and anti-rabbit antibodies, and subsequently exposed to

the avidin-peroxidase complex. Finally, diaminobenzidine was added to serve as substrate. Samples were counterstained with Mayer's Hematoxylin. Negative controls were obtained by omitting primary antibodies.

Determination of nitrites

Nitrites were analyzed using the Griess method.[17] The animals were housed in individual metabolic cages and their urine was collected for 24 h.

Measurement of cGMP

Tissues or cells were studied under basal conditions and after treatment with SNP (10^{-4}M, 15 min), with or without ODQ (10^{-6}M, 30 min). They were washed with buffer A (Tris 20 mmol/L, NaCl 130 mmol/L, KCl 5 mmol/L, sodium acetate 10 mmol/L, glucose 5 mmol/L, pH 7.45) and incubated for 15 min with the same buffer containing 2.5 mmol/L Ca^{2+} and IBMX 10^{-4} mol/L. The medium was aspirated and 1 ml of ice-cold ethanol was added to the plates and maintained at 4°C for 30 min. Cell or tissue extracts were centrifuged for 20 min at 2000 x *g* and the supernatant was evaporated to dryness. cGMP levels were determined with a commercial [125I]-cGMP radioimmunoassay kit. Tissues were weighed, and protein concentration was determined by BCA assay.

Transient transfection and Luciferase assays

A total of 3.5 x 10^5 cells/well were plated in six-well plates 24 h before transfection. The cells in each well were then incubated 4 h at 37ºC with 2 ml Opti-MEM medium containing complexes of 2 μL Lipofectamine, 0.1 μg human PKG reporter[18] and 0.01 μg Renilla luciferase reporter as an internal control. The transfected cells were then incubated with complete growth medium for 24 h and were later washed with PBS and lysed. Finally, firefly luciferase activity of the PKG reporter was measured with a luminometer (FB12 Berthold luminometer) and normalized against the Renilla luciferase activity using the dual-luciferase reporter assay system. Protein concentration was determined by BCA.

Statistical methods

The number of experiments performed is reflected in the "Figure Legends." As this number was under 10, non-parametric statistics were used for comparisons (Friedman and Wilcoxon tests for paired data, and Kruskal-Wallis or Mann-Whitney tests for non-

paired data). The data are expressed as the mean ± SEM. A $p < 0.05$ was considered statistically significant.

RESULTS

Data obtained by either direct cannulation of anesthetized animals, or tail-cuff measurements and telemetry in conscious animals revealed that H-Ras$^{-/-}$ mice showed lower systolic, diastolic, and mean arterial pressure than H-Ras$^{+/+}$ mice. Heart rate measurements taken in anesthetized animals or by telemetry were significantly lower in H-Ras lacking mice, whereas they increased in these same animals when the tail-cuff method was used (Table 1).

Figure 1 includes the results obtained when the NO-sGC-PKG pathway was stimulated. The intraperitoneal administration of ACh, SNP, and dbcGMP induced a significant reduction of SAP in both the H-Ras$^{-/-}$ mice and their respective controls (Panels A to F). However, after the administration of SNP and dbcGMP, the SAP values show a tendency towards being significantly lower in the H-Ras lacking animals. The differences observed were statistically significant when mice received SNP and the tail-cuff method was used (Panel C), and with both tail-cuff measurements and telemetry after dbcGMP treatment (Panel E and F). The results of an opposite experimental approach are collected in Figure 2. The blockade of NO with L-NAME, sGC with ODQ, and PKG with DT-3 increased SAP in both groups of animals, and the differences in blood pressure disappeared (Panels A to F).

As these pharmacological experiments suggested an up-regulation of the NO-sGC-PKG pathway in the vascular walls of the H-Ras$^{-/-}$ mice, we measured the aortic protein content of eNOS, the α subunit of sGC, and PKG I. As shown in Figure 3, all of these proteins were elevated when the animals lacked the H-Ras isoform. Moreover, these quantitative changes in the protein content determined functional modifications in knock-out mice. Thus, they excreted a lower amount of nitrites in urine (Figure 4, Panel A), their aortic ring synthesized higher amounts of cGMP when treated with SNP (Figure 4, Panel B), and VASP phosphorylation increased in their aortic walls (Figure 4, Panel C).

To gain insight into the mechanisms responsible for the changes observed in the H-Ras$^{-/-}$ animals, some experiments were performed on MEFs from these mice. As occurred in their vascular walls, the protein content of sGC (α) and PKG I was

significantly higher in the MEFs of the H-Ras knock-out animals (Figure 5, panel A). These H-Ras lacking cells, stimulated by SNP, showed greater cGMP synthesis (Figure 5, panel B) and VASP phosphorylation (Figure 5, Panel C) than their respective controls. Moreover, when B-Br-cGMP was used as a stimulus, they also showed increased VASP phosphorylation (Figure 5, Panel D) when compared with control cells. After confirmation that the changes observed in the sGC-PKG pathway were comparable in aortic walls and in MEFs, preliminary experiments were performed to evaluate the relationship between H-Ras deficiency and increased PKG I content. As shown in Figure 6, a lack of H-Ras induced significant activation in the human PKG I 2 Kb promoter region. When MEFs were transiently transfected with serial deletions of human PKG-I promoter, it was observed that promoter activity disappeared with the 0.6 kb fragment.

DISCUSSION

The analysis of blood pressure in mice is a relatively complex procedure, which is done mainly by using the classic indirect method based on sphygmomanometers. A direct intra-arterial pressure measurement requires the animals to be anesthetized, with the subsequent possible interferences of anesthesia. These methodological problems increase when the expected changes in blood pressure are not very relevant. Thus, to study the changes in AP in H-Ras deficient mice, an approach was used that combined these two techniques and added telemetry,[12-15] a procedure that allows certain hemodynamic parameters in animals to be measured in conditions that may be considered almost completely physiological. Regardless of the procedure performed, H-Ras$^{-/-}$ mice showed a lower AP than control animals, with a reduction in SAP ranging from 7 to 13 mmHg. As expected, SAP was higher when the tail-cuff method was used, and lower in anesthetized animals. The only parameter that differed among the three measurement procedures was heart rate. In general, H-Ras deficient mice showed a decreased heart rate when compared to control mice. However, when the animals' blood pressure was measured with a sphygmomanometer, their heart rate increased, probably due to increased stress and because this procedure seems to be less appropriate for analyzing heart rate.

Lower blood pressure could be explained by: decreased circulating volume, deficient cardiac function, or slightly higher vasodilation. In turn, this increase in vasodilation may be the consequence of deficient vasoconstriction or even increased vascular relaxation. Sufficient data suggests that Ras activation is an essential molecular switch that triggers many of the signal transductions and substance functions, such as angiotensin II in vascular smooth muscle cells.[9,19-21] Thus, the development of hypotension in H-Ras$^{-/-}$ mice could be explained by an inappropriate vascular response to vasoconstrictor mediators. Most studies that analyze the relationships between angiotensin II and Ras, however, are devoted to the analysis of cardiovascular remodeling.[22] Therefore, we set out to test the possibility that increased vasodilation could be responsible, at least in part, for the changes detected in AP. A three-step analysis was performed to evaluate this hypothesis. First, the pharmacological responses to different modulators of the NO-sGC-PKG pathway were tested. Second, direct measurements of the different components of this pathway were made. Finally, the functional integrity of these components was assessed.

H-Ras deficient mice showed an increased hypotensive response to the agonist in this system, particularly SNP and dbcGMP. Their low blood pressure levels reached values comparable to those of the control animals when NOS, sGC, or PKG were blocked. The protein content of eNOS, sGC (α), or PKG I was higher in H-Ras$^{-/-}$ mice than in their controls. The metabolic activity of these enzymes was increased, as was suggested by increased urinary nitrite excretion, increased SNP-stimulated vascular cGMP synthesis, and elevated phosphorylated VASP in aortic tissue. Taken together, these results strongly support the up-regulation of the NO-sGC-PKG pathway in H-Ras deficient mice and the functional relevance of this up-regulation in the genesis of the hypotension observed in these animals. However, parts of the present results do not appear to be thoroughly explained. In particular, the *in vivo* response to ACh was similar in both groups of animals. If an over-expression of eNOS were relevant as a pathogenic mechanism, a greater reduction in AP in H-Ras$^{-/-}$ mice would be expected. This was not the case, but, in contrast, a NOS blockade completely normalized blood pressure in the knock-out animals. The *in vivo* analysis of cardiovascular responses is not always a simple procedure, as the homeostatic responses may mask the primary functional changes attributable to a particular mediator, which could have been the case when treating the animals with ACh. Thus, a more careful analysis needs to be performed in

order to assess the relevance of eNOS in the pathogenesis of H-Ras$^{-/-}$ mouse hypotension.

The complexity of the analysis of the *in vivo* results led us to perform a number of experiments in the cellular system, MEFs from H-Ras deficient and control mice. Obviously, eNOS cannot be studied in these cells, but the differences in sGC and PKG were assessable. The results were completely comparable to those obtained in mice: The lack of H-Ras induced the over-expression of sGC (α) and PKG I, and these over-expressed enzymes were functionally active. An additional advantage of these experiments on cells is that they allowed us to better understand the relationships between Ras deficiency and enzyme over-expression. A preliminary analysis performed for PKG I suggests that the changes in the protein content could be related to an increased activation of the transcription of the gene due to an increased promoter activity. A particular region of the PKG promoter has been identified as being responsible for this activation, and forthcoming studies will try to establish the importance of Ras in the regulation of this region.

Unfortunately, our results can not be compared with previous studies, since no similar experimental approaches have previously been performed. However, as was stated in the Introduction, it was expected that H-Ras deficiency would determine effects opposite to those observed with H-Ras over-expression[11,24] and the present experiments confirm this hypothesis. Our results suggest that the mechanisms involved in hypertension-linked H-Ras over-expression are very complex, and must be carefully analyzed. Moreover, they suggest that H-Ras could be considered a therapeutic target in the field of hypertension treatment.

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Conflict of interest

The authors state no conflict of interest.

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FIGURE LEGENDS

Fig 1. Consequences of the activation of the NO-cGMP-PKG pathway in mice lacking H-Ras activity (-/-). Animals were treated with acetylcholine (ACh, Panels A and B), sodium nitroprusside (SNP, Panels C and D), and dibutyryl cGMP (dbcGMP, Panels E and F). Systolic arterial pressure (SAP) SAP was measured using two different methods: tail-cuff sphygmomanometer (left part of the Figure, Panels A, C, and E), and telemetry (right part of the Figure, Panels B, D, and F). Data are the mean ± SEM of 10 independent animals. Black bars represent basal SAP and the gray bars represent the SAP after treatments. $^{*}P$ <0.05 vs. control animals (+/+) $^{\#}P < 0.05$ vs. basal SAP.

Fig 2. Consequences of the inhibition of the NO-cGMP-PKG pathway in mice lacking H-Ras activity (-/-). Animals were treated with L-NAME (Panels A and B), ODQ (Panels C and D), and DT-3 (Panels E and F). Systolic arterial pressure (SAP) was measured using two different methods: tail-cuff sphygmomanometer (left part of the Figure, Panels A, C, and E), and telemetry (right part of the Figure, Panels B, D, and F). Data are the mean ± SEM of 10 independent animals. Black bars represent basal SAP and the gray bars represent the SAP after treatments. $^{*}P < 0.05$ vs. control animals (+/+) $^{\#}P < 0.05$ vs. basal SAP.

Fig 3. Protein content of endothelial nitric oxide synthase (eNOS, Panels A and B), soluble guanylyl cyclase (sGC α, Panels C and D), and protein kinase G (PKG I, Panels E and F) in aorta from mice lacking H-Ras activity (-/-). The protein content was evaluated by both immunoblotting and immunohistochemistry. In the Western blot experiments (Panels A, C, and E), a representative blot is shown in each case, including the blot of a constitutive protein (α-actin). The results of the densitometric analysis (corrected by the α-actin content) of eight independent experiments are shown as bar graphs. In these graphs, data are expressed as a percentage of the control (%C) and are the mean ± SEM. In the immunohistochemistry experiments (Panels B, D and F), a representative microphotograph is shown in each case. $^{*}P < 0.05$ vs. control animals (+/+).

Fig 4. Functional evaluation of the NO-cGMP-PKG pathway in mice lacking H-Ras activity (-/-). Urine nitrite excretion (Panel A), sodium nitroprusside-stimulated

(SNP) cyclic GMP synthesis (cGMP) by aortic rings (Panel B), and phosphorylated vasodilator-stimulated phosphoprotein (VASP) aortic content (ratio P-VASP/VASP) were analyzed in mice lacking H-Ras activity (-/-). For VASP, a representative blot is shown. The results of the densitometric analysis of the different experiments are shown as bar graphs and expressed as a percentage of the control (%C). Data are the mean ± SEM of 8 independent experiments **P* < 0.05 vs. control animals (+/+).

Fig 5. The cGMP-PKG pathway in mouse embryonic fibroblasts (MEF) from mice lacking H-Ras activity (-/-). The protein content of soluble guanylyl cyclase (sGC α) and protein kinase G (PKG I) (Panel A) was evaluated by immunoblotting. A representative blot is shown in each case, including the blot of a constitutive protein (α-actin). The results of the densitometric analysis (corrected by the α-actin content) of 6 independent experiments is shown as a bar graph. In these graphs, data are expressed as a percentage of the control (%C) and are the mean ± SEM **P* < 0.05 vs. control cells. Sodium nitroprusside-stimulated (SNP) cyclic GMP synthesis (cGMP) (Panel B) and phosphorylated vasodilator-stimulated phosphoprotein (VASP) content (ratio P-VASP/VASP) (Panel C), as well as 8-bromo-cyclic GMP (8 Br cGMP)-dependent P-VASP content (ratio P-VASP/VASP) (Panel D) were analyzed in these cells. The SNP experiments were performed in the presence of an sGC inhibitor, ODQ. For VASP, a representative blot is shown. The results of the densitometric analysis of the different experiments are shown as bar graphs and expressed as a percentage of the control (%C). In the Panels B to D, data are the mean ± SEM of 6 independent experiments. Black bars represent basal conditions, the gray bars represent the SNP treatment and the stripped bars represent the ODQ plus SNP treatment. **P* < 0.05 vs. control cells. ***P* < 0.05 vs. control cells under the same treatment.

Fig 6. Analysis of the PKG I promoter activity in mouse embryonic fibroblasts (MEF) from mice lacking H-Ras activity (-/-). MEFs from H-Ras deficient (-/-) and control (+/+) mice were transfected with the 2 kb promoter region of the human PKG I and serial deletions of this region, linked to a luciferase reporter element. Luciferase activity was normalized to Renilla activity in order to measure promoter activity. Data are representative of at least 5 independent experiments. The results are expressed as a

percentage of the control basal transcriptional activity and are the mean ± SEM *$P <$ 0.05 vs. control cells.

Table 1. Arterial pressure (AP) in mice lacking H-Ras activity (H-Ras$^{-/-}$). AP was measured by three different methods, direct intra-arterial recording in anesthetized animals (A), tail-cuff sphigmomanometer (B), and telemetry (C). Systolic (SAP), diastolic (DAP), and mean (MAP) arterial pressures are given in each case, as well as heart rate (HR). Data are expressed in mmHg (AP), and beats/min (HR). Results are shown as mean ± SEM. *P < 0.05 vs H-Ras$^{+/+}$.

(A) **Anesthetized animals**

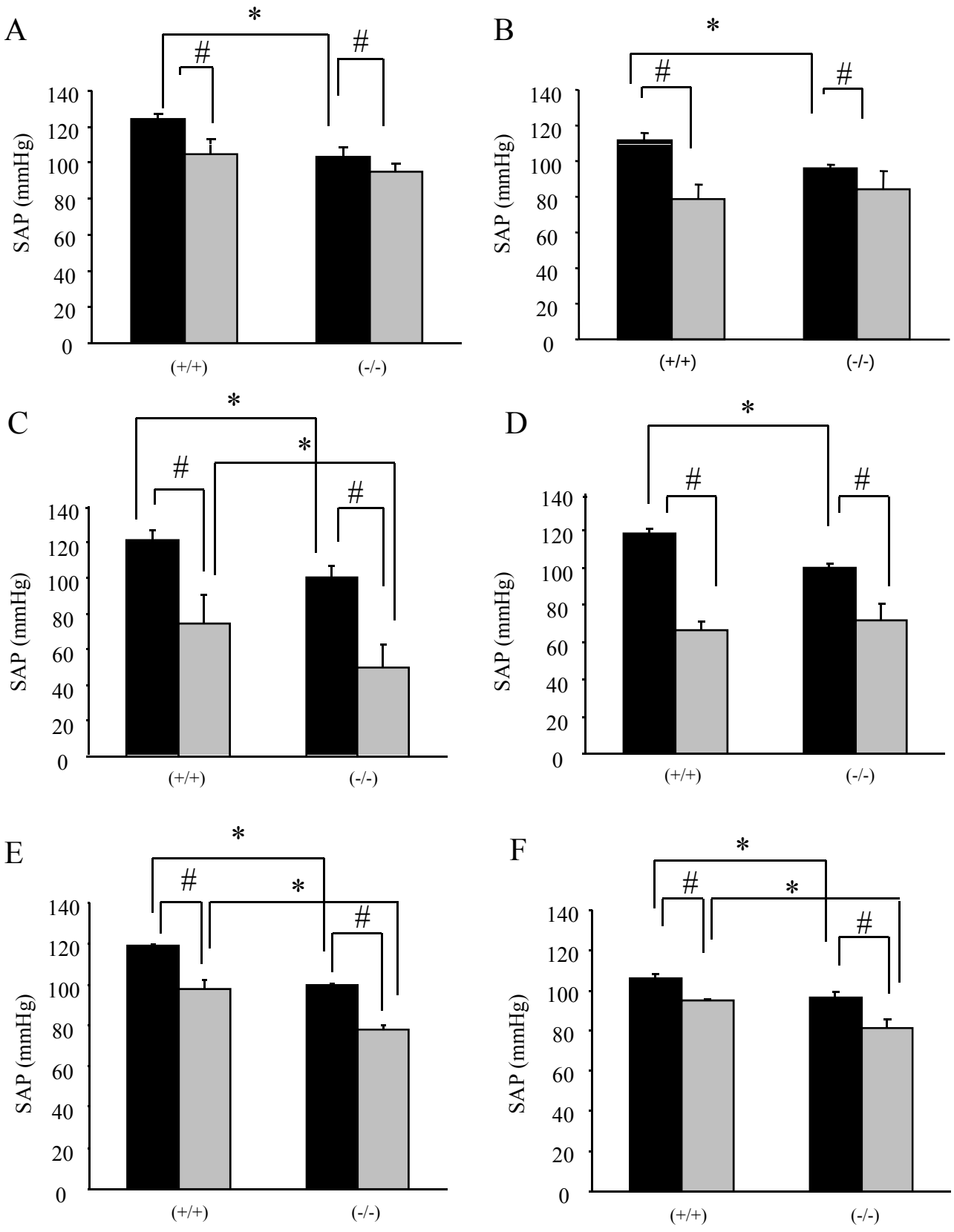
	SAP	DAP	MAP	HR	n
H-Ras$^{+/+}$	96 ± 1	71 ± 2	82 ± 2	487 ± 19	10
H-Ras $^{-/-}$	89 ± 4*	58 ± 2*	73 ± 3*	380 ± 24*	10

(B) **Conscious animals, tail-cuff**

	SAP	DAP	MAP	HR	n
H-Ras$^{+/+}$	118 ± 1	83 ± 2	94 ± 1	598 ± 34	9
H-Ras $^{-/-}$	98 ± 1*	66 ± 2*	77 ± 1*	641 ± 13*	7

(C) **Conscious animals, telemetry**

	SAP	DAP	MAP	HR	n
H-Ras$^{+/+}$	111 ± 2	97 ± 3	102± 2	603 ± 11	7
H-Ras $^{-/-}$	98 ± 2*	84 ± 3*	91 ± 3*	510 ± 29*	7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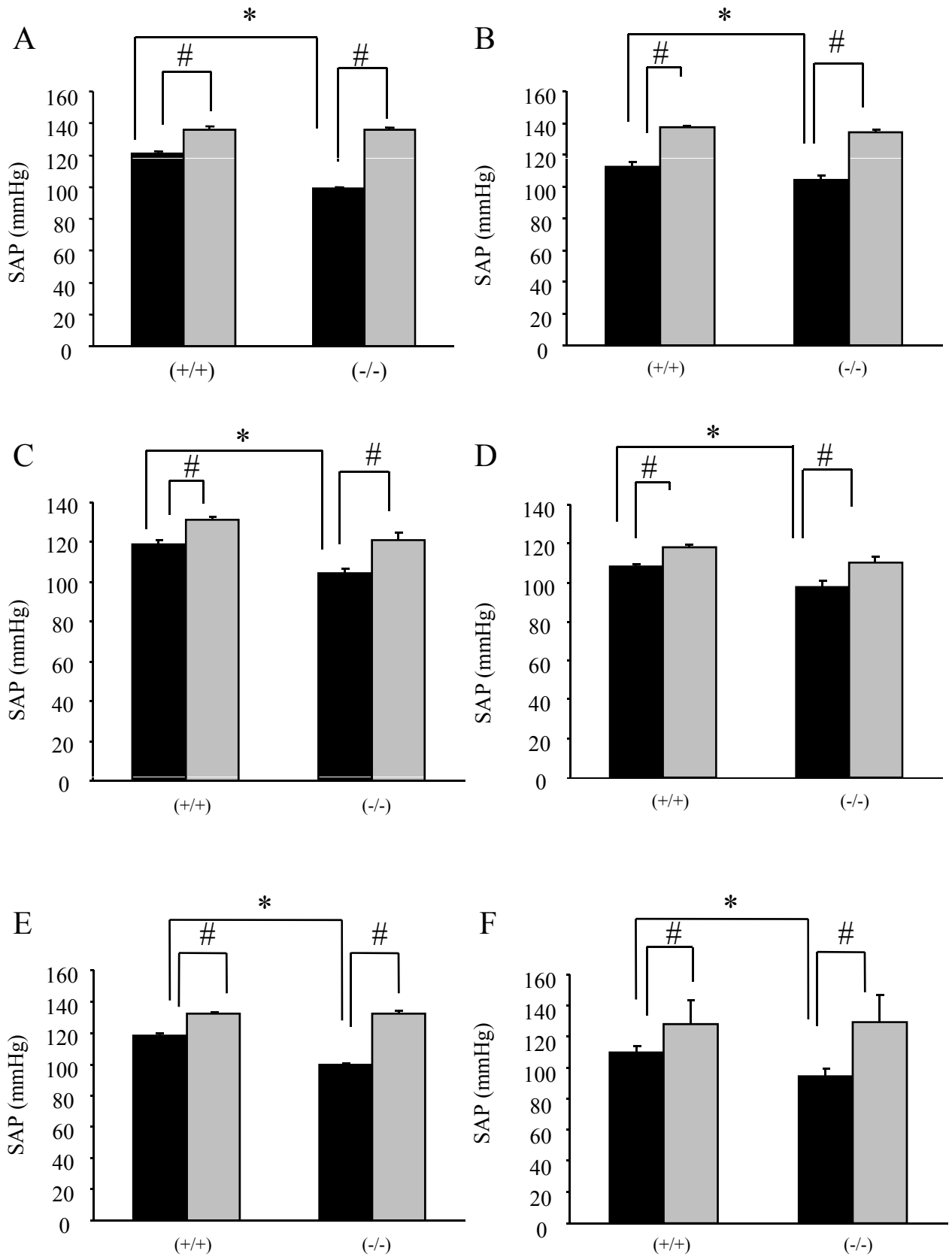
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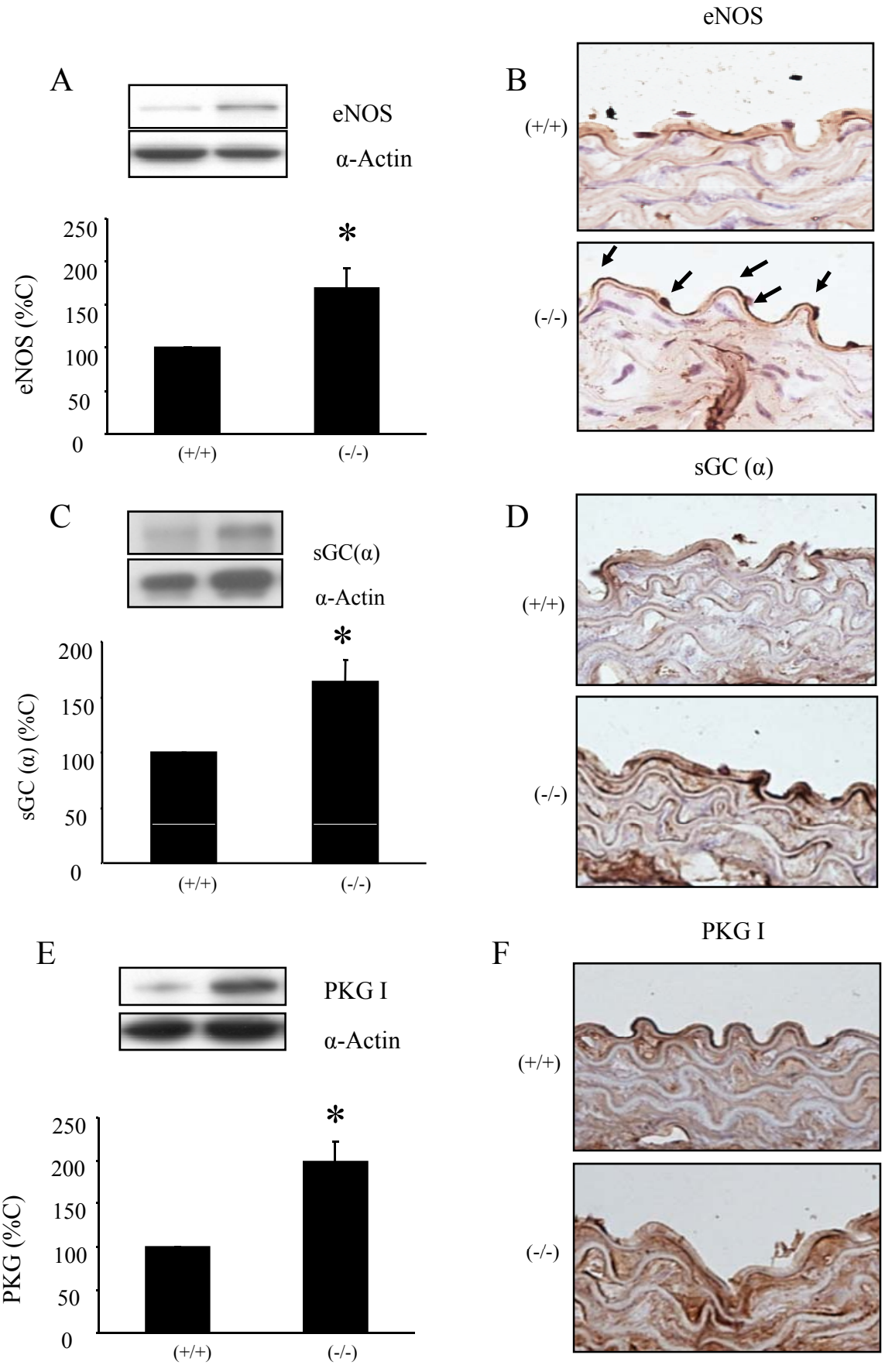
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C
sGC(α)
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sGC (α) (%C)
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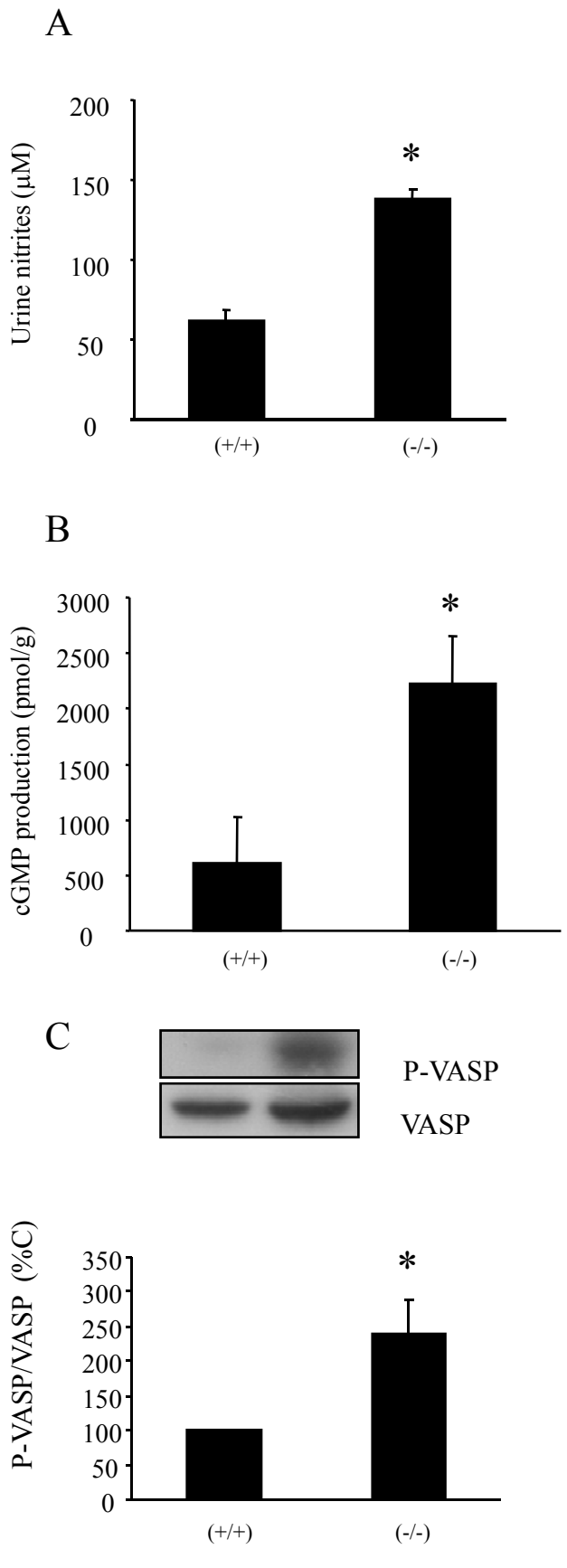
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1000
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cGMP production (pmol/g)
*
(+/+)
(-/-)
C
P-VASP
VASP
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200
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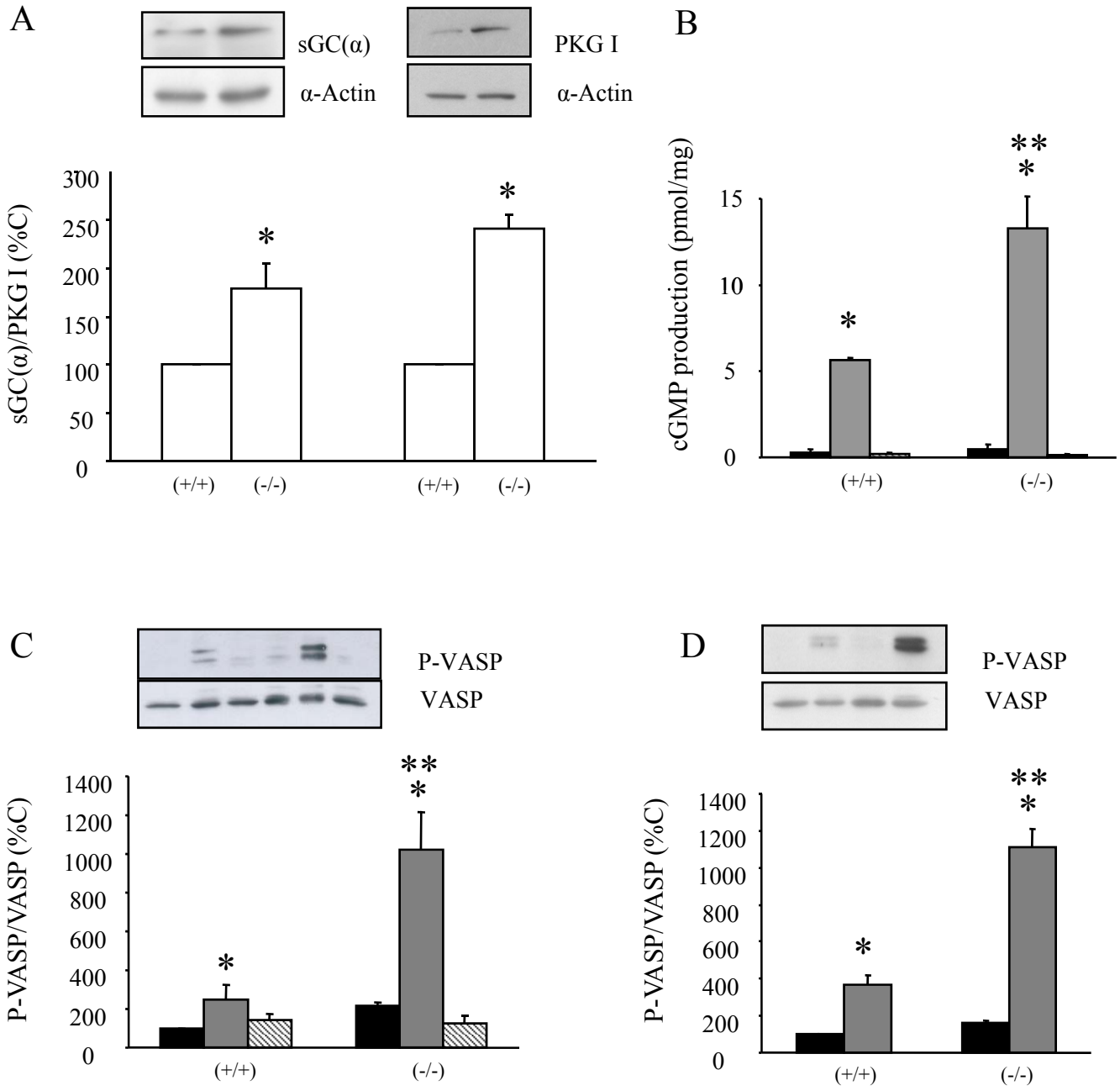
*
(+/+)
(-/-)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PKG I
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sGC(α)/PKG I (%C)
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50
0
(+/+)
(-/-)
(+/+)
(-/-)
B
cGMP production (pmol/mg)
15
10
5
0
(+/+)
(-/-)
C
P-VASP
VASP
P-VASP/VASP (%C)
1400
1200
1000
800
600
400
200
0
(+/+)
(-/-)
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VASP
P-VASP/VASP (%C)
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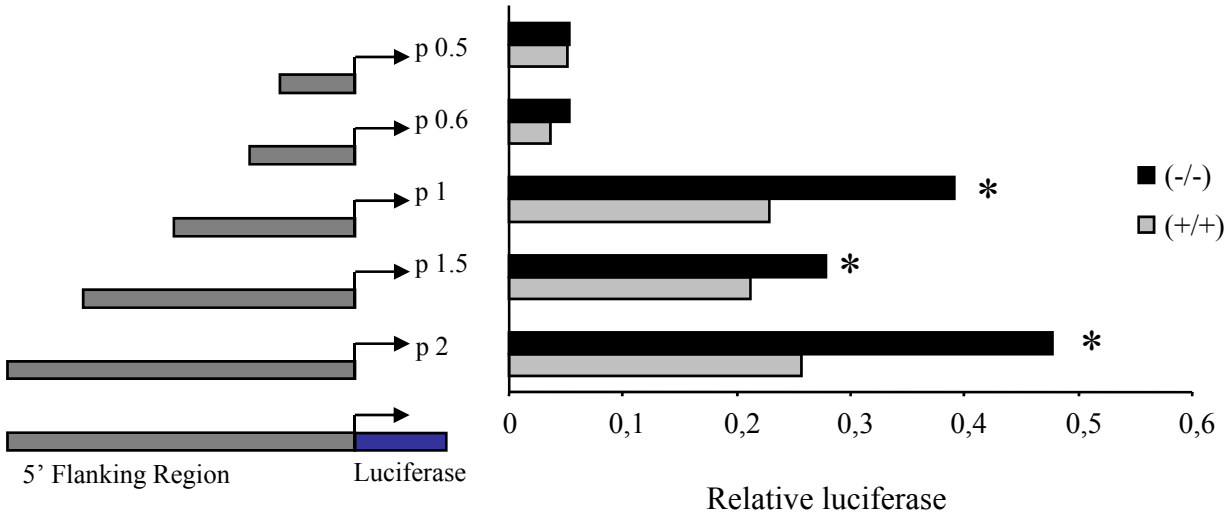
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p 2
5' Flanking Region
Luciferase
(-/-)
(+/+)
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0,1
0,2
0,3
0,4
0,5
0,6
Relative luciferase

SUMARIO DE RESULTADOS

SUMARIO DE RESULTADOS

1. El tirofiban incrementa el contenido proteico de GCs β1 en células contráctiles de rata y humanas a través de la interacción de los dominios RGD con la integrina β1.

2. El tirofiban incrementa el contenido proteico de GCs β1 en la pared vascular de ratas Wistar favoreciendo la respuesta vasorelajante a donadores del NO.

3. El tirofiban revierte la disminución de GCs β1 mediada por el tratamiento crónico con dinitrato de isosorbide y la taquifilaxia al tratamiento.

4. El tirofiban incrementa el efecto antihipertensivo de los donadores del NO en ratas SHR disminuyendo el contenido de GCs β1.

5. La fibronectina incrementa el contenido proteico y actividad de PKG Iα en células mesangiales humanas a través de la interacción con la integrina β1.

6. El fibronectina aumenta la actividad transcripcional del promotor humano de PKG I en células mesangiales humanas.

7. La fibronectina y el tirofiban incrementan el contenido proteico de PKG en células musculares lisas de aorta de rata.

8. El tirofiban incrementa el contenido proteico de PKG en la pared vascular de ratas Wistar. Además, el pretratamiento con tirofiban en ratas Wistar produce un aumento en la respuesta vasorelajante tras la administración de dibutiril GMPc.

9. Los ratones deficientes en H-Ras, en condiciones basales, muestran menores valores de presión arterial que los ratones control.

10. Los ratones deficientes en H-Ras presentan un mayor contenido proteico y actividad de eNOS, GCs (α) y PKG la pared vascular que los ratones control.

11. Los fibroblastos embrionarios procedentes de ratones H-Ras presentan un mayor contenido proteico y actividad de GCs (α) y PKG así como una mayor actividad transcripcional del promotor de PKG frente a las células control.

COMENTARIOS GENERALES

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La matriz extracelular (MEC) proporciona un soporte crítico a las células en su proceso de organización para la formación de tejidos. Recientemente se han realizado numerosas descripciones sobre la importancia de la matriz en el proceso organizativo vascular o renal. Igualmente se ha descrito un número importante de funciones biológicas como la adhesión, migración y proliferación celular en las que la MEC interviene interaccionando con receptores de membrana tipo integrinas (*Hynes, 2002*).

Nuestro grupo de investigación ha demostrado en los últimos años que la MEC juega un papel fundamental en la regulación del fenotipo de las células contráctiles, en concreto modulando la expresión de componentes de la vía de señalización del NO-GCs-PKG. De este modo, cuando se cultivaron células endoteliales en colágeno I se observó un decremento en la expresión de eNOS (*Gónzalez-Santiago y col., 2002*). Efectos similares se obtuvieron en la expresión de GCs en células mesangiales en colágeno I (*De Frutos y col., 2005*). Sin embargo, el RGDS, tetrapéptido que contiene la secuencia RGD, específica de la fibronectina, incrementaba la expresión de la subnunidad β1 de la GCs (*Díez-Marqués y col., 2005*). En todos estos estudios, las proteínas de matriz ejercían sus efectos a través de la interacción con los receptores tipo integrina de las células.

Los datos presentados en esta tesis confirman nuestra primera hipótesis de partida, es decir, que la matriz extracelular ejerce un papel clave en la regulación de la expresión de las proteínas GCs y PKG. Cambios en el contenido proteico y actividad de la GCs se han relacionado con la disfunción endotelial en determinadas enfermedades cardiovasculares como la hipertensión, aterosclerosis y diabetes (*Ruetten y col., 1999; Melichar y col., 2004*). Por ello, en el primer bloque de resultados, se quiso evaluar la capacidad de análogos del RGD, como el tirofiban, para modular el contenido vascular de GCs en animales de experimentación en condiciones basales y situaciones fisiopatológicas. El tirofiban no sólo incrementó el contenido proteico de GCs sino que además, redujo la taquifilaxia observada tras el tratamiento de donadores crónicos del NO en ratas Wistar.

Como modelo genético de hipertensión sistémica utilizamos ratas espontáneamente hipertensas (SHR), que presentan un menor contenido proteico de GCs respecto a las ratas control Wistar Kyoto (*Ruetten y col., 1999*). Tras el tratamiento con tirofiban se observó un incremento el contenido proteico de GCs en los vasos de las ratas SHR y se favoreció la respuesta hipotensora frente a donadores de NO. La importancia de este trabajo reside en que es la primera vez que se describen las consecuencias funcionales del aumento "*in vivo*" de la GCs mediado por las proteínas de matriz extracelular.

La GCs cataliza la conversión del GTP en CMPc, cuyo efector principal es la PKG, que juega un papel fundamental en el proceso de contracción-vasorelajación. En determinadas situaciones patológicas como hipertensión y aterosclerosis tardía se ha observado una disminución en el contenido o actividad de esta enzima, lo que sugiere que PKG podría estar involucrada en la disfunción vascular de las enfermedades cardiovasculares (*Pfeifer y col., 1998; Sausbier y col., 2000; Melichar y col., 2004*).

El segundo bloque de resultados se centró en la regulación de PKG por proteínas de matriz. Teniendo en cuenta los antecedentes del laboratorio estudiamos el efecto de la fibronectina en la modulación de PKG en células contráctiles y animales de experimentación. La fibronectina modula la expresión de PKG Iα a través de la interacción con integrinas, concretamente la subunidad β1 y la posterior fosforilación de Akt. Es un hecho ampliamente descrito la fosforilación de Akt tras la activación de integrinas (*Gibson y col., 2005; Ruiz-Torres y col., 2007; Kotha y col., 2008*). La fibronectina incrementó el contenido proteico y actividad de PKG Iα en células mesangiales humanas. Además se observó un aumento en la expresión de ARNm y actividad transcripcional del promotor de PKG. Para estudiar la relevancia de estos datos "*in vivo*" se administró dibutiril GMPc (dbGMPc) en ratas pretratadas o no con tirofiban, observando una mayor respuesta vasorelajante con el pretratamiento con tirofiban. Todos estos resultados apoyan la utilidad del tirofiban y por extensión la modulación de integrinas, en el tratamiento de enfermedades cardiovasculares.

El tercer bloque de resultados se basó en el estudio de la regulación de la presión arterial en ratones deficientes en H-Ras. Aunque H-Ras está asociado a diversas patologías como cáncer de vejiga, próstata, tiroides (*Mo y col., 2007*) y síndrome de

Costello (*Schuhmacher y col., 2008*) también se ha relacionado esta proteína con el mecanismo de contracción-relajación vascular (*Griendling y col., 1997; Haruhiko y col., 2006*). Existen trabajos previos que sugieren que Ras tiene un papel importante en la respuesta celular a angiotensina II, pero no se ha demostrado concluyentemente una relación entre esta proteína y la presión arterial. Recientemente se ha generado un ratón que sobreexpresa H-Ras constitutivamente y presenta hipertensión e hipertrofia pero no se ha descrito el mecanismo molecular implicado (*Schuhmacher y col., 2008*).

Los resultados obtenidos en esta tesis demostraron nuestra segunda hipótesis: los ratones deficientes en H-Ras presentan el efecto opuesto a la los ratones que sobreexpresan H-Ras, es decir, son hipotensos. Este efecto puede deberse principalmente a un déficit de la respuesta vasoconstrictora o un incremento de la respuesta vasodilatadora. La administración de ACh, SNP y dbGMPc incrementó la respuesta hipotensora en los ratones deficientes en H-Ras; sin embargo el tratamiento con diferentes inhibidores de los distintos componentes de la vía del NO-GCs-PKG como el L-NAME, ODQ y DT-3 revertió el efecto, igualando las presiones arteriales sistólicas en ambos grupos de animales. Además se observó un aumento en el contenido proteico y actividad de eNOS, GC (α) y PKG I en los animales deficientes en H-Ras respecto a los ratones control. Existe un estudio que demuestra la regulación de la producción de NO endotelial por la vía H-Ras-PI3K/Akt-eNOS *"in vivo"* de forma que el descenso de la actividad transcripcional de H-Ras produjo un aumento en la expresión de eNOS total (*Jeon y col., 2004*). Este trabajo apoya nuestros resultados que demuestran que los ratones deficientes en H-Ras presentan un aumento de la vía de señalización del NO-GCs-PKG.

CONCLUSIONES

CONCLUSIONES

Las proteínas de matriz extracelular, concretamente la fibronectina, y determinados activadores de integrinas, como el tirofiban, regulan la expresión y actividad de la GCs β1 y PKG Iα en células contráctiles y animales de experimentación.

El tirofiban podría emplearse, en conjunción con otros fármacos vasodilatadores, como herramienta terapéutica en las enfermedades cardiovasculares.

La proteína H-Ras juega un papel fundamental en la regulación de la presión arterial. Su déficit produce un aumento en la vía de señalización NO-GCs-PKG.

H-Ras podría ser considerado como una diana terapéutica en el tratamiento de la hipertensión.

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