UNIVERSITAT AUTÒNOMA DE BARCELONA
Departament de Bioquímica i Biologia Molecular

MUERTE CELULAR DURANTE LA REPERFUSIÓN MIOCÁRDICA.

MECANISMOS Y NUEVAS DIANAS TERAPEÚTICAS.

Memoria presentada por Javier Inserte Igual para optar al grado de Doctor en Ciencias por la Universitat Autònoma de Barcelona

Director:

DAVID GARCIA-DORADO GARCIA, Doctor en Medicina por la Universidad Complutense de Madrid

Tutor:

MARGARITA SENTIS VILALTA, Doctora en Farmacia por la Universitat de Barcelona.

Servicio de Cardiología. Hospital Universitari Vall d'Hebron.

Barcelona, Diciembre 2003

David Garcia-Dorado García, Doctor en Medicina y Jefe de Sección del Hospital Universitari Vall d'Hebron de Barcelona, y Margarita Sentís Vilalta, Doctora en Farmacia, Jefe de Servicio de Bioquímica y Hematología del Hospital Materno Infantil Vall d'Hebron de Barcelona y Profesora Titular de la Facultat de Medicina de la Universitat Autònoma de Barcelona,

CERTIFICAN: Que la presente tesis "MUERTE CELULAR DURANTE LA REPERFUSIÓN MIOCÁRDICA. MECANISMOS Y NUEVAS DIANAS TERAPEÚTICAS", presentada por Javier Inserte Igual para aspirar al grado de Doctor en Ciencias, ha sido realizada bajo nuestra dirección.

Dr. David Garcia-Dorado Dra. Margarita Sentís

Barcelona, 1 de Diciembre de 2003

A Mireia

A Clara y Xavi

ÍNDICE

ABREVIATURAS UTILIZADAS

ANP: factor natriurético atrial

GCm: guanilato ciclasa de membrana o particulada

GCs: guanilato ciclasa soluble

GMPc: 3',5'-guanosín monofosfato cíclico

ICAM-1: molécula de adhesión intercelular

KB-R7943: 2-[2[4-nitrobenziloxil]phenil]etil isotiourea metanosulfonato

NCX: intercambiador Na^{+}/Ca^{2+}

NO: óxido nítrico

NOS: sintasa del óxido nítrico

O_2^{-}: ión superóxido

ODQ: 1*H*-[1,2,4]oxadiazolo[4,3-α]quinoxalin-1-ona

OH·: radical hidroxilo

PCR: polimerase chain reaction

PKG: proteína quinasa dependiente de GMPc

PTM: poro de transición mitocondrial

RLO: radicales libres de oxígeno

SNAP: S-nitroso-N-acetil penicilamina

RESUMEN

La restauración del flujo sanguíneo es condición indispensable para la supervivencia del miocardio sometido a isquemia. Sin embargo, paradójicamente la reperfusión desencadena la muerte de células que habían sobrevivido al periodo isquémico. En este fenómeno, conocido como daño letal por reperfusión, los miocitos reperfundidos pueden morir de manera inmediata por necrosis o más tardíamente por necrosis o apoptosis.

La contribución de la muerte por apoptosis al total de la muerte celular continúa siendo objeto de debate. En la primera parte de esta tesis se ha investigado si las alteraciones metabólicas que ocurren durante un episodio isquémico actúan como estímulo iniciador de la apoptosis o, alternativamente, producen la inhibición de los mecanismos que desencadenan el proceso apoptótico. Los resultados obtenidos indican que la simulación de isquemia/reperfusión en miocitos aislados y corazón aislado no induce muerte por apoptosis. Por el contrario, la menor susceptibilidad de los miocitos a desarrollar apoptosis por exposición a agentes externos proapoptóticos (H_2O_2 y donadores de NO) respecto a miocitos normóxicos sugiere que la isquemia miocárdica genera un efecto protector frente a la apoptosis. Este mecanismo protector se encuentra asociado a un equilibrio claramente antiapoptótico del programa génico implicado en la regulación de la apoptosis.

La mayor parte de la muerte celular que tiene lugar durante la reperfusión se produce durante los primeros minutos en forma de necrosis. Esta ha sido tradicionalmente atribuida a la sobrecarga de Ca^{2+} citosólico que ocurre durante la deprivación energética. Con la reperfusión, la reactivación de la síntesis de ATP en presencia de unos niveles elevados de Ca^{2+} provoca una activación exagerada de la contractilidad de las miofibrillas dando lugar a un acortamiento extremo y rotura del sarcolema, fenómeno que se conoce como hipercontractura. La muerte provocada por la hipercontractura se ve favorecida por la fragilidad del citoesqueleto y sarcolema secundaria a un episodio isquémico previo, y por el edema celular causado por la abrupta normalización de la osmolaridad extracelular y la entrada de Na^+ al citosol. En la segunda parte de esta tesis se han estudiado dos nuevos mecanismos de prevención de la muerte inmediata por reperfusión dirigidos a disminuir por vías distintas el desarrollo de la hipercontractura. El primero actúa inhibiendo la función contráctil durante los primeros minutos de la reperfusión mediante la estimulación de la síntesis de GMPc. El segundo favorece la normalización del Ca^{2+} citosólico al inhibir la forma reversa del intercambiador Na^+/Ca^{2+} (NCX) durante el inicio de la reperfusión:

1. En corazones aislados de rata se produce durante la isquemia miocárdica una disminución en los niveles intracelulares de GMPc. La estimulación de la guanilato ciclasa soluble con L-arginina de manera previa a un protocolo de hipoxia/reoxigenación o la estimulación de la guanilato ciclasa de membrana con el factor natriurético urodilatina durante los primeros minutos de la reperfusión, aumenta los niveles de GMPc miocárdico presentes en el inicio de la reperfusión, reduce la muerte celular y mejora la recuperación funcional. El mecanismo de protección está mediado, al menos en parte, por una mayor susceptibilidad del miocardio reperfundido a la acción inhibitoria que el GMPc ejerce sobre la contracción. Este efecto permite reducir la fuerza contráctil generada al reestablecerse con la reperfusión el metabolismo celular, atenuando con ello el desarrollo de hipercontractura.

2. El segundo mecanismo se basa en la hipótesis de que durante el inicio de la reperfusión las condiciones electroquímicas en las que se encuentran los miocitos predicen un funcionamiento del NCX en modo reverso (entrada de Ca^{2+}). Esta entrada adicional de Ca^{2+} puede contribuir a la sobrecarga de Ca^{2+} citosólico que tiene lugar durante el periodo isquémico y, por tanto, a la hipercontractura. La inhibición del NCX con una nueva droga altamente específica, KB-R7943, durante los primeros minutos de la reperfusión apoyan esta hipótesis: KB-R7943 inhibe el NCX, favorece la recuperación de los niveles de Ca^{2+} y disminuye las oscilaciones de Ca^{2+} durante la reperfusión reduciendo la tasa de hipercontractura en miocitos aislados sometidos a protocolos que simulan isquemia/reperfusión. En corazones aislados atenúa la hipercontractura y liberación enzimática y mejora la recuperación funcional y, en un modelo porcino sometido a oclusión coronaria transitoria, reduce el tamaño del infarto a concentraciones que no tienen efectos hemodinámicos en condiciones normóxicas. Sin embargo, una vez reestablecido el transporte en sentido directo (salida de Ca^{2+}), la inhibición del NCX puede tener efectos perjudiciales.

En conclusión, los resultados obtenidos en estos estudios relativizan la muerte por apoptosis tras isquemia/reperfusión y acentúan la importancia terapéutica de aquellas estrategias destinadas a prevenir el desarrollo de hipercontractura como modo de limitar el daño letal por reperfusión.

INTRODUCCIÓN

I- INTRODUCCIÓN

Las consecuencias de un periodo de isquemia miocárdica transitoria abarcan desde trastornos funcionales reversibles como son fallo de la función contráctil o arritmias a la muerte celular masiva. Aunque las alteraciones funcionales transitorias son de gran importancia clínica, la muerte celular es la manifestación más relevante del daño miocárdico debido a isquemia/reperfusión y su extensión constituye el principal factor determinante de supervivencia y calidad de vida en aquellos pacientes que han sufrido un infarto agudo de miocardio [1].

En general, la isquemia que acontece durante un síndrome coronario agudo es debida a la obstrucción trombótica de una arteria coronaria epicárdica [2]. Esta situación presenta dos principales componentes patofisiológicos, por un lado una reducción del aporte de oxígeno y nutrientes y, por otro, una acumulación de catabolitos generados en el área isquémica. La restauración del flujo coronario tras una isquemia inferior a 20 minutos permite la recuperación de la función contráctil sin señales bioquímicas o estructurales de daño tisular. Sin embargo, a medida que la duración del episodio isquémico aumenta, el tiempo que se requiere para la recuperación funcional se incrementa con mucha rapidez, incluso en ausencia de muerte celular detectable [3]. Si la reperfusión continúa retrasándose la muerte celular se produce a un ritmo que crece exponencialmente con el tiempo de isquemia y que al sobrepasar los 90 minutos abarca prácticamente todo el área en riesgo [4].

El reestablecimiento del flujo sanguíneo es indispensable para limitar la muerte del miocardio sometido a una oclusión coronaria y es el objetivo a conseguir ya sea siguiendo un tratamiento trombolítico o mediante técnicas percutáneas de intervención intracoronaria [5,6]. Sin embargo, numerosos estudios experimentales han proporcionado pruebas convincentes de que, si bien es esencial para la supervivencia miocárdica, la reperfusión es capaz por sí misma de producir un daño adicional, lo que se conoce como daño por reperfusión [7,8]. El daño por reperfusión puede ser reversible, constituido por fenómenos como las arritmias de reperfusión y el aturdimiento miocárdico, o irreversible (daño letal por reperfusión), sin duda el más interesante desde un punto de vista terapéutico.

El daño letal por reperfusión se define como el daño causado por la reinstauración del flujo sanguíneo capaz de producir la muerte de células que sólo fueron dañadas de manera reversible durante el periodo isquémico previo. Esto significa que las alteraciones celulares que

tienen lugar durante la fase de isquemia son un prerrequisito para que se produzca daño letal por reperfusión pero no son por si mismas causa suficiente para inducir muerte celular. Por tanto, un criterio que define este tipo de muerte es que puede prevenirse con tratamientos administrados exclusivamente en el momento de la reperfusión [8]. El fenómeno del daño por reperfusión adquiere especial importancia en aquellas situaciones clínicas en las que no ha podido realizarse una rápida revascularización del miocardio isquémico. Dada la importancia de limitar la extensión de la necrosis miocárdica, es importante desarrollar estrategias que eviten este daño letal y potencien el efecto beneficioso de la reperfusión sobre el tamaño del infarto.

Esta tesis se centra únicamente en la muerte de miocitos asociada a isquemia/reperfusión por ser el tipo celular que determina en última consecuencia la función cardiaca y porque la potente capacidad contráctil así como el alto grado de interconexión celular que poseen los hacen altamente susceptibles al daño por reperfusión.

1. Alteraciones celulares durante la isquemia y reperfusión miocárdicas implicadas en el daño letal por reperfusión.

1.1. Homeóstasis catiónica.

El Ca^{2+} citosólico juega un papel fundamental en la traducción de señales intracelulares en todos los tipos celulares. La contracción del músculo cardiaco requiere rápidas elevaciones en su concentración por periodos breves pero continuos, la actividad de gran cantidad de enzimas implicados en transducción de señales, proteasas y fosfolipasas dependen de su concentración [9]. Por ello, es fundamental que los niveles de Ca^{2+} citosólico estén controlados de una manera precisa y sean capaces de aumentar y disminuir rápidamente generando señales transitorias. El gradiente electroquímico que presenta el Ca^{2+} a través del sarcolema es del orden de 10^4 y su mantenimiento es un proceso activo altamente dependiente de energía. La depleción energética que tiene lugar durante un periodo isquémico provoca que los miocitos pierdan el control de la homeóstasis de Ca^{2+} y que ello tenga consecuencias dramáticas que se manifestarán sobretodo durante la reperfusión.

1.1.1. Cambios durante isquemia

Durante los primeros minutos de un periodo isquémico cesa la síntesis de ATP. El ATP existente se hidroliza por múltiples ATPasas involucradas en la actividad contráctil y en el mantenimiento de la homeostasis iónica, especialmente la Na^+/K^+-ATPasa [10], y como

consecuencia de ello aumentan los niveles de ADP y P_i. La activación de la glucolisis anaeróbica mantiene una síntesis residual de ATP, dando lugar a una sobreproducción de lactato y protones. Esto provoca una rápida acidosis con una caída del pH intracelular que pasa de 7.4 a 6.4 en pocos minutos [11] inhibiendo con ello la deficitaria producción de ATP. Una de las principales consecuencias de la acidificación celular es la estimulación de transportadores sarcolemales alcalinizadores: el cotransportador Na^+/HCO_3^- y el intercambiador Na^+/H^+ [12]. Estos dos sistemas atenúan la progresión de la acidosis intracelular pero llevan asociados la entrada de un ión Na^+ por cada H^+ extraído. La imposibilidad de la célula de corregir esta entrada de Na^+ debido a la inhibición de la Na^+/K^+-ATPasa que tiene lugar como consecuencia de la depleción energética [13] da lugar a un aumento progresivo en la concentración citosólica de Na^+ [14]. El aumento en los niveles de Na^+ se ha relacionado con efectos deletéreos sobre el metabolismo celular que inducirían disfunción contráctil [15] y también con la pérdida neta de K^+, causante de fibrilaciones durante la reperfusión [16]. Pero sobretodo, la acumulación de Na^+ es de una importancia crítica en la patofisiología del daño celular causado por isquemia/reperfusión debido al efecto directo que tiene la pérdida del gradiente transmembrana de Na^+ sobre la actividad del intercambiador Na^+/Ca^{2+}.

Intercambiador Na^+/Ca^{2+}

El intercambiador Na^+/Ca^{2+} (NCX) sarcolemal desempeña un papel fundamental en la regulación de la homeóstasis del Ca^{2+}. La isoforma predominante en el corazón, NCX1, representa la principal ruta de extrusión de Ca^{2+} intracelular y junto a la captación de Ca^{2+} por el retículo sarcoplasmático a través de la Ca^{2+}-ATPasa sarcoplasmática y, en menor medida, a la extrusión de Ca^{2+} mediada por la Ca^{2+}-ATPasa del sarcolema, constituye el principal mecanismo celular de regulación de la concentración citosólica de Ca^{2+}. El intercambio catiónico se realiza con una estequiometría de 3 Na^+:1 Ca^{2+}, generando una corriente de carga positiva cuyo sentido depende de la diferencia entre el potencial transmembrana y el potencial de reversión del transportador. El potencial de reversión está determinado por los potenciales electroquímicos de Na^+ y Ca^{2+} y, por tanto, por las concentraciones intra y extracelulares de Na^+ y Ca^{2+} [17]. En condiciones fisiológicas, durante la diástole, el gradiente transmembrana de Na^+ existente (concentración extracelular ~ 140 mM, concentración intracelular ~15 mM) determina un potencial de reversión mayor (~ -50 mV) que el potencial de reposo transmembrana (~ -80 mV) produciéndose la salida de Ca^{2+} y entrada de Na^+ a través del transportador. Esta representa la vía más importante de extrusión del Ca^{2+} que entra en la célula durante la sístole. Durante la despolarización sistólica, el potencial de membrana alcanza durante milisegundos

valores positivos y pasa a ser mayor que el potencial de reversión dando lugar a un funcionamiento en modo reverso del NCX (salida de Na^+ de la célula y entrada de Ca^{2+}). A pesar de ello, el breve periodo de actividad de este modo de transporte hace que el Ca^{2+} que entra por el intercambiador sea poco importante. Sin embargo, durante el periodo de isquemia, la despolarización progresiva de la membrana y la sobrecarga de Na^+ provocan un funcionamiento continuo de la forma reversa del transportador dando lugar a una entrada neta de Ca^{2+} que se mantiene hasta que la célula vuelve a reestablecer el gradiente transmembrana de Na^+. El efecto añadido de la inhibición de la extrusión de Ca^{2+} a través de las Ca^{2+}-ATPasas del sarcolema y del retículo sarcoplasmático debido a la depleción energética, provoca que durante el periodo isquémico se produzca un aumento en la concentración citosólica de Ca^{2+} [18].

1.1.1.2 Cambios durante la reperfusión

Al disponer las células nuevamente de oxígeno se reestablece la síntesis de ATP. En las mitocondrias que resultan dañadas se encuentra alterado el proceso oxidativo dando lugar a una sobreproducción de radicales libres de oxígeno (RLO) [19]. A pesar de que las concentraciones de ATP y fosfocreatina puedan necesitar horas para recuperar los niveles basales, la energía libre de intercambio de ATP, indicador de la disponibilidad energética, se recupera pocos segundos después de la llegada del oxígeno [20].

Durante los primeros minutos de la reperfusión se producen cambios dramáticos en la homeóstasis catiónica. El lavado extracelular de H^+ y la actividad del intercambiador Na^+/H^+ y cotransportador Na^+/HCO_3^- permite una rápida corrección de la acidosis intracelular. Tras periodos breves de isquemia, la concentración intracelular de Na^+ sólo se encuentra moderadamente elevada y puede retornar rápidamente a los valores basales con la inmediata reactivación de la Na^+/K^+-ATPasa. Sin embargo, después de tiempos prolongados de isquemia, la concentración de Na^+ permanece elevada durante minutos debido a una entrada adicional de Na^+ a través de la actividad del intercambiador Na^+/H^+ y cotransportador Na^+/HCO_3^- asociada a la corrección del pH y a la inhibición transitoria de la Na^+/K^+-ATPasa [21]. A esto hay que añadir la entrada masiva de Na^+ que se produce a través de uniones tipo *gap* (gap junctions) desde miocitos adyacentes que han sufrido hipercontractura y rotura de la membrana sarcolemal [22].

Hipercontractura

De manera inmediata a la restauración de la disponibilidad de oxígeno, se reactiva la Ca^{2+}-ATPasa ligada al retículo sarcoplasmático. En esta primera fase, el secuestro de Ca^{2+} por parte del retículo provoca una primera caída en los niveles de Ca^{2+} citosólico. Sin embargo, si se excede la capacidad acumulativa de la organela, se produce un ciclo continuo de liberaciones y recaptaciones de Ca^{2+} dando lugar a una fase de oscilaciones en los niveles de Ca^{2+} citosólico [23]. Estas oscilaciones espontáneas tienden a finalizar a medida que la célula normaliza los niveles intracelulares de Ca^{2+} a través del NCX. Para que esto ocurra es esencial la reanudación de la actividad Na^+/K^+-ATPasa y la recuperación del gradiente transmembrana de Na^+ [24]. Sin embargo, en las células que recuperan la síntesis de ATP y normalizan el pH citosólico antes de haber podido eliminar la sobrecarga citosólica de Ca^{2+}, se produce una activación exagerada y de forma sostenida de la actividad contráctil causando un acortamiento celular irreversible que daña a las estructuras del citoesqueleto debido a que esta fuerza contráctil excede la capacidad elástica del sarcómero y del citoesqueleto. Esta incontrolada contracción del cardiomiocito dependiente de Ca^{2+} se conoce como **hipercontractura** [8] (**Figura 1**).

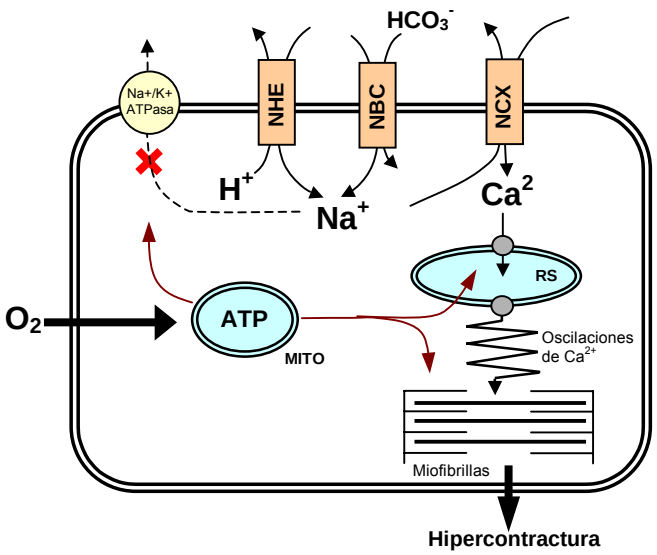

Figura 1. Esquema del desarrollo de la hipercontractura. Durante el episodio isquémico, los miocitos acumulan Na^+ como consecuencia de la inhibición de la bomba de Na^+ y la activación del intercambiador Na^+/H^+ (NHE) y cotransportador Na^+/HCO_3^- (NBC). Con la pérdida del gradiente transmembrana de Na^+, el intercambiador Na^+/Ca^{2+} (NCX) pasa a actuar en forma reversa provocando una sobrecarga de Ca^{2+}citosólico. Con la reenergización de la célula durante la reperfusión, el retículo sarcoplasmático (RS) acumula Ca^{2+}. Su saturación genera movimientos cíclicos de entrada y salida (oscilaciones de Ca^{2+}) que causan, una vez normalizado el pH, una activación exagerada de las miofibrillas y con ello la hipercontracción del miocito.

Se ha demostrado que las oscilaciones espontáneas de Ca^{2+} que tienen lugar durante el inicio de la reperfusión contribuyen al desarrollo de la hipercontractura y a la génesis de arritmias asociadas a la reperfusión [23]. Esta influencia de las oscilaciones de Ca^{2+} sobre la hipercontractura es resultado del aumento en la susceptibilidad que presentan los miocitos reperfundidos al Ca^{2+} [25]. Las células sometidas a depleción-repleción energética pueden desarrollar hipercontractura con concentraciones de Ca^{2+} que no tendrían este efecto en células normóxicas. La causa de este aumento en la susceptibilidad al Ca^{2+} parece residir en el aumento en la fragilidad del citoesqueleto que tiene lugar con la isquemia/reperfusión [26].

1.2 Edema celular

La pérdida del control del volumen celular es un mecanismo esencial en la muerte celular por necrosis isquémica (oncosis). El contenido de agua en el miocardio aumenta precozmente con un periodo isquémico severo [27] debido a la acumulación de metabolitos con gran capacidad osmótica. Sin embargo, tras una fase inicial de formación de edema celular, muchos de los productos generados durante la fase isquémica pueden salir de la célula y acumularse en el espacio extracelular disminuyendo el gradiente osmótico. Como resultado, el aumento de volumen celular durante un episodio isquémico reversible es despreciable.

Por el contrario, la reperfusión después de un periodo prolongado de isquemia se acompaña de un brusco aumento en el contenido miocárdico de agua. El mecanismo de formación de este edema parece ser múltiple. Por un lado, la rápida sustitución del fluido intravascular hiperosmótico por sangre normosmótica genera un gradiente osmótico entre el espacio intra y extravascular. El agua se desplaza hacia el espacio intersticial reduciéndose su osmolaridad y creando un nuevo gradiente osmótico a través de las membranas de miocitos viables que arrastran la alta osmolaridad interna generada durante la oclusión miocárdica. El resultado es el desarrollo de edema celular cuya magnitud depende principalmente de la duración y severidad del episodio isquémico previo [28]. Además del gradiente osmótico otra de las causas que favorecerían la formación de edema es la rápida corrección de los desequilibrios iónicos que tienen lugar durante la deprivación energética y la acumulación de Na^+. Este último factor se ha demostrado en estudios en los que la inhibición de los transportadores Na^+/H^+ y Na^+/HCO_3^- provoca una reducción del contenido miocárdico de H_2O [29].

1.3 Activación enzimática y fragilidad celular

Con la isquemia/reperfusión miocárdica se producen alteraciones en la composición lipídica de la membrana, degradación de proteínas sarcolemales y pérdida de unión entre sarcolema y citoesqueleto resultando en un aumento de la fragilidad celular [26]. Las células, aunque conservan la integridad de su sarcolema, tienen disminuida su capacidad para soportar una sobrecarga mecánica. Este aumento de la fragilidad celular se ha demostrado en experimentos en los que la aplicación de una sobrecarga osmótica en corazones aislados mediante la perfusión de una solución hipoosmótica provocaba la disrupción de la membrana celular y liberación enzimática sólo si previamente eran sometidos a isquemia [30].

La elevación de la concentración citosólica de Ca^{2+} durante el periodo isquémico no sólo resulta determinante en el desarrollo de la hipercontractura sino que también provoca la activación de sistemas enzimáticos dependientes de Ca^{2+}. La activación de fosfolipasas Ca^{2+} dependientes, principalmente la fosfolipasa A2, se ha propuesto como un importante mecanismo en la génesis de los cambios sarcolemales asociados a los episodios isquémicos [31]. La hidrólisis de fosfolípidos y la acción detergente de productos anfipáticos (lisofosfolípidos) producto de su actividad, altera la composición y propiedades físicas de la membrana celular. Los lisofosfolípidos se acumulan en el miocardio isquémico y tienen múltiples efectos sobre diferentes proteínas de carácter estructural, canales sarcolemales y uniones tipo *gap* [32, 33]. Adicionalmente, durante la reperfusión se genera una gran cantidad de RLO capaces de peroxidar ácidos grasos poliinsaturados haciéndolos más susceptibles a la acción de la fosfolipasa A2 [34].

Las calpaínas, proteasas no lisosomales calcio dependientes, son activadas como consecuencia del aumento de Ca^{2+} intracelular que tiene lugar durante la depleción energética. Estas proteasas tienen como sustrato proteínas que contribuyen a la integridad sarcolemal como son la fodrina y la distrofina, así como miofilamentos contráctiles y canales iónicos [35]. Dado que *in vitro*, la actividad de la calpaína es máxima a pH neutro, su papel durante el periodo isquémico parece quedar bastante limitado como consecuencia del pH ácido intracelular. Sin embargo, su actividad aumenta de manera dramática durante la reperfusión contribuyendo tanto al aumento en la fragilidad y rotura del sarcolema [36, 37], como a la alteración de la contractilidad por degradación de proteínas implicadas en el mecanismo contráctil [38].

1.4 Alteraciones mitocondriales

A pesar de que la reactivación de la respiración mitocondrial durante la reperfusión es un prerrequisito para la recuperación celular, esta no determina por si sola la supervivencia celular. Paradójicamente, los miocitos que mueren durante la reperfusión necesitan haber recuperado su capacidad metabólica. Con la reperfusión, la mitocondria además de ser la fuente intracelular más importante de producción de RLO, también puede contribuir a las alteraciones en el Ca^{2+} intracelular y al desencadenamiento de procesos apoptóticos.

La inhibición de la cadena respiratoria por falta de oxígeno provoca una reducción de la ubiquinona a ubisemiquinona, forma que reacciona con el oxígeno aportado con la reperfusión generando RLO. Estos RLO tienen efectos directos sobre componentes de la cadena respiratoria, especialmente sobre el complejo I y complejo III afectando con ello a la síntesis de ATP [39], pero también actúan alterando la permeabilidad de la membrana mitocondrial [34].

Tras la restauración del potencial de membrana mitocondrial, la captación de Ca^{2+} por la mitocondria contribuye a la normalización del Ca^{2+} citosólico. Sin embargo, la sobrecarga de Ca^{2+} mitocondrial resultante tiene como efecto adverso la abertura del poro de transición mitocondrial (PTM) [40]. El PTM es un megacanal con un poro no selectivo de difusión de moléculas. En condiciones fisiológicas se abre de manera limitada y reversible contribuyendo a la modulación de cambios en el Ca^{2+} citosólico. Una abertura descontrolada da lugar a la despolarización de la mitocondria, pérdida del gradiente de H^+ y de la síntesis de ATP, y liberación de Ca^{2+} mitocondrial al citosol. Cuando esto ocurre en una fracción importante de las mitocondrias se produce la muerte de la célula. La abertura del PTM se puede inducir experimentalmente con concentraciones de Ca^{2+} citosólico elevadas, estrés oxidativo, y depleción de ATP, condiciones todas ellas presentes durante el periodo isquémico, pero en cambio se inhibe con la acidosis [41]. Hay pruebas obtenidas a partir del uso de ciclosporina A, inhibidor del PTM, que sugieren que este permanecería cerrado durante el periodo isquémico y sería la normalización del pH durante la reperfusión la que permitiría su abertura [42].

La abertura del PTM también provoca la entrada de agua a la matriz mitocondrial, produciendo un edema lo suficientemente severo como para romper la membrana externa y liberar el citocromo c, elemento determinante en la activación de la cascada de caspasas, enzimas implicados en el desencadenamiento de la muerte por apoptosis [43]. La liberación del citocromo c puede producirse de manera independiente a la abertura del PTM por formación de

un poro en la cara externa de la membrana mitocondrial debido a la oligomerización de proteínas pro-apoptóticas como Bax [44]. La mitocondria también es capaz de liberar proteínas que actúan como inductoras de la apoptosis por mecanismos independientes del citocromo c, asociadas a la activación de caspasas (Apaf-1) [45] o con capacidad para translocarse directamente al núcleo sin implicación de caspasas como el factor inductor de apoptosis, AIF [46].

2. Mecanismos de muerte celular durante la isquemia y reperfusión miocárdica.

No existe una definición de muerte celular universalmente válida. Hasta hace relativamente poco tiempo se pensaba que la necrosis era el único modo de muerte celular. En 1994 aparece por primera vez el término apoptosis en relación con el miocardio isquémico y reperfundido [47].

Por necrosis se entiende el tipo de muerte celular causado por una alteración severa de la homeóstasis celular (muerte accidental) [48]. Se caracteriza por una rotura de la membrana plasmática y de las organelas intracelulares incluyendo mitocondrias. La fragmentación del DNA es anárquica. La liberación del contenido intracelular desencadena una respuesta inflamatoria y la fagocitosis de estos elementos sólo se produce cuando se alcanza una cierta acumulación de células inflamatorias. Las lesiones resultantes contienen grupos de células necróticas contiguas.

La muerte por apoptosis está caracterizada por una serie típica de eventos morfológicos: inicialmente se produce un encogimiento de la célula y su núcleo así como una condensación de la cromatina, una posterior fragmentación y formación de cuerpos apoptóticos (material celular resultante de la fragmentación rodeado de membrana plasmática), desanclaje del resto de tejido y una rápida fagocitosis por células vecinas como macrófagos o células del parénquima, todo ello sin dar lugar a inflamación. La degradación internucleosomal del DNA por DNAsas endógenas es un proceso ATP dependiente que da lugar a fragmentos de 180-200 pares de bases y que constituye el distintivo bioquímico de la apoptosis. Este proceso de muerte celular está ejecutado de una manera organizada, su desencadenamiento supone desequilibrios en el balance existente entre la expresión de genes pro-apoptóticos y anti-apoptóticos, y su ejecución refleja vías moleculares específicas [49]. La degradación proteolítica mediada por proteasas conocidas como caspasas desempeña un papel clave en el desarrollo de la muerte por apoptosis.

A pesar de que los dos mecanismos de muerte celular difieren entre si parece existir un cierto solapamiento durante las primeras fases de ambos procesos. La liberación del citocromo c mitocondrial se considera un claro nexo de unión entre los dos mecanismos. Su liberación al citosol desencadena la activación de la cascada de caspasas pero también produce la pérdida de la fosforilación oxidativa y reduce la síntesis de ATP [50]. Se ha observado que las calpaínas, activadas con la elevación citosólica de Ca^{2+} que tiene lugar durante el periodo isquémico e implicadas principalmente en la muerte por necrosis, tienen también como sustratos pro-caspasas que pasarían a un estado activo al ser hidrolizadas por estas [38, 51]. Estudios en los que se ha inhibido la calpaína no sólo han observado una reducción del tamaño del infarto sino también una disminución en la muerte por apoptosis [52]. De la misma manera, proteínas que contribuyen a la integridad de la membrana como la fodrina y que son degradadas por la calpaína también son sustrato de determinadas caspasas [35].

2.1 Muerte celular durante isquemia

Una isquemia miocárdica severa sostenida provoca la muerte inevitable de los miocitos. Sin embargo, la muerte por necrosis es un fenómeno tardío durante isquemia: se observa mucho más tarde que el momento en el que la reperfusión deja de prevenir la muerte de los miocitos circunscritos al área en riesgo [7].

En un episodio isquémico, la producción de radicales libres, el aumento en la concentración de Ca^{2+} así como otras alteraciones pueden actuar como potentes estímulos para la iniciación de la apoptosis. Sin embargo, dado que el programa apoptótico es dependiente de energía, todavía no está claro si la isquemia miocárdica es suficiente para completar el proceso apoptótico o si se necesita de la reperfusión. La decisión que toma la célula entre muerte por necrosis o apoptosis parece depender de la concentración de ATP. Se ha sugerido que una isquemia severa con depleción de ATP mayor del 70% es incompatible con la apoptosis [53]. Aunque se ha demostrado activación de los primeros estadios de la vía apoptótica durante el periodo isquémico [54] la mayoría de los estudios ven la necesidad de la reperfusión para poder completar el proceso apoptótico [47] . Esto apoyaría la hipótesis de que la apoptosis podría desencadenarse por estímulos generados durante el episodio isquémico y completarse con la re-energización que tiene lugar con la reperfusión. En cualquier caso, parece claro que la apoptosis juega un papel poco importante en la muerte celular durante isquemias con una duración suficiente para que el miocardio en riesgo sea susceptible de ser salvado con la reperfusión.

2.2 Muerte celular durante la reperfusión.

La muerte de los cardiomiocitos se observa desde los primeros minutos de la reperfusión miocárdica. Esta muerte celular que tiene lugar de manera inmediata a la reperfusión incluye rotura del sarcolema y liberación enzimática, y está asociada a una excesiva activación contráctil que resulta en un acortamiento celular anormal con una marcada alteración de la morfología celular y que da lugar a un patrón histológico característico conocido como necrosis en bandas de contracción [55, 56]. Los cardiomiocitos también pueden morir de manera más tardía por apoptosis o necrosis debida a la toxicidad asociada a la reacción inflamatoria que tiene lugar durante la reperfusión [57-59].

2.1.1 Muerte celular inmediata

Hay pruebas sólidas demostrando que la muerte inmediata por reperfusión es la forma de muerte celular más importante cuando la reperfusión tiene lugar dentro de un margen suficiente como para salvar miocardio. El patrón histológico característico de este tipo de muerte, la necrosis en bandas de contracción, puede observarse a los 5 minutos de la reperfusión, alcanza su extensión máxima enseguida y permanece inalterable después [60], constituyendo aproximadamente el 90% del total de la necrosis miocárdica [61].

La hipercontractura es el principal determinante de la necrosis en bandas de contracción [8, 55]. Estudios realizados en el miocardio *in situ* han demostrado que la extensión de la necrosis en bandas de contracción tras una oclusión coronaria transitoria se encuentra estrechamente correlacionada con la magnitud de la reducción en la longitud diastólica del miocardio reperfundido [62]. El análisis histológico de estas áreas de necrosis en bandas de contracción confirma que están formadas por miocitos hipercontraídos y que estos representan la mayor parte de la masa del infarto resultante de una isquemia severa seguida de reperfusión [63].

En contra de lo que sucede en el miocardio *in situ*, en miocitos aislados, la hipercontractura por si sola no produce rotura de la membrana y muerte celular a pesar de dañar de manera irreversible las estructuras del citoesqueleto [20]. Esta diferencia ha sido explicada por la ausencia en el modelo de miocitos aislados de las restricciones físicas que impone la interacción mecánica célula-célula pero también por la falta de un edema osmótico importante debido a que el gran espacio extracelular que supone la placa de cultivo evita cambios apreciables en la osmolaridad. Esta observación a llevado a sugerir que la pérdida de viabilidad celular que tiene lugar con la reperfusión es resultado de la sobrecarga mecánica impuesta por

la hipercontractura, el edema celular y la interacción física entre células adyacentes producida sobre un cardiomiocito cuya resistencia mecánica se encuentra disminuida debido al aumento en la fragilidad celular, tanto de su sarcolema como de su citoesqueleto [64] (**Figura 2**)

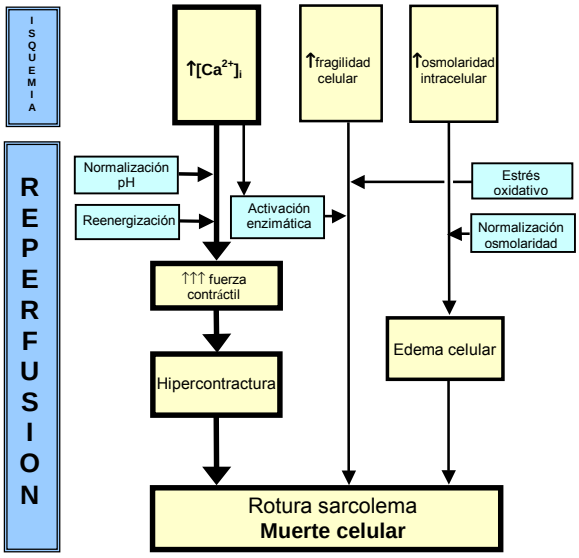

Figura 2 Esquema ilustrativo de los factores que contribuyen a la muerte de los miocitos que tiene lugar de manera inmediata a la reperfusión. (Modificado de Piper HM *et al* [8]).

Las interacciones célula-célula a través de uniones tipo *gap* (gap juntions) contribuyen a la propagación de la hipercontractura. Esta observación se ha demostrado en pares de miocitos aislados interconectados entre si donde el cierre de las uniones tipo *gap* con heptanol previene su propagación [65]. El paso de Na^+ de células hipercontraídas a través de las uniones *gap* a células adyacentes y su subsiguiente intercambio por Ca^{2+} vía modo reverso del NCX sería la causa responsable de la propagación de la hipercontractura [22]. Esta nunca excede de los límites del área en riesgo, probablemente debido a que las células presentan una menor fragilidad y pueden desarrollar hipercontractura sin rotura del sarcolema. La propagación de la hipercontractura a través de uniones *gap* contribuiría a explicar la geometría continua y tamaño final del infarto [61].

Atenuación de la hipercontractura

Experimentalmente, las estrategias destinadas a reducir la hipercontractura han demostrado ser altamente eficaces en la limitación de la necrosis miocárdica. Este objetivo puede conseguirse de diferentes maneras:

Primero, la hipercontractura puede prevenirse con protocolos que actúan inhibiendo la maquinaria contráctil de la célula durante los primeros minutos de la reperfusión. El uso del inhibidor de la ATPasa de la actomiosina 2,3-butanediona monoxima, previene la hipercontractura *in vitro* y reduce el tamaño del infarto en modelos *in situ* [56, 66]. Sin embargo, esta droga es altamente tóxica debido a su actividad fosfatasa inespecífica con lo que su aplicación terapéutica esta descartada. A nivel experimental, la prolongación de la acidosis intracelular durante el inicio de la reperfusión tiene un efecto protector frente a la necrosis asociado al efecto inhibitorio que el pH ácido ejerce sobre la contractilidad [67]. En miocitos aislados la inhibición simultánea del intercambiador Na^+/H^+ y del cotransportador Na^+/HCO_3^- retrasa la normalización del pH [68]. Sin embargo, la inhibición únicamente del intercambiador Na^+/H^+ durante la reperfusión no prolonga la acidosis y ha resultado ser ineficaz en la prevención de la hipercontractura en estudios experimentales [69] y clínicos [70]. Recientemente, la elevación de los niveles intracelulares de GMPc en miocitos aislados ha sido capaz de reducir la tasa de hipercontractura [71] por un mecanismo asociado, al menos en parte, a una reducción en la sensibilidad de las miofibrillas por el Ca^{2+}. Estas estrategias de inhibición de la contractilidad durante el inicio de la reperfusión, permiten que las células recuperen su metabolismo mientras la actividad contráctil permanece inhibida. La recuperación de la síntesis de ATP activa los mecanismos que llevan a reestablecer el control catiónico, eliminándose así la posibilidad de desarrollar hipercontractura.

Segundo, la hipercontractura se puede inhibir reduciendo la sobrecarga de Ca^{2+} citosólico. Los protocolos realizados con este fin han buscado disminuir la acumulación de Ca^{2+} que tiene lugar durante la depleción energética. Una extensa evidencia preclínica indica que la inhibición del intercambio Na^+/H^+ antes de la reperfusión tiene un efecto beneficioso sobre el miocardio sometido a isquemia transitoria. El uso de inhibidores específicos como cariporide ha sido efectivo en reducir la necrosis miocárdica, arritmias y aturdimiento en una amplia variedad de especies animales y condiciones experimentales [72,73]. Si embargo, como se ha mencionado anteriormente, el bloqueo del intercambio Na^+/H^+ durante el inicio de la reperfusión no ha ofrecido efectos beneficiosos [69] con lo que su posible aplicación clínica queda limitada. De una manera clásica se ha propuesto que el mecanismo por el que la inhibición del transportador

Na^+/H^+ resulta beneficiosa supone la disminución en la acumulación de Na^+ que, de manera indirecta, se traduciría en una menor sobrecarga de Ca^{2+} citosólico al reducirse el intercambio por Ca^{2+} a través de la forma reversa del NCX. Sin embargo, recientemente se ha sugerido que los inhibidores del transportador Na^+/H^+ podrían ejercer parte de sus efectos a nivel mitocondrial [74]. Una forma más directa de limitar la entrada de Ca^{2+} es actuando sobre la forma reversa del NCX. La ausencia hasta hace poco de inhibidores lo suficientemente selectivos para este transportador había impedido hacer esta aproximación. Con la reciente aparición de una nueva droga altamente selectiva, KB-R7943, se ha demostrado que la inhibición del NCX durante el episodio isquémico reduce de manera significativa la elevación de Ca^{2+} citosólico y la muerte celular asociada a isquemia/reperfusión en diferentes modelos experimentales [75]. A pesar de estos resultados no se tienen pruebas de la efectividad de esta estrategia aplicada en el momento de la reperfusión.

Tercero, la inhibición de las oscilaciones espontáneas de Ca^{2+} entre citosol y retículo sarcoplasmático que tienen lugar durante el inicio de la reperfusión previene el desarrollo de hipercontractura en cardiomiocitos aislados. La reducción de estas oscilaciones se puede conseguir experimentalmente interfiriendo los ciclos de captación y liberación de Ca^{2+} con tapsigargina (bloqueante de la Ca^{2+}-ATPasa del retículo sarcoplasmático) y con rianodina (bloqueante de los canales de liberación de Ca^{2+} del retículo) [66]. El uso de sustancias más inespecíficas, como el halotano, un anestésico volátil administrado durante la reperfusión previene las oscilaciones y limita la hipercontractura en miocitos aislados [23].

2.2.2 Muerte celular tardía

Parte de la muerte celular que se produce durante la reperfusión sucede de manera tardía, durante las horas siguientes a la reinstauración del flujo. Además de muerte por necrosis, se produce muerte por apoptosis que podría ser consecuencia de la respuesta inflamatoria que acompaña al insulto isquémico pero también la manifestación final del proceso apoptótico desencadenado por las alteraciones metabólicas que tienen lugar durante la isquemia miocárdica.

Respuesta inflamatoria

La respuesta inflamatoria asociada a un episodio isquémico conlleva la llegada de células sanguíneas al miocardio reperfundido y la liberación de productos altamente nocivos que pueden afectar la viabilidad de miocitos que han sobrevivido a los primeros minutos de la

reperfusión. Tras un periodo de isquemia, las células del endotelio microvascular liberan citoquinas y moléculas altamente activas como PAF e IL-8, pasan a expresar en su superficie receptores de adhesión (P-selectina y E-selectina) y disminuyen la síntesis de NO. Los leucocitos atraídos al miocardio reperfundido se unen inicialmente a través de la L-selectina al endotelio activado e inician su rodadura sobre la superficie endotelial. En una siguiente fase se produce la activación de los neutrófilos por factores quimiotácticos y una adhesión firme al endotelio dependiente principalmente del complejo formado por las integrinas CD11/CD18 y de la molécula endotelial de adhesión intercelular (ICAM-1). ICAM-1, presente de manera constitutiva en la superficie endotelial, sufre una marcada sobreexpresión por estimulación con citoquinas. Los leucocitos adheridos al endotelio liberan ROS, NO, ácido hipocloroso y diversos enzimas proteolíticos, pueden obstruir capilares o migrar a través del endotelio por diapédesis [57]. Infiltrados en el miocardio podrían adherirse a miocitos [76]. *In vitro*, se ha demostrado adhesión de leucocitos a miocitos a través de ICAM-1 expresado en miocitos e integrinas CD11b en neutrófilos [77]. A pesar de que la acumulación de neutrófilos se correlaciona bien con el tamaño de infarto [78], la demostración de un papel determinante en la muerte celular permanece abierta. Mientras que en unos estudios los tratamientos encaminados a disminuir la acumulación de leucocitos ha sido efectiva en la reducción del infarto [78,79] en otros ha resultado ser inefectiva [80,81]. Varias pruebas sugieren que en el contexto de una oclusión coronaria transitoria la contribución de la infiltración de leucocitos en la muerte celular es marginal. La cuantificación de la infiltración por inmunohistoquímica y actividad mieloperoxidasa indican un aumento a partir de la tercera hora de reperfusión, tiempo tras el cual el área de necrosis es próxima al tamaño final. Por otro lado, la relación entre duración de isquemia y tamaño de infarto es similar en experimentos realizados en ratas *in situ* y en corazones de rata aislados y perfundidos con una solución carente de células sanguíneas. Recientemente, se ha demostrado que la depleción genética de las moléculas de adhesión ICAM-1 y P-selectina inhibe la unión leucocito-endotelio sin que ello tenga consecuencias en el tamaño del infarto medido tras 24 horas de reperfusión [82].

Las plaquetas se acumulan en gran cantidad en el miocardio [83]. Esta acumulación parece depender de la adhesión local de plaquetas al endotelio activado [83,84]. A pesar de que, por un lado pueden adherirse a leucocitos formando agregados leucoplaquetarios y obstruir microvasos y, por otro, liberar sustancias tóxicas como tromboxano A2, factor activador plaquetar y serotonina, las consecuencias de la acumulación plaquetar en la muerte celular durante la reperfusión es poco conocida. Experimentalmente existen estudios describiendo

tanto efectos deletéreos como beneficiosos [85,86]. Recientemente se ha sugerido que las plaquetas activadas por isquemia y con expresión de P-selectina, pero no las que se encuentran inactivas, contribuyen a aumentar el daño por reperfusión [87].

Muerte celular por apoptosis

En los últimos años numerosos estudios han demostrado la coexistencia de muerte por necrosis y apoptosis en la isquemia/reperfusión miocárdica [47,88]. Estudios histológicos en pacientes con infarto agudo de miocardio han detectado miocitos apoptóticos situados en los bordes de la zona infartada pero no en el centro del infarto [88]. Esta localización en el tejido perinecrótico ha llevado a sugerir una participación de la apoptosis en el remodelado ventricular y en la progresión hacia el fallo cardiaco [89]. Sin embargo, su contribución a la extensión final del infarto continúa siendo controvertida [90]. Algunos estudios indican que este tipo de muerte, a pesar de estar presente en el miocardio reperfundido, sólo representa un pequeño porcentaje del total de la muerte celular [91,92]. En otros la interrupción del mecanismo apoptótico con inhibidores de caspasas ha contribuido a atenuar el daño por isquemia/reperfusión [90]. Sin embargo, dado que la actividad de determinadas caspasas puede contribuir también a la muerte por necrosis, estos resultados no descartan la posibilidad de que parte de la reducción en la extensión del infarto sea independiente del bloqueo de la apoptosis [35, 93]. Recientemente, la sobreexpresión de la proteasa inductora de apoptosis caspasa-3 provocó un aumento del tamaño del infarto y una peor recuperación funcional en ratones sometidos a un protocolo de isquemia transitoria y reperfusión [94] mientras que la sobreexpresión del gen anti-apoptótico Bcl-2 produjo el efecto contrario [95].

Durante la reperfusión se generan gran cantidad de RLO y NO, se producen citoquinas pro-inflamatorias y daño mitocondrial (obertura del poro de transición mitocondrial), todos ellos factores con demostrada capacidad para inducir apoptosis en diferentes modelos experimentales.

La reperfusión miocárdica lleva consigo la formación de RLO en cantidades mayores a las producidas en condiciones fisiológicas [19, 96]. En miocitos provienen principalmente de las mitocondrias en forma de anión superóxido ($O_2^{\cdot-}$) generado como subproducto resultante del transporte de electrones mitocondrial, pero también se producen por la oxidación del ácido araquidónico en forma de radicales hidroxilo (·OH) [97, 98]. Otras fuentes importantes de formación de RLO durante la reperfusión son los neutrófilos polimorfonucleares infiltrados en el

miocardio reperfundido y los generados en células endoteliales por la oxidación de la xantina al producirse durante la depleción energética la transformación de xantina dehidrogenasa en xantina oxidasa [99]. Por otro lado, el miocardio sometido a isquemia/reperfusión podría presentar una mayor susceptibilidad a los efectos adversos de los RLO que el miocardio normal debido a la disminución de su potencial antioxidante. Durante isquemia se ha descrito una reducción en la actividad de la superóxido dismutasa, de la catalasa y en los niveles de la forma reducida del glutation [100, 101]. El uso de sustancias con propiedades antioxidantes o la adición de superóxido dismutasa inhibe los niveles intracelulares de RLO y reduce la muerte por apoptosis [102, 103]. Los mecanismos que inducen apoptosis mediada por RLO todavía no están claramente establecidos aunque aparentemente están relacionados con la obertura del poro de transición mitocondrial y la liberación del citocromo c [104].

El NO presenta un papel dual en el desarrollo de apoptosis. Puede proteger a las células o inducir apoptosis dependiendo de su concentración. NO es una molécula extremadamente activa y de vida corta. Su concentración depende de la del oxígeno dado que las dos moléculas reaccionan para producir peroxinitrito. El peroxinitrito es un radical altamente reactivo que puede reaccionar con diferentes biomoléculas, liberar otros radicales tóxicos como el radical hidroxilo o servir de sustrato para la regeneración de NO. Así, el NO puede actuar secuestrando anión superóxido y a su vez es inactivado con diferentes resultados posibles. Sin embargo, a altas concentraciones desencadena procesos apoptóticos por mecanismos dependientes [105, 106] e independientes de GMPc mediados por peroxinitrito, incluyendo la abertura del poro mitocondrial de transición [107]. En el miocardio reperfundido, se pueden producir grandes cantidades de NO por infiltración de macrófagos, y por expresión de la forma inducible de la NO sintasa en miocitos y células endoteliales microvasculares [108].

Miocitos, células endoteliales, neutrófilos y plaquetas liberan diversas citoquinas en respuesta al insulto isquémico con capacidad para inducir apoptosis. Hay datos que indican que TNFα y IL1β inducirían apoptosis por activación de NOS inducible y liberación de NO [107, 109].

HIPÓTESIS Y OBJETIVOS

II- HIPÓTESIS Y OBJETIVOS

1. Hipótesis

La hipótesis de este estudio es que la muerte celular que se produce durante la reperfusión tiene lugar esencialmente por necrosis durante los primeros minutos de la reperfusión siendo el papel de la muerte por apoptosis poco relevante.

Esta muerte inmediata por reperfusión puede prevenirse con tratamientos efectuados únicamente durante el inicio de la reperfusión dirigidos a disminuir la sobrecarga mecánica causada por la hipercontractura.

2. Objetivos

1. Estudiar la posibilidad de que las alteraciones metabólicas que tienen lugar durante un episodio de isquemia miocárdica modifiquen los mecanismos que regulan la apoptosis disminuyendo la susceptibilidad de los cardiomiocitos a factores inductores de apoptosis y que se generan en exceso durante la reperfusión, como son los RLO y NO.

En primer lugar se han usado como modelo experimental miocitos aislados sometidos a un protocolo que simula isquemia y expuestos a H_2O_2 durante la reoxigenación como fuente generadora de RLO y se ha comparado la curva dosis-efecto (porcentaje de células que desarrollan apoptosis) con la obtenida en miocitos control. En este mismo modelo se ha caracterizado la dependencia de la respuesta apoptótica a la defensa antioxidante variando los niveles internos de la forma reducida del glutation.

La capacidad del NO para inducir apoptosis en cardiomiocitos y cómo esta se ve modificada por el insulto isquémico se ha estudiado en miocitos aislados sometidos a isquemia simulada y en corazones aislados sometidos a isquemia y reperfundidos en presencia del donador de NO SNAP. Finalmente se ha analizado el efecto que tiene la isquemia/reperfusión tanto en el modelo de miocitos aislados como en corazones aislados sobre la expresión de genes pro- y anti-apoptóticos cuantificada por PCR.

2. Demostrar que la muerte celular inmediata por reperfusión tras una isquemia transitoria se puede reducir con estrategias aplicadas en el inicio de la reperfusión encaminadas a disminuir la hipercontractura a) inhibiendo la contractilidad durante el inicio de la reperfusión mediante la estimulación de la síntesis de GMPc; b) inhibiendo la entrada de Ca^{2+} durante los primeros minutos de la reperfusión.

a) La primera parte del objetivo planteado deriva de estudios que demuestran que la estimulación de la síntesis de GMPc tiene efectos inhibitorios sobre la contractilidad celular [72, 110] y reduce el desarrollo de hipercontractura en miocitos aislados sometidos a hipoxia/reoxigenación [72]. Esta idea nos ha llevado a considerar la estimulación de la síntesis de GMPc durante el inicio de la reperfusión como posible actuación destinada ha reducir la muerte inmediata por reperfusión a través de la atenuación de la hipercontractura.

a1. Inicialmente se caracterizaron las variaciones en los niveles de GMPc en un modelo de corazón aislado de rata sometido a un protocolo de hipoxia/reoxigenación. Se estudió el efecto que tiene sobre el daño por reoxigenación la estimulación de la guanilato ciclasa soluble por suplementación del medio de perfusión con L-arginina.

a2. Posteriormente se analizó en un modelo de corazón aislado sometido a isquemia/reperfusión el efecto que tiene sobre el daño por reperfusión y la contractilidad del miocardio el aumento en la síntesis de GMPc mediada por la estimulación de la guanilato ciclasa particulada. Para ello se administró el análogo del péptido natriurético atrial urodilatina durante los primeros minutos de la reperfusión.

b) Para abordar la segunda parte de este objetivo se ha estudiado la posible participación de la forma reversa del intercambiador Na^+/Ca^{2+} durante el inicio de la reperfusión en la sobrecarga de Ca^{2+} citosólico y su contribución al desarrollo de hipercontractura y muerte por reperfusión. Esto se ha conseguido inhibiendo el intercambiador Na^+/Ca^{2+} durante los primeros minutos de la reperfusión con un inhibidor altamente específico, KB-R7943. En el estudio se emplearon diferentes modelos: 1) miocitos aislados sometidos a modelos que simulan isquemia/reperfusión. En este modelo se ha calculado la IC_{50} de KB-R7943 y los efectos de la inhibición del intercambiador Na^+/Ca^{2+} sobre la sobrecarga de Ca^{2+} citosólica, las oscilaciones de Ca^{2+} e hipercontractura que tienen lugar durante la reenergización; 2) corazones de rata aislados sometidos a isquemia/reperfusión en los que se ha determinado el efecto de la

inhibición del transportador Na^+/Ca^{2+} sobre la recuperación funcional y necrosis miocárdica durante la reperfusión y se han caracterizado las dosis y tiempos óptimos de aplicación de la droga; 3) finalmente, se ha evaluado la relevancia terapéutica del tratamiento en un modelo porcino sometido a isquemia miocárdica transitoria.

MÉTODOS Y RESULTADOS

1. Contribución de la apoptosis a la muerte por reperfusión:

***Influence of simulated ischemia on apoptosis induction by oxidative stress in adult cariomyocytes of rats*.** J Inserte, G Taimor, B Hoffstaetter, D Garcia-Dorado, HM Piper. *Am J Physiol*. 2000; 278:H94-H99.

Inhibition of apoptotic responses after ischemic stress in isolated hearts and cardiomyocytes. B Hoffstaetter, G Taimor, J Inserte, D Garcia-Dorado, HM Piper. Basic Res Cardiol. 2002; 97:479-488.

Am. J. Physiol. Heart Circ. Physiol.
278: H94–H99, 2000.

Influence of simulated ischemia on apoptosis induction by oxidative stress in adult cardiomyocytes of rats

J. INSERTE,[2] G. TAIMOR,[1] B. HOFSTAETTER,[1] D. GARCIA-DORADO,[2] AND H. M. PIPER[1]
[1]*Physiologisches Institut, Justus-Liebig-Universität Giessen, 35392 Giessen, Germany; and* [2]*Servicio de Cardiologia, Hospital General Vall d' Hebron, 08035 Barcelona, Spain*

Inserte, J., G. Taimor, B. Hofstaetter, D. Garcia-Dorado, and H. M. Piper. Influence of simulated ischemia on apoptosis induction by oxidative stress in adult cardiomyocytes of rats. *Am. J. Physiol. Heart Circ. Physiol.* 278: H94–H99, 2000.—Oxidative stress may cause apoptosis of cardiomyocytes in ischemic-reperfused myocardium. We investigated whether ischemia-reperfusion modifies the susceptibility of cardiomyocyte induction of apoptosis by oxidative stress. Ischemia was simulated by incubating isolated cardiomyocytes from adult rats in an anoxic, glucose-free medium, pH 6.4, for 3 h. Annexin V-fluorescein isothiocyanate/propidium iodide staining and the detection of DNA laddering were used as apoptotic markers. H_2O_2 (7.5 μmol/l) induced apoptosis in 20.1 ± 1.8% of cells under normoxic conditions but only 14.4 ± 1.6% ($n = 6$, $P < 0.05$) after ischemia-reoxygenation. This partial protection of ischemic-reoxygenated cells was observed despite a reduction in their cellular glutathione content, from 11.4 ± 1.9 in normoxic controls to 2.9 ± 0.8 nmol/mg protein ($n = 3$, $P < 0.05$). Elevation of end-ischemic glutathione contents by pretreatment with 1 mmol/l *N*-acetylcysteine entirely protected ischemic-reoxygenated cells against induction of apoptosis by H_2O_2. In conclusion, ischemia-reperfusion can protect cardiomyocytes against induction of apoptosis by exogenous oxidative stress. This endogenous protective effect is most clearly demonstrated when control and postischemic cardiomyocytes are compared at similar glutathione levels.

simulated ischemia-reoxygenation; hydrogen peroxide; glutathione

BOTH NECROSIS AND APOPTOSIS have been shown to contribute to cell death induced by myocardial ischemia-reperfusion (9, 13). Apoptosis is a process of transcriptionally regulated, programmed cell death. The mechanisms by which ischemia-reperfusion may initiate programmed cell death are not well known. Recently, we have demonstrated that, in isolated adult cardiomyocytes from rats, conditions of simulated ischemia-reoxygenation cannot induce apoptosis (26). It seems therefore reasonable that exogenous factors in ischemic-reperfused myocardium might contribute to the induction of apoptosis. Reactive oxygen species (ROS) may play a role. ROS are indeed able to induce apoptosis in various cell types (16, 24, 25), and they are generated in excess in reperfused myocardium (3, 8, 18, 31). Cardiomyocytes represent only one of the potential sources of ROS. Sources exogenous to cardiomyocytes include neutrophils with a high capacity for producing ROS (2).

There are reasons to expect that ischemia can increase the susceptibility of cardiomyocytes to apoptosis induced by exogenous ROS. This is because ischemia reduces cellular levels of glutathione, superoxide dismutase, catalase, and other components of the cellular antioxidant defense (1, 7). It seems also possible, however, that ischemia disturbs the cellular metabolism in such a way that the transcriptionally regulated processes of programmed cell death are in fact impaired compared with normoxic control conditions.

The purpose of this study was to investigate experimentally whether ischemia influences the susceptibility of cardiomyocytes to apoptosis induced by exogenous ROS. Isolated rat cardiomyocytes were used as an experimental model because in this model the influence of nonmyocyte cells is excluded. The cells were exposed to H_2O_2, a generator of ROS, under normoxic control conditions and after simulated ischemia. The dependency of the apoptotic response on the cellular antioxidant defense was studied by variation of cellular glutathione contents. The results show that, in the absence of other external factors, the susceptibility of cardiomyocytes to apoptosis induced by exogenous oxidative stress is reduced after ischemic conditions.

METHODS

Cell isolation and short-term culture. Ventricular heart muscle cells were isolated from adult male Wistar rats (200–250 g) and plated in medium 199 (Sigma, St. Louis, MO) on 60-mm culture dishes preincubated overnight in medium 199 with 4% FCS (GIBCO), as described previously (20). Four hours after plating, the dishes were washed with a modified phosphate-free Tyrode medium (in mmol/l: 140 NaCl, 3.6 KCl, 1.2 $MgSO_4$, 1 $CaCl_2$, and 20 HEPES, pH 7.4). As a result of medium change, nonattached cells were removed, resulting in cultures of 85 ± 3% quiescent rod-shaped cells.

Simulated ischemia and reoxygenation. For simulated ischemia, dishes were filled with 1 ml of the modified Tyrode medium at pH 6.4, gassed with 100% N_2, and incubated at 37°C in gas-tight chambers in an atmosphere of 100% N_2 for 3 h. Reoxygenation was performed by addition of 1 ml of CCT medium (modified medium 199 including Earle's salts, 5 mmol/l creatine, 2 mmol/l L-carnitine, 5 mmol/l taurine, 100 IU/ml penicillin, 100 mg/ml streptomycin, and 10 mmol/l cytosine-β-D-arabinofuranoside, pH 7.4). Time-matched normoxic controls were obtained with the use of air-equilibrated, instead of N_2-saturated, modified Tyrode medium at pH 7.4.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Experimental protocol. Three sets of experiments were performed. In the first set of experiments, the induction of apoptosis by different concentrations of H_2O_2 under normoxic conditions versus simulated ischemia-reoxygenation was compared. In these experiments, the cardiomyocytes were exposed to 3 h of simulated ischemia or normoxia and then reoxygenated by adding 1 ml of CCT medium supplemented with H_2O_2 at final concentrations of 0, 1, 5, 7.5, 10, or 15 μmol/l.

The second set of experiments was performed to test the effect of *N*-acetylcysteine (NAC) pretreatment on glutathione content. NAC is a sulfhydryl group donor that is easily transported into the cells where it is deacetylated and increases the thiol pool, primarily by reduction of glutathione, in a dose-dependent manner (5). NAC, at final concentration of 0.01, 0.1, or 1 mmol/l, was added during the 4-h incubation period in medium 199. Next, cardiomyocytes were washed, subjected to 3 h of either simulated ischemia or normoxia, and reoxygenated by adding 1 ml of CCT medium.

Finally, in the third set of experiments, the effect of NAC pretreatment on the induction of apoptosis by H_2O_2 was analyzed under normoxic conditions and after simulated ischemia-reoxygenation. In these experiments, dishes were preincubated for 4 h with medium 199 with or without NAC at final concentrations of 0.01, 0.1, or 1 mmol/l, exposed to either normoxia or simulated ischemia for 3 h, and reoxygenated by adding 1 ml of CCT medium with or without 10 μmol/l of H_2O_2.

Determination of creatine phosphate contents. Experiments were terminated by addition of 1 ml of $HClO_4$ (1.2 mol/l) to the contents of the culture dishes. After neutralization, perchloric acid extracts of cultures were analyzed for creatine phosphate (CrP; see Ref. 15).

Analysis of genomic DNA. Six hours after intervention, i.e., either 3 h of simulated ischemia or addition of H_2O_2, DNA was extracted as described by Tanaka et al. (27). In a previous study (26), this time interval has been shown to be sufficient for detection of DNA laddering in isolated cardiomyocytes. Briefly, cardiomyocytes were harvested by centrifugation at 2,800 *g* for 5 min. After resuspension in lysis buffer (100 mmol/l NaCl, 10 mmol/l Tris·HCl, 25 mmol/l EDTA, 0.5% SDS, and 100 mg/ml proteinase K, pH 8.0), myocytes were incubated for 3 h at 37°C. After phenol/chloroform extraction and ethanol precipitation, DNA was dissolved in TE buffer (10 mmol/l Tris·HCl and 1 mmol/l EDTA, pH 8.0) and incubated with 5 mg/ml DNase-free RNase for 2 h at 37°C. Again, the DNA was precipitated and resuspended in TE buffer. Concentrations were measured spectrophotometrically. Each DNA (5 μg) was electrophoretically separated on 1.5% agarose gels and stained with ethidium bromide.

Fluorescence staining of cardiomyocytes. Apoptosis and necrosis were identified by means of double fluorescence staining with annexin V-FITC/propidium iodide. Apoptotic cells translocate phosphatidylserine from the inner site of the plasma membrane to the outer surface (6) while the membrane remains physically intact. Apoptotic cells therefore stain with annexin V-FITC, which binds with high affinity to phosphatidylserine, resulting in a green fluorescence when exited at 450–480 nm, and exclude propidium iodide, a DNA dye unable to pass the plasma membrane. Necrotic cells have lost the physical integrity of their plasma membrane and stain therefore both with annexin V-FITC/propidium iodide, which fluoresces in the red when exited at 510–550 nm. Cells that are neither apoptotic nor necrotic do not stain with either dye (29).

For fluorescent staining, 10 μl of annexin V-FITC (Boehringer Ingelheim) and 1 ng propidium iodide were added to the culture medium (2 ml) 2 h after intervention, i.e., either 3 h of simulated ischemia or addition of H_2O_2. It was shown before that this time interval is sufficient for apoptosis detection in cardiomyocytes (26). Cultures were then incubated for 10 min at 37°C in the dark and then analyzed by fluorescence microscopy. For quantification of apoptosis and necrosis, ~300 randomly distributed cells per dish were counted. The small number of necrotic cells at *time 0* was subtracted from all following counts.

Analysis of glutathione content. After 3 h of simulated ischemia, modified Tyrode medium was discarded. The culture dishes were washed two times with ice-cold potassium phosphate buffer (0.2 mol/l, pH 7.1) containing 2 mmol/l EDTA and were taken up in 340 μl of this buffer. After homogenization in a glass potter tube with a glass pestle at 4°C, and sonication for 15 s at 30 W (4°C), the cellular homogenate was deproteinized by adding 60 μl of sulfosalicylic acid (30%). The homogenate was centrifuged (5 min, 3,000 *g*) at 4°C, the supernatant was used for assays of GSH and GSSG, and the pellet was used for protein determination (4).

Total glutathione content was quantified by using the glutathione reductase technique (11), which monitors the reduction of 5,5′-dithio-bis(2-nitrobenzoic acid) at 412 nm. Pure GSH dissolved in 4.5% sulfosalicylic acid solution was assayed for the purpose of calibration. Concentrations of 1, 2, 3, and 4 nmol GSH/cuvette were used.

GSSG was assayed enzymatically after derivatization of GSH with 2-vinylpyridine. Pure GSSG dissolved in 4.5% of sulfosalicylic acid solution was assayed under identical conditions to ensure correct calibration. Concentrations of 0.2, 0.4, 0.6, 0.8, and 1.0 nmol GSSG/cuvette were used. GSH was calculated as total glutathione minus GSSG.

Protein analysis. Protein content was assayed in the acid precipitates with Coomassie brilliant blue according to the method of Bradford (4), using BSA as standard.

Statistics. Data are given as means ± SE from *n* different culture preparations. Comparisons involving multiple groups were performed by means of ANOVA. Statistical significance of difference between groups was assessed by Student-Newman-Keuls test. A critical *P* value of 0.05 was used for all tests; values above $P = 0.05$ were regarded as not significant (NS).

RESULTS

High-energy phosphates during simulated ischemia and reoxygenation. Under normoxic conditions, cardiomyocytes contained 20.1 ± 2.6 nmol CrP/mg protein. After 3 h of simulated ischemia, the content of CrP was reduced to 9.7 ± 3.4 nmol/mg protein ($n = 3$, $P < 0.05$). After 1 h of reoxygenation, cells had completely recovered their CrP (19.4 ± 5.1 nmol/mg protein). Pretreatment of cardiomyocytes with 1 mmol/l NAC did not alter cellular contents of CrP either under normoxic conditions or after 3 h of simulated ischemia (21.1 ± 7.6 and 9.7 ± 3.3 nmol/mg protein, respectively, P = NS with respect to corresponding values in nontreated cells).

Induction of apoptosis by oxidative stress under normoxia and simulated ischemia-reoxygenation. H_2O_2 induced apoptosis in a dose-dependent manner in cardiomyocytes under normoxic control conditions and after simulated ischemia-reoxygenation (Fig. 1*A*). The basal number of apoptotic cells in the absence of H_2O_2 was the same in both experimental groups (7.2 ± 1.4%

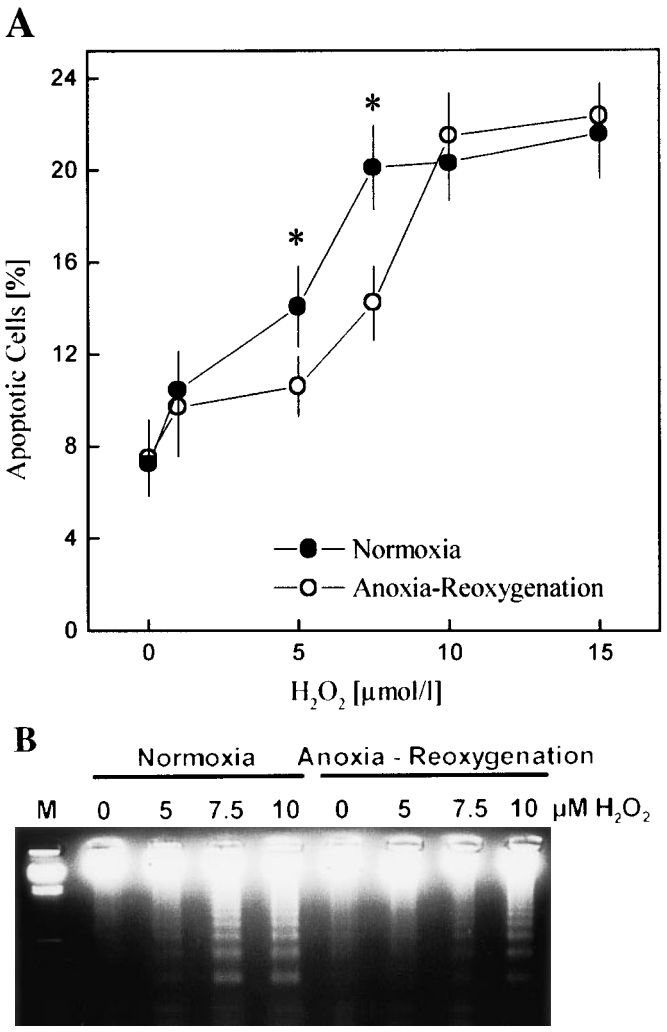

Fig. 1. Induction of apoptosis by H_2O_2 under normoxic conditions and after simulated ischemia-reoxygenation. H_2O_2 was added to the culture medium of normoxic cells (Normoxia) and of cells reoxygenated after 3 h of simulated ischemia (Anoxia-Reoxygenation) at a final concentration of 1, 5, 7.5, and 10 µmol/l. *A*: quantification of apoptotic cells by annexin V-FITC/propidium iodide staining 2 h after H_2O_2 addition or after 2 h of reoxygenation, given in percentage of all cells. Data are means ± SE of 6 independent preparations. *Differences from normoxic cells with $P < 0.05$. *B*: DNA laddering. DNA was extracted 6 h after H_2O_2 addition or after 6 h of reoxygenation. Five micrograms per lane were separated on 1.5% agarose gels. Marker (M) was λ-DNA, *Hin*d III digested. DNA was stained by ethidium bromide.

in normoxia and 7.5 ± 1.7% after simulated ischemia-reoxygenation). The relative apoptotic response to addition of H_2O_2 was different among the groups, however. At low doses of H_2O_2, cells under normoxic conditions were more prone to develop apoptosis than after simulated ischemia. In normoxic cells, H_2O_2 added at a concentration of 7.5 µmol/l induced a maximum degree of apoptosis. In cells exposed to reoxygenation, the maximum level of apoptosis was not reached before 10 µmol/l H_2O_2 were applied. At this concentration, the maximal percentage of apoptosis achieved in either group was the same (21.5 ± 0.7 and 20.3 ± 0.6%, respectively, $n = 6$, NS).

DNA "laddering," indicative of apoptotic internucleosomal DNA fragmentation, was confirmation of the results obtained with annexin V-FITC/propidium iodide staining (Fig. 1*B*). The intensity of the ladders increased progressively with increasing concentrations of H_2O_2. In the normoxic group, DNA laddering could be seen after exposure to 7.5 µmol/l H_2O_2, whereas in the group with simulated ischemia-reoxygenation DNA laddering was visible only at higher concentrations of H_2O_2 (10 µmol/l).

Effect of NAC pretreatment on glutathione levels. Three hours of simulated ischemia produced a 3.9-fold decrease of total glutathione contents compared with normoxic controls (11.4 ± 1.1 and 2.9 ± 0.4 nmol/mg protein, respectively, $n = 3$, $P < 0.05$; Fig. 2*A*). The ratio of GSH to GSSG, which is indicative of the redox state of the cells, also decreased during simulated ischemia from 17.6 ± 3.5 to 4.3 ± 0.9 ($n = 3$, $P < 0.05$; Fig. 2*B*). Pretreatment with NAC of cardiomyocytes subjected to 3 h of simulated ischemia produced a dose-dependent increase of the end-ischemic glutathione levels and of the ratio of GSH to GSSG. Pretreatment at a concentration of 1 mmol/l attenuated the drop below the normoxic control level of cellular glutathione contents after 3 h of simulated ischemia (8.4 ± 0.8, $n = 3$, NS). Normoxic cells pretreated with 1 mmol/l NAC did not significantly increase their glutathione content (Fig. 2*A*).

Effect of NAC pretreatment on apoptosis induced by oxidative stress. When normoxic cells pretreated with 1 mmol/l NAC were subjected to 10 µmol/l H_2O_2, the number of cells becoming apoptotic was not different compared with the absence of NAC pretreatment (18.8 ± 1.5 and 18.7 ± 1.4%, respectively, $n = 6$, NS). In contrast to this behavior of normoxic cells, cardiomyocytes exposed first to simulated ischemia and then to H_2O_2 were less prone to develop apoptosis when pretreated with NAC (Fig. 3*A*). The effect of NAC pretreatment was dose dependent. The attenuation of H_2O_2-induced apoptosis became significant in cells pretreated with 0.01 mmol/l NAC (18.2 ± 0.5 vs. 20.95 ± 1.9% apoptotic cells, $n = 6$, $P < 0.05$). After pretreatment with 1 mmol/l NAC, ischemic-reoxygenated cardiomyocytes were entirely unresponsive to apoptosis induction with 10 µmol/l H_2O_2 (9.0 ± 0.3% apoptotic cells compared with 9.1 ± 0.7% among pretreated ischemic-reoxygenated cells without exposure to H_2O_2, $n = 6$, NS).

The percentage of necrotic cardiomyocytes in normoxic preparations was 7.3 ± 2.1%, without differences between groups. After simulated ischemia-reoxygenation, the percentage of necrotic cells increased significantly to 36.4 ± 3.6% ($P < 0.05$), without significant influence of NAC or H_2O_2 treatment (Fig. 3*A*).

Comparable results were obtained by DNA agarose electrophoresis (Fig. 3*B*). DNA ladders were clearly visible in the normoxic groups subjected to 10 µmol/l H_2O_2, with or without 1 mmol/l NAC pretreatment. No DNA laddering was observed when cardiomyocytes were treated with 1 mmol/l or 100 µmol/l NAC before being subjected to simulated ischemia and reoxygenation in the presence of 10 µmol/l H_2O_2.

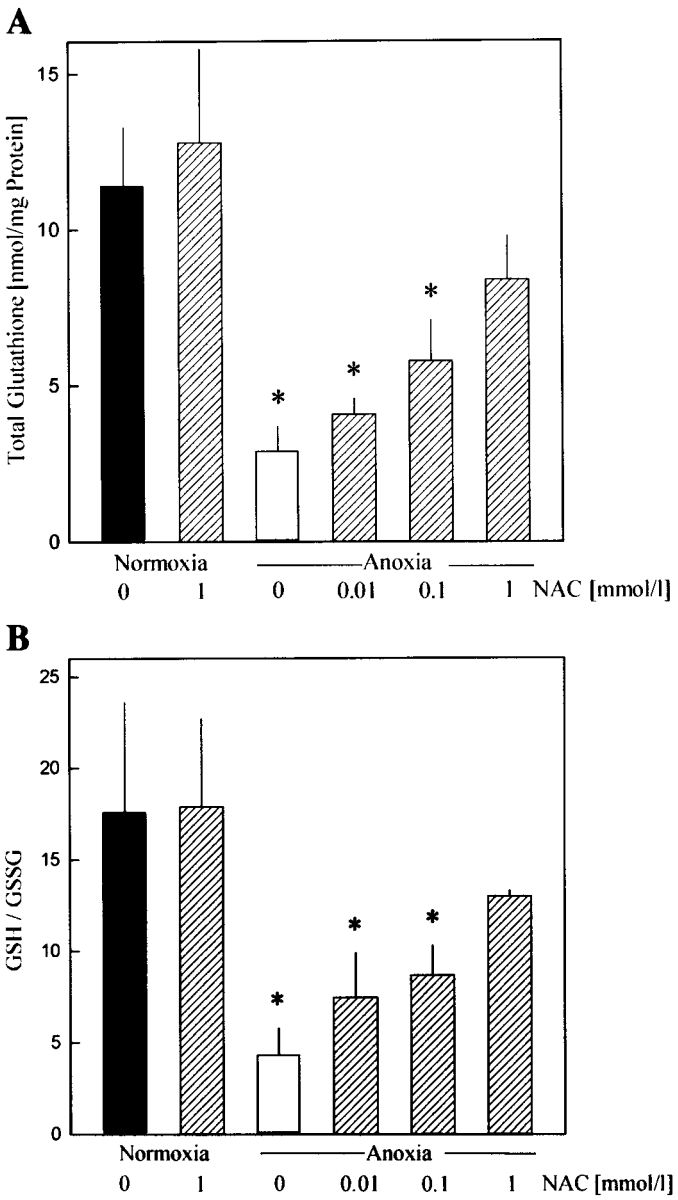

Fig. 2. Glutathione contents after simulated ischemia. *A*: total glutathione contents; *B*: ratio of GSH to GSSG. Cardiomyocytes were preincubated with 0, 0.01, 0.1, and 1 mmol/l *N*-acetylcysteine (NAC) for 4 h. Preincubation was followed either by normoxia or by 3 h of simulated ischemia (Anoxia). Thereafter, glutathione contents were determined. Data are means ± SE of 3 independent preparations. *Differences from nonpretreated normoxic cells with $P < 0.05$.

DISCUSSION

The central question of the present study was whether the susceptibility of cardiomyocytes to apoptosis induced by exogenous oxidative stress would be changed after exposure to simulated ischemia-reoxygenation. The main finding is that cardiomyocytes are less susceptible to the induction of apoptosis by H_2O_2 after simulated ischemia-reoxygenation. Full protection against H_2O_2-induced apoptosis is achieved if the ischemic loss of glutathione is prevented.

It has been demonstrated by studies from our laboratory and other laboratories that fundamental aspects of ischemia-reperfusion injury can be simulated in an isolated cardiomyocyte model and that this model allows causal analysis in greater depth than possible in intact tissue (12, 14, 22, 23, 28). We showed previously that, for isolated cardiomyocytes devoid of the influences by the surrounding tissue, conditions of simulated ischemia (up to 18 h) and reoxygenation (up to 12 h) were not sufficient to induce apoptosis (26). Only the progressive development of necrosis was observed. The lack of development of apoptosis in cardiomyocytes in response to simulated ischemia and reoxygenation is in contrast to the ability of exogenous factors to induce apoptosis in these cells.

In the present study, the prooxidant H_2O_2 was used at low concentrations, which caused apoptosis but not necrosis of cardiomyocytes. To determine apoptosis in the cell population, the cells were stained with annexin

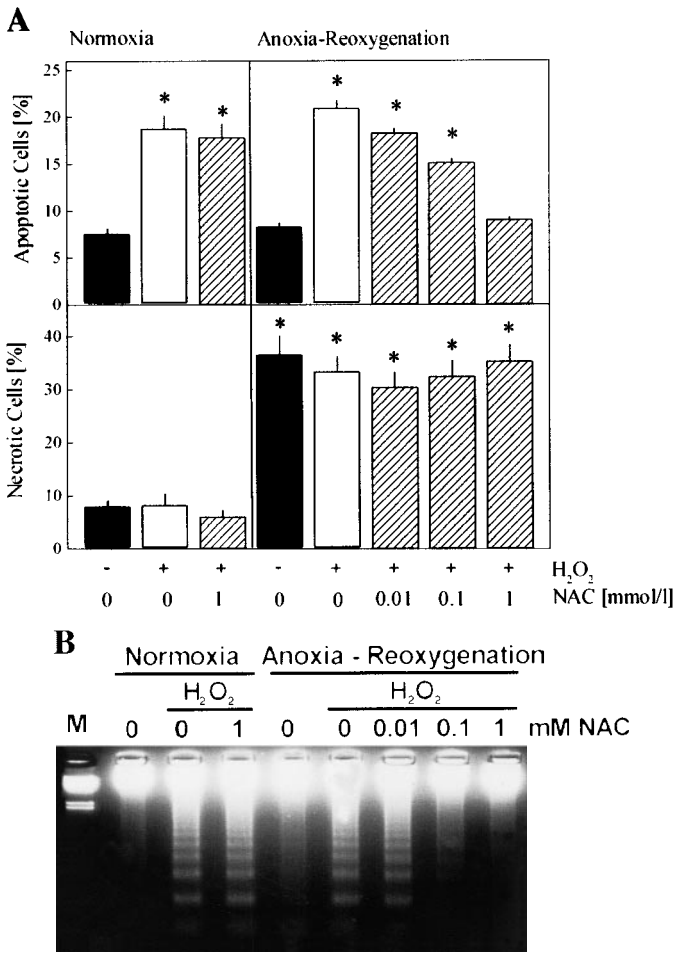

Fig. 3. Apoptosis and necrosis in normoxic and anoxic/reoxygenated cardiomyocytes after adjustment of cellular glutathione contents. Cardiomyocytes were preincubated with 0, 0.01, 0.1, and 1 mmol/l NAC for 4 h. Preincubation was followed by normoxia or 3 h of simulated ischemia. Thereafter, 10 µmol/l H_2O_2 were added to the culture medium of normoxic cells (Normoxia) and of cells reoxygenated after 3 h of simulated ischemia (Anoxia-Reoxygenation). *A*: quantification of apoptotic and necrotic cells by annexin V-FITC/propidium iodide staining 2 h after H_2O_2 addition or after 2 h of reoxygenation, given in percentages of all cells. Data are means ± SE of 6 independent preparations. *Differences from nonpretreated normoxic control with $P < 0.05$. *B*: DNA laddering. DNA was extracted 6 h after H_2O_2 addition or after 6 h of reoxygenation. Five micrograms per lane were separated on 1.5% agarose gels. Marker (M) was λ-DNA, *Hin*d III digested. DNA was stained by ethidium bromide.

V-FITC/propidium iodide. As an additional parameter for apoptosis, internucleosomal DNA cleavage was assayed by the DNA-laddering phenomenon after gel electrophoretic separation of DNA. In agreement with our previous experience, both methods gave basically the same results.

During simulated ischemia-reoxygenation, cardiomyocytes lost more than one-half of their glutathione contents, and in the remaining pool the ratio of GSH versus GSSG was markedly diminished. This finding is consistent with results from many other studies demonstrating the loss of GSH in energy-depleted myocardial cells (1, 6). Because GSH is a central element in the cellular antioxidant defense, its loss renders cells more prone to oxidative stress. In part of the experiments, cardiomyocytes were pretreated with the glutathione precursor NAC before ischemia. This has been shown to be an efficient procedure to increase myocardial glutathione content (5). In NAC-pretreated cells, the glutathione content at the end of simulated ischemia was much higher than in nontreated cells and came close to the glutathione content of normoxic control cells. Concomitantly, the ratio of GSH to GSSG at the end of simulated ischemia was increased in NAC-pretreated cells.

The degree of the postischemic apoptotic response to H_2O_2 was related in a dose-dependent manner to the magnitude of the end-ischemic cellular glutathione pool. This indicates that the induction of apoptosis is related to the extent of antioxidant defense (Fig. 4). This finding is consistent with the observation that apoptosis is elicited in a dose-dependent manner by the prooxidant H_2O_2. The importance of these results is only revealed if the ability of H_2O_2 to induce apoptosis is compared between ischemic-reoxygenated and normoxic cardiomyocytes, each with high glutathione levels. This comparison uncovers that, at a given antioxidative defense, ischemic-reoxygenated cardiomyocytes are much less susceptible to H_2O_2-induced apoptosis. In fact, reoxygenated cells are completely protected against H_2O_2-induced apoptosis if their end-ischemic glutathione reserves are brought to a close-to-normal level.

The antiapoptotic protective effect of an ischemic exposure resembles the protection provided by ischemic preconditioning. Furthermore, both phenomena could share the same common mechanisms. For example, ROS generated upon postischemic reperfusion could precondition the heart via activation of the mitogen-activated protein kinase cascade (17). However, the phenomenon described here presents conceptual differences with ischemic preconditioning. Ischemic preconditioning is defined as the protection provided by a brief ischemic episode against a subsequent period of ischemia (30). In the phenomenon described here, ischemia protects myocytes against exogenous noxious stimuli during reperfusion. The findings in the present study should therefore be distinguished from recent observations showing that repetitive brief episodes of ischemia (5 min) protect rat hearts against apoptosis induced by a subsequent 30-min ischemic period (19). They should also be distinguished from the protection afforded by brief metabolic inhibition against apoptosis induced by prolonged metabolic inhibition in isolated cardiomyocytes (10). In these cases, reduced apoptosis clearly appears as another manifestation of classic ischemic preconditioning.

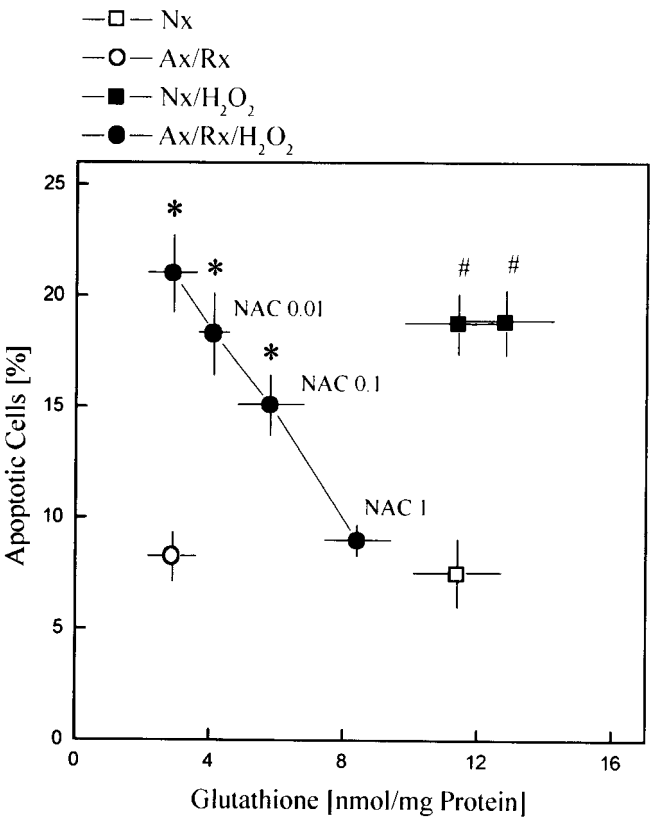

Fig. 4. Relationship between percentage of apoptotic cells and cellular glutathione contents. Glutathione contents, which were measured in normoxic (Nx) cultures (□, ■) or directly after 3 h of simulated ischemia (○, ●), are related to the percentage of apoptotic cells in normoxic (□, ■) or anoxic (Ax)-reoxygenated (Rx; ○, ●) cultures. □ and ○, No. of apoptotic cells in nontreated controls; ■ and ●, amount of apoptotic cells after addition of 10 μmol/l H_2O_2. In normoxic and anoxic-reoxygenated cultures, H_2O_2 induces the same amount of apoptosis. However, in anoxic cells, glutathione levels are reduced. When preincubated with NAC (0.01, 0.1, and 1 mmol/l), which results in an increase of glutathione contents in anoxic and reoxygenated cells, anoxic-reoxygenated cardiomyocytes progressively lose their responsiveness to apoptosis induction by H_2O_2. *Differences from anoxic/reoxygenated control with $P < 0.05$. #Difference from normoxic control with $P < 0.05$.

Various studies performed during the last few years in different models have convincingly demonstrated the occurrence of apoptotic cell death in ischemic-reperfused myocardium (10, 13, 21), but the relative importance of apoptotic cell death compared with necrosis has remained controversial. These observations have raised questions about the nature of inducers of apoptosis and mechanisms leading to programmed cell death in ischemic or ischemic-reperfused myocardium. Apoptosis might be directly initiated by the sublethal injury of cardiomyocytes in an energy-depleted state or, alternatively, by exogenous apoptosis-promoting factors originating from blood or nonmyocytes in ischemic-reperfused tissue. We have shown in a previous (26) and in the present study that neither the metabolic depression of ischemia nor the process of metabolic alterations upon reoxygenation are sufficient causes for induction

of apoptosis in adult cardiomyocytes once these are separated from exogenous influences. The inducers of apoptosis in ischemic-reoxygenated myocardium must therefore be sought among factors exogenous to cardiomyocytes. The results of the present study indicate that the reoxygenated cardiomyocyte responds less than control, normoxic myocytes to such exogenous inducers of apoptosis.

The phenomenon described in this study, i.e., that prolonged ischemia renders cardiomyocytes less susceptible to prooxidant-induced apoptosis, may correspond to a new form of endogenous protection induced by ischemia. It must be left to future studies to identify the mechanisms of this form of protection and to establish their relationship with the mechanisms of protection against ischemia-reperfusion involved in classic ischemic preconditioning.

This study was partially supported by the Commission of the European Union, BIOMED II Program (PL95–1254).

Address for reprint requests and other correspondence: J. Inserte, Hospital General, Laboratorio B Cardiol. Exp., Pg. Vall d Hebron, 119–129, 08035 Barcelona, Spain (E-mail: inserte@hg.vhebron.es).

Received 4 March 1999; accepted in final form 3 August 1999.

REFERENCES

1. **Arduini, A., A. Mezzetti, E. Porreca, D. Lapenna, J. De Julia, L. Marzio, G. Polidoro, and F. Cuccurollo.** Effect of ischemia and reperfusion on antioxidant enzymes and mitochondrial inner membrane proteins in perfused rat hearts. *Biochim. Biophys. Acta* 970: 113–121, 1988.
2. **Badchi, D., D. K. Das, R. M Engelman, M. R. Prasad, and R. Subramanian.** Polymorphonuclear leucocytes as potential source of free radicals in the ischemic-reperfused myocardium. *Eur. Heart J*. 11: 800–813, 1990.
3. **Bolli, R., M. O. Jeroudi, B. S. Patel, C. M. DuBose, E. K. Lai, R. Roberts, and P. B. McCay.** Direct evidence that oxygen derived free radicals contribute to postischemic myocardial dysfunction in the intact dog. *Proc. Natl. Acad. Sci. USA* 86: 4695–4699, 1989.
4. **Bradford, M. M.** A rapid and sensitive method for the quantitation of microgramquantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*. 72: 248–254, 1976.
5. **Ceconi, C., S. Curello, A. Cargnoni, R. Ferrari, A. Albertini, and O. Visioli.** The role of glutathione status in the protection against ischemic and reperfusion damage: effects of *N*-acetyl cysteine. *J. Mol. Cell. Cardiol*. 20: 5–13, 1988.
6. **Fadok, V. A., D. R. Voelker, P. A. Campbell, J. J. Cohen, D. L. Bratton, and P. M. Henson.** Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *J. Immunol*. 148: 2207–2216, 1992.
7. **Ferrari, R., C. Ceconi, S. Curello, C. Guarinieri, C. M. Calderera, A. Albertini, and O. Visoli.** Oxygen mediated myocardial damage during ischemia and reperfusion: role of the cellular defences against oxygen toxicity. *J. Mol. Cell. Cardiol*. 17: 937–945, 1985.
8. **Garlick, P. B., M. J. Davies, D. J. Hearse, and T. F. Slater.** Direct detection of free radicals in the reperfused rat using electron spin resonance microscopy. *Circ. Res*. 61: 757–760, 1987.
9. **Gottlieb, R. A., K. O. Burleson, R. A. Kloner, B. M. Babior, and R. L. Engler.** Reperfusion injury induces apoptosis in rabbit cariomyocytes. *J. Clin. Invest*. 94: 1621–1628, 1994.
10. **Gottlieb, R. A., D. L. Gruol, J. Y. Zhu, and R. L. Engler.** Preconditioning in rabbit cardiomyocytes: role of pH, vacuolar proton ATPase, and apoptosis. *J. Clin. Invest*. 97: 2391–2398, 1996.
11. **Griffith, O. W., and A. Meister.** Potent and specific inhibition of glutathione synthesis by buthionine sulfoximine (S-nButyl homocysteine sulfoximine). *J. Biol. Chem*. 254: 7558–7560, 1979.
12. **Hohl, C., A. Ansel, R. Altschuld, and G. P. Brierley.** Contracture of isolated rat heart cells on anaerobic and aerobic transition. *Am. J. Physiol. Heart Circ. Physiol*. 242: H1022–H1030, 1982.
13. **Kajstura, J., W. Cheng, K. Reiss, W. A. Clark, E. H. Sonnenblick, S. Krajewski, J. C. Reed, G. Olivetti, and P. Anversa.** Apoptotic and necrotic myocytes cell deaths are independent contributing variables of infarct size in rats. *Lab. Invest*. 74: 86–107, 1996.
14. **Ladilov, Y., B. Siegmund, and H. M. Piper.** Protection of reoxygenated cardiomyocytes against hypercontracture by inhibition of Na^+/H^+ exchange. *Am. J. Physiol. Heart Circ. Physiol*. 268: H1531–H1539, 1995.
15. **Lamprecht, J. W., P. Stein, F. Heinz, and H. Weisser.** Creatinphosphate. In: *Methoden der Enzymatischen Analyse*, edited by H. U. Bergmeyer. Weinheim, Germany: Verlag Chemie, 1974, p. 1825–1829.
16. **Li, P.-F., R. Dietz, and H. Rüdiger.** Reactive oxygen species induce apoptosis of vascular smooth muscle cell. *FEBS Lett*. 404: 249–252, 1997.
17. **Maulik, N., M. Watanabe, Y. L. Zu, C. K. Huang, G. A. Cordis, J. A. Schley, and D. K. Das.** Ischemic preconditioning triggers the activation of MAP kinases and MAPKAP kinase 2 in rat hearts. *FEBS Lett*. 396: 233–237, 1996.
18. **McCord, J. M.** Oxygen free radicals in post-ischemic tissue injury. *N. Engl. J. Med*. 312: 159–164, 1985.
19. **Piot, C. A., D. Padmanaban, P. C. Ursell, R. E. Sievers, and C. L. Wolfe.** Ischemic preconditioning decreases apoptosis in rat hearts in vivo. *Circulation* 96: 1598–1604, 1997.
20. **Piper, H. M., I. Probs, P. Schwartz, J. F. Hütter, and P. G. Spieckermann.** Culturing of calcium stable adult cardiac myocytes. *J. Mol. Cell. Cardiol*. 14: 397–412, 1982.
21. **Saraste, A., K. Pulkki, M. Kallakoji, K. Henriksen, M. Parvinen, and L. M. Voipio-Pulkki.** Apoptosis in human acute myocardial infarction. *Circulation* 95: 320–323, 1997.
22. **Siegmund, B., A. Koop, T. Klietz, P. Schwartz, and H. M. Piper.** Sarcolemmal integrity and metabolic competence of cardiomyocytes under anoxia-reoxygenation. *Am. J. Physiol. Heart Circ. Physiol*. 258: H285–H291, 1990.
23. **Siegmund, B., R. Zude, and H. M. Piper.** Recovery of anoxic-reoxygenated cardiomyocytes from severe Ca^{2+}-overload. *Am. J. Physiol. Heart Circ. Physiol*. 263: H1262–H1269, 1992.
24. **Slater, A. F., C. S. Nobel, and S. Orrenius.** The role of intracellular oxidants in apoptosis. *Biochim. Biophys. Acta* 1271: 59–62, 1995.
25. **Stangel, M., U. K. Zettl, E. Mix, J. Zielasek, K. V. Toyka, H. P. Hartung, and R. Gold.** H_2O_2 and nitric oxide-mediated oxidative stress induce apoptosis in rat skeletal muscle myoblasts. *J. Neuropathol. Exp. Neurol*. 55: 36–43, 1996.
26. **Taimor, G., H. Lorenz, B. Hofstaetter, K.-D. Schlüter, and H. M. Piper.** Induction of necrosis but not apoptosis after anoxia and reoxygenation in isolated adult cardiomyocytes of rat. *Cardiovasc. Res*. 41: 147–156, 1999.
27. **Tanaka, M., H. Ito, S. Adachi, H. Akimoto, T. Nishikawa, T. Kasajima, F. Marumo, and M. Hiroe.** Hypoxia induces apoptosis with enhanced expression of fas antigen messenger RNA in cultured neonatal rat cardiomyocytes. *Circ. Res*. 75: 426–433, 1994.
28. **Vander Heide, R. S., J. P. Angelo, R. A. Altschuld, and C. E. Ganote.** Energy dependence of contraction band formation in perfused hearts and isolated adult cardiomyocytes. *Am. J. Pathol*. 125: 55–68, 1986.
29. **Vermes, I., C. Haanen, H. Steffens-Nakken, and C. A. Reutelingsperger.** A novel assay for apoptosis flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled annexin V. *J. Immunol. Methods* 184: 39–51, 1995.
30. **Yellon, D. M, G. F. Baxter, D. Garcia-Dorado, G. Heusch, and M. S. Sumeray.** Ischaemic preconditioning: present position and future directions. *Cardiovasc. Res*. 37: 21–33, 1998.
31. **Zweier, J. L., J. T. Flaherty, and M. L. Weisfeldt.** Direct measurement of free radical generation following reperfusion of ischemic myocardium. *Proc. Natl. Acad. Sci. USA* 84: 1404–1407, 1987.

Basic Res Cardiol 97: 479 – 488 (2002)
DOI 10.1107/s00395-002-0373-8

ORIGINAL CONTRIBUTION

Bettina Hofstaetter
Gerhild Taimor
Javier Inserte
David Garcia-Dorado
Hans Michael Piper

Inhibition of apoptotic responses after ischemic stress in isolated hearts and cardiomyocytes

Received: 20 March 2002
Returned for 1. revision: 8 April 2002
1. Revision received: 30 April 2002
Returned for 2. revision: 21 May 2002
2. Revision received: 29 May 2002
Returned for 3. revision: 29 May 2002
3. Revision received: 6 June 2002
Accepted: 12 June 2002

Dr. G. Taimor (✉) · B. Hofstaetter
H. M. Piper
Physiologisches Institut
Justus-Liebig-Universität Gießen
Aulweg 129
35392 Gießen, Germany
Tel.: +49-641/9947246
Fax: +49-641/9947239
E-Mail:
gerhild.taimor@physiologie.med.uni-giessen.de

J. Inserte · D. Garcia-Dorado
Servicio de Cardiologia
Hospital General Universitari
Vall d'Hebron 119–129
Barcelona 08035, Spain

Abstract Recent findings on the induction of anti-apoptotic gene expression in ischemic/reperfused hearts encouraged us to investigate whether ischemic/reperfused hearts may be protected against apoptosis induction. To analyze this hypothesis we performed studies on isolated perfused hearts of rat. For apoptosis induction, hearts were perfused with the NO donor (±)-S-nitroso-N-acetylpenicillamine (SNAP, 10 μM) for 30 minutes. Four hours thereafter apoptosis was detected by DNA laddering and TUNEL assay. Under normoperfusion SNAP induced 5.5 ± 1.4 TUNEL-positive myocytes per tissue section (vs. 1.8 ± 0.5 in controls). But when hearts were subjected to 20 minutes of no flow ischemia, which was sufficient for energy depletion of the hearts without inducing severe necrotic or apoptotic cell death, reperfusion in the presence of SNAP did not induce apoptosis. To analyze if this mode of protection is a property of the cardiomyocytes, we performed corresponding experiments on ventricular cardiomyocytes of rat. Again, under normoxic conditions SNAP (100 μM) increased the number of TUNEL-positive cells to 12.6 ± 4.9 % (vs. 5.4 ± 0.7 % in controls). But when SNAP was added after 3 h of simulated ischemia, which was sufficient for energy depletion of the cells without inducing apoptotic cell death, the number of apoptotic cells did not increase. The ischemia-induced protection of hearts and cardiomyocytes goes along with an increased expression of several anti-apoptotic genes, mainly of the bcl-2 family. This indicates that ischemic conditions induce an anti-apoptotic gene program in cardiomyocytes, which may also be responsible for the observed anti-apoptotic actions in the intact ischemic/reperfused myocardium.

Key words Anoxia – Bcl-2 – IAP – mitochondria – nitric oxide

Introduction

During the past few years it has become apparent that, besides necrotic cell death, apoptosis is also induced in ischemic/reperfused myocardium (1). The causal mechanisms and the relative importance of apoptosis for the loss of viable cardiomyocytes are not yet entirely clear.

It has been shown that ischemia alone can induce apoptosis in the heart, which can be accelerated by reperfusion (9). Whether this reperfusion injury triggers apoptosis in already ischemic injured cells or whether it contributes to cell death of non-injured cells has not been proven yet. But the burst of free radicals in reperfused myocardium, like oxygen radicals or nitric oxide (NO), are discussed as contributing to apoptosis induction in reperfusion.

In spite of this, it is also known that ischemia induces antagonistic "endogenous" mechanisms of protection. Recent reports indicate that in ischemic/reperfused myo-

cardium not only pro-apoptotic forces are activated: Olivetti et al. (18) and Misao et al. (16) demonstrate the induction of the anti-apoptotic gene bcl-2 in samples of human failing hearts or after myocardial infarction. *In vivo* occlusion of coronary artery in rats induced mcl-1 expression in non-ischemic regions of the heart (14). Bcl-2-like proteins predominantly act at mitochondria and protect against the release of cytochrome c into the cytosol, a triggering process for activation of caspases, which represent key enzymes in apoptotic signaling (7). After transient, non-lethal ischemia in a swine model, in addition to other cell survival genes, the anti-apoptotic gene IAP-1 (inhibitors of apoptosis) is found increased (6). IAP-like proteins are intracellular inhibitors of caspases (8). Therefore, a number of anti-apoptotic genes are activated in ischemic hearts.

In the present study we investigated if an anti-apoptotic effect can be directly demonstrated in an intact heart. For this purpose isolated hearts were challenged with NO as an inducer of apoptosis. NO was chosen, because its increased release in ischemic/reperfused myocardium represents a likely cause for apoptosis after myocardial infarction (26). The capacity of NO to provoke apoptosis was compared under normoxic conditions and under ischemia/reperfusion; the expression of the pro- and anti-apoptotic marker genes was analyzed. We then hypothesized that the results obtained on the whole heart were due to properties of the cardiomyocytes. We therefore performed corresponding experiments with isolated cardiomyocytes, a model in which an anti-apoptotic postischemic effect has already been described (11, 23).

Methods

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85–23, revised 1996).

Studies in isolated perfused rat heart

Adult male Sprague-Dawley rats (300 – 350 g) were anesthetized with thiopental sodium (200 mg/kg) and killed by cervical dislocation. Hearts were excised, arrested in ice-cold saline and retrogradely perfused with Krebs-Henseleit bicarbonate buffer at a constant flow of 10 ml/min (in mmol/L: NaCl 140, $NaHCO_3$ 24, KCl 2.7, KH_2PO_4 0.4, $MgSO_4$ 1, $CaCl_2$ 1.8, glucose 5; equilibrated with 95% O_2 – 5% CO_2, pH 7.4). Myocardial temperature was kept at 37 °C throughout the experiments.

Experimental protocols are illustrated in Fig. 1. Experiments were started with a 30 min normoxic equilibration period. In order to induce ischemia, the perfusion was stopped for 20 or 30 minutes in some experiments. Thereafter, normoxic perfusion was resumed (reperfusion). SNAP (10 μM) was added to perfusates either immediately with onset of reperfusion or at the respective time point in normoxic controls. SNAP remained in the perfusates for 30 min. Perfusion was continued for 270 min after addition of SNAP in all experiments or at the given time points in control experiments. TUNEL assay and DNA extraction were performed at the end of the perfusion times.

Left ventricular pressure

Left ventricular (LV) pressure was monitored by means of a fluid-filled balloon located in the tip of a Cordis 5 F catheter connected to a pressure transducer (Baxter 43600F) inserted into the LV. The intraventricular balloon was slowly inflated to achieve an initial end-diastolic pressure (LVEDP) between 8 and 12 mmHg (5, 28), which results in maximal LV developed systolic pressure (LVdevP) defined as LV systolic pressure minus LVEDP. The balloon volume was kept constant thereafter.

Enzyme release

In samples taken from the coronary effluent every 10 min during equilibration and normoxic perfusion lactate dehydrogenase (LDH) activity was assayed by spectrophotometric analysis according to (3). During reperfusion, samples were taken after 1, 2, 3, 4, 6, 8, 10, 12, 15, 20 min and every 10 min thereafter. Total tissue activities were obtained from tissue homogenates which were lysed by addition of TritonX-100 (1% final concentration). Results were expressed in % of total activity per g of dry weight.

Studies in isolated cardiomyocytes

The procedure of isolating ventricular cardiomyocytes from hearts of male Wistar rats (200 – 250 g) was conducted as previously described (20). The cells were plated in CCT medium (modified medium 199 containing Earle's salts, 2 mM L-carnitine, 5 mM taurine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10 μM cytosine-β-D-arabinofuranoside, pH 7.4) on culture dishes. Cells were allowed to attach for 3 h at 37 °C before washing with modified Tyrode's medium (140 mM NaCl, 3.6 mM KCl, 1.2 mM Mg_2SO_4, 1 mM $CaCl_2$ and 20 mM N-2-(hydroxyethyl)-piperazine-N'-2-ethanesulfonic acid, pH 7.4).

All incubations were performed at 37 °C. To simulate ischemic conditions, the washing medium was replaced

by 1 ml of modified anoxic Tyrode's medium (equilibrated with 100 % N_2, pH adjusted to 6.4) per 60 mm dish and incubated in an atmosphere of 100 % N_2 in gas tight chambers. After 3 h of anoxia, cells were reoxygenated for different time periods exchanging the anoxic with CCT medium equilibrated with 140 mmHg O_2 (pH 7.4).

A stock solution of the spontaneous NO donor SNAP (10 mM) was prepared in DMSO. 10 µl of this solution per ml medium was applied to each dish as indicated to give a final concentration of 100 µM SNAP. Control dishes were incubated with vehicle. After onset of reoxygenation, SNAP was added to the reoxygenation media immediately. Control experiments were performed adding SNAP to the normoxic control media without intervening anoxia. TUNEL assay or DNA extraction was performed after an 8 h incubation period with or without SNAP in CCT medium. Experimental protocols are illustrated in Fig. 1.

TUNEL assay

Isolated cardiomyocytes were fixed with acetone (–20 °C, 12 h) and 4 % paraformaldehyde (10 min at room temperature). From isolated hearts, cross-sectional midventricular slices were embedded in paraffin and cut into 4 µm-sections (Leica RM2145 microtome). Fixed cells or tissue sections were incubated at 37 °C for 1.5 h with 5 U terminal desoxynucleotidyl-transferase, 1.5 mM $CoCl_2$, 0.2 M potassium cacodylate, 25 mM Tris-HCl, 0.25 % BSA and 0.5 nM Biotin-16-dUTP (Boehringer Mannheim, Germany) after proteinase K treatment. In negative controls dUTP-biotin was omitted, positive controls were pre-treated with DNAse A. For TUNEL staining, biotin-streptavidin Texas Red (Amersham) was used on cells and 3,3'-diaminobenzidine on tissue sections. The latter were counterstained with eosin. For analysis of isolated cells, 300 randomly distributed cells for each experimental condition were counted. For analysis of the tissue sections, 12 photographs (magnification 40x) of each section were taken in a fixed blind pattern. The number of cardiomyocytes with clear nuclear staining, indicating apoptosis, was expressed in mean number per tissue section, respectively. Each tissue section represents 106 cells on average.

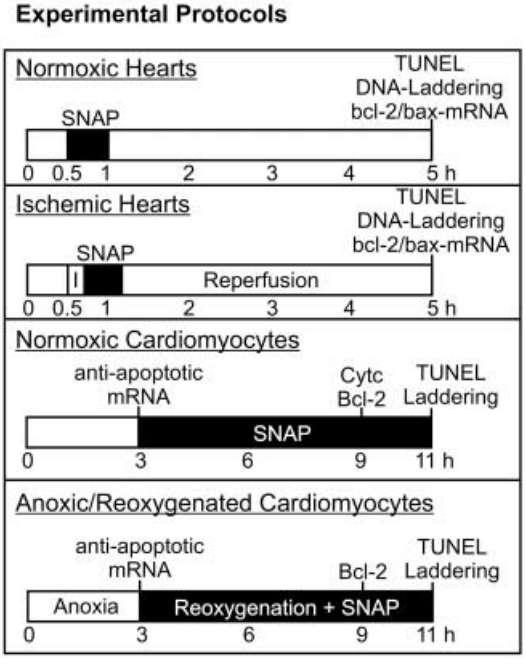

Fig. 1 Experimental protocols for studies on isolated hearts and myocytes. The figure illustrates times of normoxic, ischemic and reperfusion conditions. Also, time points at which mRNA-, protein- and apoptosis-detection were performed are indicated. **I** No-flow ischemia. Black bars: presence of SNAP. In controls SNAP was not added.

Analysis of genomic DNA

For DNA extraction cells were incubated in lysis buffer (100 mM NaCl, 10 mM Tris/Cl, 25 mM EDTA, 0.5 % SDS and 100 µg/ml proteinase K, pH 8.0) for 3 h at 37 °C. DNA was extracted with phenol/chloroform and precipitated in ethanol. RNA was removed by digestion with DNAse-free RNAse (5 µg/ml) for 2 h at 37 °C. 5 µg DNA of each sample was electrophoretically separated on agarose gels (1.5 %) and stained with ethidium bromide.

Determination of creatine phosphate contents

Adding 1 ml 1.2 M $HClO_4$ to the cultures terminated the experiments. Protein was determined in the acid precipitates according to Bradford (4) using bovine serum albumin as a standard. After neutralization, perchloric acid extracts were analyzed for creatine phosphate (CrP) (13).

Semi-quantitative RT-PCR analysis

Total RNA from cardiomyocytes and ventricular tissue was extracted with RNA-Clean (AGS, Heidelberg, Germany) as described by the manufacturer. Reverse transcriptase reactions were performed at 37 °C for 1 h in a final volume of 10 µl containing 1µg RNA, 100 ng oligo$(dT)_{15}$, 1 mM dNTPs, 8 U RNAse Inhibitor and 60 U M-MLV reverse transcriptase. Polymerase chain reaction (PCR) was performed with 1.5 µl cDNA, 1.5 µM of primer pairs, 0.4 mM dNTPs, 1.5 mM $MgCl_2$, and 1 U Taq-Polymerase in a final volume of 10 µl. Primer sequences and amplified fragment length are listed in

Table 1 Primers for RT-PCR

Gene	Primer sequence	Fragment length	Accession-No.
Mcl-1	5′-CTCGAGTGATGACCCATGTT-3′ 5′-TTTCTGATGCCGCCTTCTAG-3′	250 bp	AF115380
Bcl-2	5′-AAGCTGAGGCAGAAGGGTTA-3′ 5′-ACCATCCAATCCTGCACTTG-3′	320 bp	NM_021850
Bcl-xl	5′-ATGAACTCTTTCGGGATGGG-3′ 5′-GATCCACAAAAGTGTCCCAG-3′	190 bp	U34963
Bcl-w	5′-AAGCTGAGGCAGAAGGGTTA-3′ 5′-ACCATCCAATCCTGCACTTG-3′	320 bp	NM_021850
IAP-1	5′-TGGCCAAGCTGATGAAGAGT-3′ 5′-CAAAGCAGGCCACTCTATCA-3′	580 bp	AF183430
Survivin	5′-GGCAGATGTACCTTAAGGAC-3′ 5′-GGTCTCCTCGAACTCTTTCT-3′	350 bp	NM_022274
XIAP	5′-ACTGTTGTACCTGCAGACAC-3′ 5′-CCATTTTGGATGCCAGGAGA-3′	310 bp	AF183429
NAIP	5′-GCAGTGAAGCCAAACGACTA-3′ 5′-TCTTCACCCGTATGTCCTAC-3′	260 bp	AJ271303
Bax	5′-CCTGAGCTGACCTTGGAGCA-3′ 5′-CCTGGTCTTGGATCCAGACA-3ę	340 bp	S76511
Actin	5′-GAAGTGTGACGTTGACATCCG-3′ 5′-TGCTGATCCACATCTGCTGGA-3′	223 bp	J00691

Table 1. For each assayed gene the number of cycles resulting in a linear amplification range was tested. PCR products were separated by polyacrylamide gel electrophoresis (5%), stained with ethidium bromide and photographed and digitized with a DDC camera (INTAS). For quantification, the density of the DNA fragments was determined using Image Quant (Molecular Dynamics, Krefeld, Germany). mRNA expression was always related to the constitutively expressed β-actin mRNA.

Preparation of cytosolic and mitochondrial protein fractions

Cardiomyocytes were homogenized in extraction buffer (20 mM HEPES, pH 7.5, 10 mM KCl, 1,5 mM $MgCl_2$, 250 mM sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF). Lysates were centrifuged briefly at 10,000 × g to pellet nuclei. Supernatants were again centrifuged at 38,000 × g to pellet mitochondria. The resulting supernatants are referred to cytosolic fractions. Cytosolic or mitochondrial fractions were separated on 12.5% SDS gels and blotted on PVDF membranes. Blots were assayed with antibodies as indicated. Separation of mitochondrial from cytosolic protein fractions was controlled by use of cytochrome oxidase antibodies. Cytosolic extracts were virtually free of cytochrome oxidase.

Materials

Medium 199 was obtained from Boehringer (Mannheim, Germany); fetal calf serum from PAA (Linz, Austria), crude collagenase from Biochrom (Berlin, Germany) and annexin V-FITC from Bioproducts (Heidelberg, Germany). (±)-S-nitroso-N-acetylpenicillamine and polyclonal anti-cytochrome c antibody were obtained from Calbiochem (Bad Soden, Germany). All chemicals for the TUNEL assay were purchased at Boehringer (Mannheim, Germany). PCR reagents were from Gibco-BRL (Eggenstein, Germany). Polyclonal anti-rat-Bcl-2 antibody was from Pharmingen (Hamburg, Germany), monoclonal anti-cytochrome oxidase subunit IV antibody from Molecular Probes (Göttingen, Germany) and secondary antibodies were from Sigma.

Statistical analysis

Data were analyzed using Microcal Origin Version 3.5 (Microcal Software Inc., Northampton MA., USA). Statistical comparisons involving more than two groups were performed using the ANOVA test and Bonferroni method. Comparisons between two groups were accomplished by means of *t*-tests for independent samples. The critical value was set at $p = 0.05$ in these tests. All values are expressed as means ± SEM.

Results

Energy reserves, enzyme loss and contractile function in ischemic and reperfused hearts

Isolated hearts were perfused at constant flow under normoxic conditions or exposed to no-flow ischemia with subsequent reperfusion. Induction of apoptosis by NO was studied after perfusion of the hearts with the NO-donor (±)-S-nitroso-N-acetylpenicillamine (SNAP, 10 μM) for 30 minutes either under normoxic conditions or when given during the first 30 minutes of reperfusion (Fig. 1). Functional parameters and enzyme release were monitored throughout the perfusion time under these conditions.

During 20 min ischemia creatine phosphate contents dropped from 22.4 ± 3.0 nmol/mg protein to 7.7 ± 0.7 ($n = 3$, $p > 0.05$). This demonstrates that energy depletion of the heart already occurred during 20 min ischemia. ATP contents dropped from 12.6 ± 1.8 nmol/mg protein to 4.9 ± 0.3 within this time ($n = 3$, $p < 0.05$). After 1 h of reperfusion CrP values recovered to 19.5 ± 0.9 nmol/mg protein, while ATP contents remained low (6.2 ± 0.2 nmol/mg protein). Heart rate and perfusion pressure

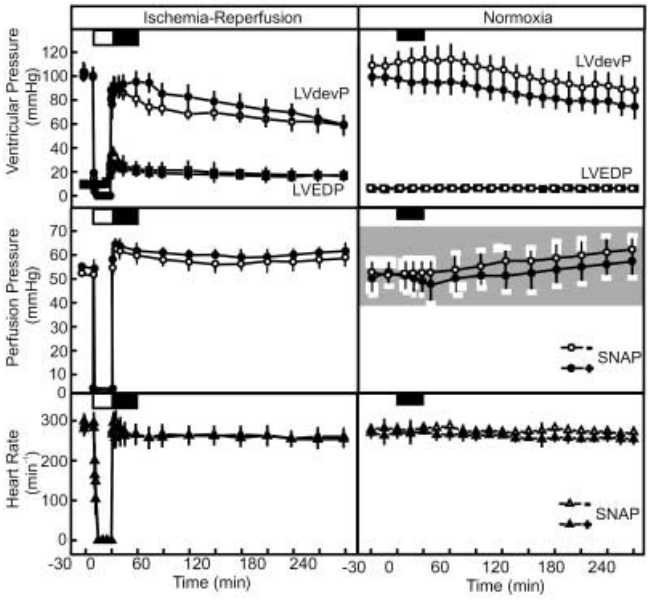

Fig. 2 Left ventricular end diastolic pressure (LVEDP), left ventricular developed pressure (LVdevP peak systolic pressure – LVEDP), perfusion pressure and heart rate in isolated rat hearts. All experiments were started with a 30-min equilibration period and continued as follows: normoxic perfusion at constant flow. The initial equilibration period was followed by 30 min exposure to SNAP (10 μM) and another 4 h perfusion, all with normoxic perfusate (closed symbols). Time matched controls were perfused under the same conditions with addition of vehicle only (open symbols). In the ischemic experiments the initial equilibration period was followed by 20 min no-flow ischemia and another 4.5 h of normoxic perfusion. During the first 30 min of reperfusion, SNAP (10 μM, closed symbols) or vehicle (open symbols) was added to the perfusate. Data are means ± SE of 3 independent experiments.

remained constant throughout the experiments except for the ischemic periods, yet it recovered quickly during reperfusion. Left ventricular developed pressure also recovered quickly upon reperfusion, although a slight decline appeared later during reperfusion. Perfusion with SNAP did not influence these functional parameters (Fig. 2).

LDH release, as an indicator of necrotic cell damage, occurred only in ischemic-reperfused hearts, amounting to 2.6 ± 0.7 % of total LDH activity released after 20 min ischemia and 4.5 h reperfusion and to 7.5 ± 0.8 % after 30 min ischemia and 4.4 h reperfusion. This indicates that significant necrotic cell death occurs only when ischemia is extended beyond 20 min ischemia. Perfusion with SNAP, either under normoxic conditions or after 20 min ischemia, did not alter the LDH release (none or 2.6 ± 0.6 % of total).

Inhibition of SNAP-induced apoptosis in isolated hearts

Perfusion of normoxic hearts with SNAP-induced apoptosis: DNA extracted from hearts 4 h after SNAP perfusion showed the typical DNA-laddering pattern in agarose gels (Fig. 3A, lane 2), which is not found in DNA from control hearts submitted to normoxic perfusion for 5 hours (Fig. 3A, lane 1). To confirm these results we additionally performed the TUNEL assay. In sections from control hearts the number of TUNEL-positive cardiomyocyte nuclei, indicative of apoptosis, was 1.8 ± 0.5 per field (Fig. 3B). Treatment of the hearts with SNAP (10 μM, applied for 30 min and followed by 4 h of perfusion) increased the number of TUNEL-positive cardiomyocyte nuclei significantly to 5.5 ± 1.4 per field (Fig. 3B).

Although longer times of ischemia (30 minutes) were sufficient to induce apoptosis in isolated perfused hearts (6.0 ± 0.2 TUNEL-positive cardiomyocyte nuclei per field, $p < 0.05$ vs. control), 20 min of ischemia followed by 4.5 h reperfusion did not induce apoptosis: DNA laddering was not found (Fig. 3A) and the number of TUNEL-positive cardiomyocyte nuclei was only slightly but not significantly elevated vs. normoxic controls (3.4 ± 1.06 per field, Fig. 3B). Perfusion of the ischemic hearts during the first 30 minutes of reperfusion with 10 μM SNAP, which was found to induce apoptosis under nor-

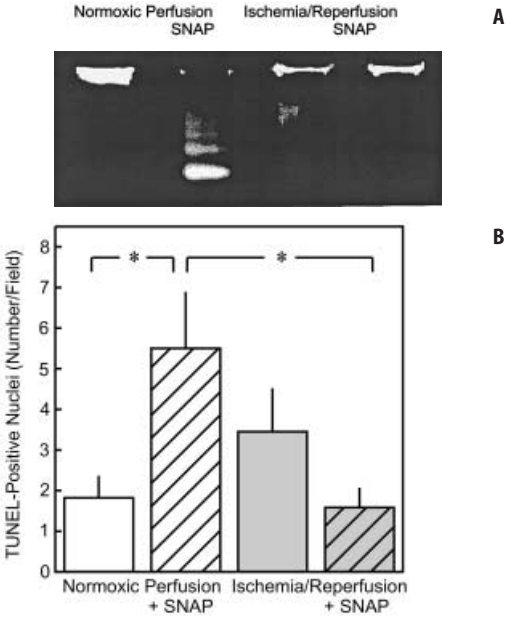

Fig. 3 Protection of ischemic hearts against NO-induced apoptosis. All experiments were started with a 30-min equilibration period and continued as follows: hearts were either perfused under normoxic conditions at constant flow for 4.5 h or 20 min. No-flow ischemia was performed followed by 4.5 h reperfusion. In normoxic perfused hearts after the equilibration period, SNAP (10 μM) was added to the perfusate for 30 min. In ischemic hearts, perfusion in the presence of SNAP started at the beginning of reperfusion. **A** DNA was extracted 4 h after exposure to SNAP or vehicle. 5 μg per lane were separated on 1.5 % agarose gels. DNA was stained by ethidium bromide. **B** Quantification of apoptotic cells, given in number of TUNEL-positive nuclei per visual field, 4 h after exposure to SNAP. Data are means ± SE of 6 independent experiments. * Differences with $p < 0.05$.

moxic conditions, was not able to induce apoptosis in the ischemic/reperfused hearts (1.6 ± 0.5 TUNEL-positive cardiomyocyte nuclei per field, Fig. 3B). This indicates induction of an anti-apoptotic effect by ischemic conditions in the heart.

Inhibition of SNAP-induced apoptosis in isolated cardiomyocytes

To analyze the ischemia-induced protection in further detail, cardiomyocytes were subjected to anoxia at a pH of 6.4 (simulated ischemia) for 3 h, followed by up to 8 h of reoxygenation (pH 7.4). Similar to the situation in isolated hearts, but with a prolonged time course, energy reserves of the cells were almost depleted within 2 h by these ischemic conditions (5.4 ± 3.3 nmol CrP/mg protein vs. 22.1 ± 2.6 nmol CrP/mg protein in normoxic controls, and 5.0 ± 0.7 nmol ATP/mg protein vs. 24.5 ± 1.0 nmol ATP/mg protein under normoxia). Also similar to reperfused hearts, upon reoxygenation a rapid restoration of creatine phosphate reserves, reaching 14.2 ± 2.9 nmol CrP/mg protein after 30 minutes and 19.6 ± 1.3, 21.2 ± 3.2 and 22.3 ± 2.9 nmol CrP/mg protein after 60, 90 and 120 minutes reoxygenation was noted, whereas only a small amount of ATP recovered (7.2 ± 0.6 nmol ATP/mg protein after 1 h of reoxygenation). Anoxia/reoxygenation did not induce apoptosis in the isolated cardiomyocytes (Fig. 4).

Addition of SNAP (100 μM) to normoxic cardiomyocytes resulted in an increased number of TUNEL-positive, apoptotic cells (12.6 ± 4.9 % TUNEL-positive cells vs. 5.4 ± 0.7 % in controls, n = 3, $p < 0.05$) (Fig. 4B). As additional sign of apoptosis, DNA laddering could be detected after SNAP treatment (Fig. 4A). However, when cells were exposed to 3 h of ischemia, the ability of SNAP to induce apoptosis thereafter had vanished (3.9 ± 1.2 % TUNEL-positive cells, Fig. 4B). Furthermore, DNA laddering was no longer found when SNAP was added after 3 h of anoxia (Fig. 4A).

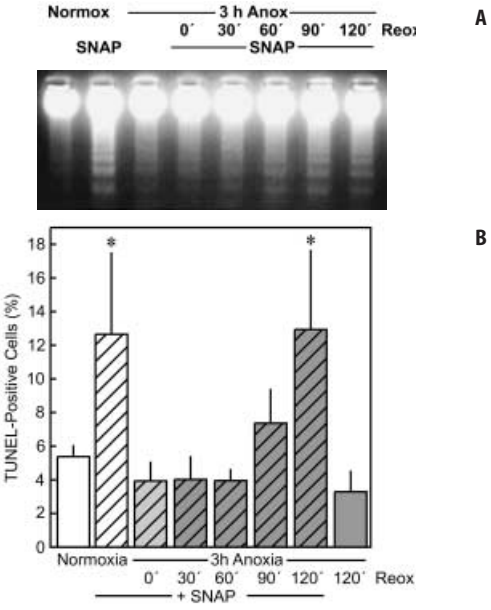

Fig. 4 Protection of anoxic cardiomyocytes against NO-induced apoptosis. In normoxic incubation, exposure to SNAP (100 μM) was started after 3 h normoxic incubation without it (lanes 1–2). Ischemia was simulated by anoxia at a pH of 6.4 for 3 h. The cells were then reoxygenated in the presence or absence of SNAP. **A** DNA was extracted 8 h after addition of SNAP or vehicle. 5 μg per lane were separated on 1.5 % agarose gels. DNA was stained by ethidium bromide. **B** Quantification of apoptotic cells was done by the TUNEL method 8 h after addition of SNAP or vehicle. Experiments with SNAP are signified by hatched columns, time-matched control experiments with vehicle addition by open columns. Data are means ± SE of 3 independent preparations. * Differences from non-treated normoxic cells with $p < 0.05$.

Expression of pro- and anti-apoptotic marker genes

The ischemia-induced protection raised the question, whether responses in apoptotic gene expression may occur under these conditions and whether such responses in intact hearts rely on the properties of the cardiomyocytes themselves. Therefore, we analyzed the expression of the prominent anti-apoptotic gene bcl-2 and of the pro-apoptotic gene bax in intact hearts and isolated cardiomyocytes. The balance between these proteins is known to be critical for pro- or anti-apoptotic reactions.

In normoxic hearts treated with SNAP, bcl-2 mRNA expression decreased by 31.3 ± 0.9 %, whereas bax mRNA increased by 71.2 ± 2.3 % compared to controls (Fig. 5), which reveals a pro-apoptotic situation on the expression level. After 20 min ischemia and 4 h reperfusion, the anti-apoptotic state of the hearts is indicated by the increase in bcl-2 mRNA by 92.3 ± 27.7 %, while bax mRNA remained unchanged. When SNAP was added during reperfusion, bcl-2 and bax mRNA levels did not change (Fig. 5).

Similar changes in the expression of bcl-2- and bax-mRNA are found in isolated cardiomyocytes. In the presence of SNAP under normoxic conditions, bcl-2 mRNA declined by 24.6 ± 3.3 %, while simultaneously bax mRNA was found increased by 66.7 ± 8.8 % (Fig. 5). When cells had been submitted to 3 h of simulated ischemia and 2 h reoxygenation, bcl-2 mRNA increased by 69.4 ± 13.2 %, whereas bax mRNA expression did not change compared to normoxic controls. This indicates an augmented anti-apoptotic defense. Addition of SNAP after simulated ischemia did not influence these bcl-2 and bax mRNA levels (Fig. 5).

Because Bcl-2 exerts its anti-apoptotic function only when located at mitochondria (11), we investigated its location under the different conditions. Interestingly, after addition of SNAP to normoxic cardiomyocytes the

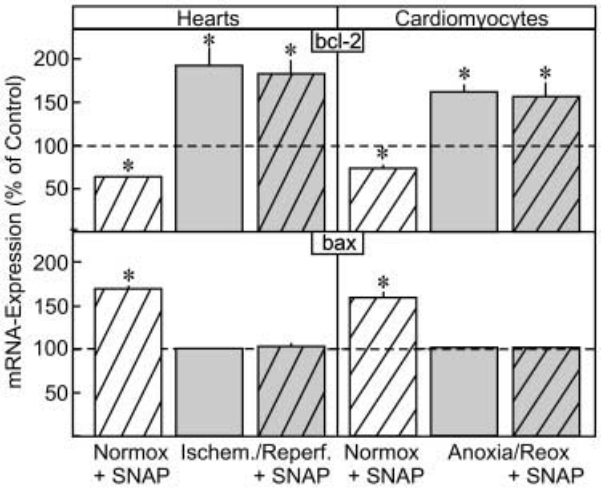

Fig. 5 Bcl-2- and bax-mRNA expression in isolated hearts and cardiomyocytes. Hearts were perfused for 30 minutes with 10 μM SNAP or vehicle either under normoxic conditions or immediately upon reperfusion after 20 minutes of ischemia. Cardiomyocytes were exposed for 2 h to 100 μM SNAP or vehicle either under normoxic conditions or immediately upon reoxygenation after 3 h of anoxia. In hearts 4 h after exposure to SNAP or vehicle and in cardiomyocytes 2 h after SNAP addition, mRNA expression was determined by RT-PCR. bcl-2- and bax-mRNA expression were always related to the constitutively expressed β-actin mRNA. Data are given as percent of normoxic controls (vehicle only) and are means of 4 independent experiments. * Difference from non-treated normoxic controls with $p < 0.05$.

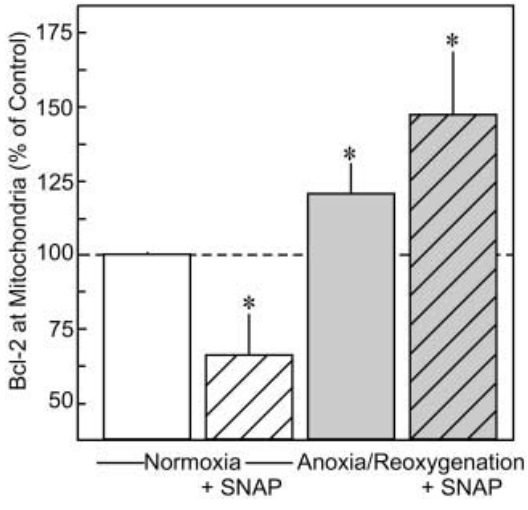

Fig. 6 Bcl-2 associated with mitochondria of isolated cardiomyocytes. Cardiomyocytes were exposed for 6 h to 100 μM SNAP or vehicle either under normoxic conditions or immediately upon reoxygenation after 3 h of anoxia. Immunodetection of Bcl-2 was performed on mitochondrial protein fractions of the cells. Experiments with SNAP are signified by hatched columns, time-matched control experiments with vehicle addition by open columns. Data are given as percent of normoxic controls (vehicle only) and are means ± SE of 8 independent experiments. * Difference from non-treated normoxic controls with $p < 0.05$.

amount of Bcl-2 protein associated with mitochondria decreased to 67.2 ± 13.9% within 6 h ($n = 8$, $p < 0.05$ vs. normoxic controls, Fig. 6). At the same time point (6 h after addition of SNAP) cytochrome c release from mitochondria into the cytosol increased by 103 ± 38.5% ($n = 5$, $p < 0.05$ vs. normoxic controls). This indicates a pro-apoptotic response. After 3 h of anoxia and reoxygenation, the amount of Bcl-2 associated with mitochondria increased to 122.1 ± 6.7% ($n = 8$, $p < 0.05$, Fig. 6). This is a sign of an anti-apoptotic state of the cells. Incubation of cardiomyocytes with SNAP during reoxygenation did not reduce the amount of Bcl-2 located at mitochondria (Fig. 6).

To test whether, besides the fine-tuning in bcl-2 and bax expression, a general anti-apoptotic program is induced in myocytes by ischemic conditions, we analyzed the mRNA-expression of auxiliary, anti-apoptotic genes of the bcl-2 and IAP families. Of the analyzed Bcl-2-family members (mcl-1, bcl-2, bcl-xl and bcl-w) all were found to be expressed in normoxic cardiomyocytes. The expression of all but bcl-w were increased after 3 h anoxia (Fig. 7). This increased mRNA expression is also reflected in the increased amount of Bcl-2 located at mitochondria (Fig. 6) and in an increase of Bcl-xl protein by 40 ± 16 % in anoxic/reoxygenated cardiomyocytes ($n=3$, $p<0.05$ vs. normoxic controls). In contrast to this, the amount of Mcl1-protein did not change after anoxic conditions. Of the investigated IAP-family members (IAP-1, survivin, XIAP and NAIP) only IAP-1- and survivin-mRNAs were detected in normoxic cardiomyocytes. After 3 h of anoxia, again only these two mRNAs were detected and IAP-1-mRNA was significantly increased (Fig. 7).

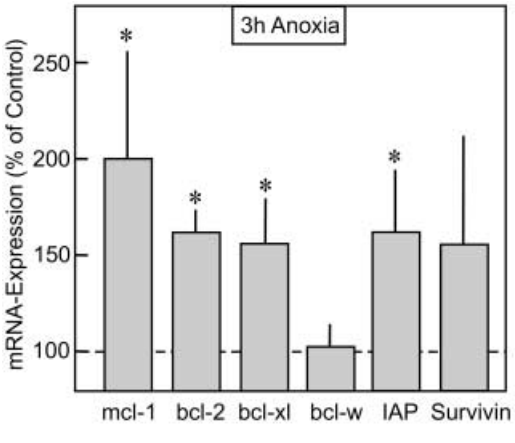

Fig. 7 Induction of anti-apoptotic gene programs in anoxic cardiomyocytes. Cardiomyocytes were exposed for 3 h of anoxia. RNA was extracted and RT-PCR was performed. mRNA expression of the analyzed genes were always related to the constitutively expressed β-actin mRNA. Data are given as percent of normoxic controls (vehicle only) and are means ± SE of 4 independent experiments. * Difference from non-treated normoxic controls (----) with $p < 0.05$.

Discussion

The main result of this study is that non-lethal periods of no-flow ischemia can provide endogenous protection of the heart against an exogenous inducer of apoptosis (NO). Since this protection is also observed in isolated cardiomyocytes, it is due to a mechanism inherent to this cell type. A whole host of anti-apoptotic genes is found upregulated in ischemic/reperfused myocardium and cells. Which of these are responsible for the observed protection against NO-induced apoptosis is still an open question.

Findings that demonstrate the induction of anti-apoptotic genes in ischemic/reperfused hearts in vivo (6, 18) encouraged us to investigate, if ischemia can protect hearts against apoptotic stimuli. To analyze this under defined conditions, we performed studies on isolated Langendorff-perfused rat hearts. In order to achieve non-lethal ischemic conditions a time period of 20 minutes no-flow ischemia was chosen for the analysis. Under these conditions necrotic cell damage was minimal, as shown by the complete functional recovery and the small release of enzymes upon reperfusion. To induce apoptosis, hearts were perfused with the NO-donor SNAP (10 μM). Perfusion with NO was chosen, because it has been shown to induce apoptosis in isolated cardiomyocytes (2, 10, 23) and it is discussed to be involved in apoptosis induction in ischemic/reperfused hearts (22, 26). We found that NO induces apoptosis in normoxic hearts. But after non-lethal ischemia hearts were protected against NO-induced apoptosis.

The relevance of these findings is stressed by the point that the concentration of the NO donor, used in the cell culture experiments (100 μM SNAP), should reflect in vivo concentrations of NO in the reperfused myocardium. This is because concentrations of nitrite/nitrate found in coronary arteries after myocardial infarction (60 μM) (27) are the same as produced by decomposition of 100 μM SNAP in buffer (50 – 70 μM) (13). In isolated hearts concentrations of SNAP (10 μM), which were sufficient for apoptosis induction, were determined and used during the 30 minutes perfusion time. In both situations, in isolated cardiomyocytes and hearts, ischemia-induced protection against NO-induced apoptosis was observed.

Three possible causes for the ischemia-induced protection may be considered:

- Since apoptosis is an active, energy-demanding process (9, 25) it might be assumed that energy depletion in ischemic cardiomyocytes is responsible for the anti-apoptotic, protective effect. But reoxygenated isolated cardiomyocytes are protected up to 1 h after anoxia. Within this time ATP levels remained low but creatine phosphate contents had already recovered. This recovery is dependent on the free energy change of ATP hydrolysis, as shown previously (21). This demonstrates that, although energy metabolism has already recovered, NO can not induce apoptosis. Therefore, other mechanisms seem to be involved in the ischemia-induced anti-apoptotic effects.
- Ischemic conditions may result in a very specific interruption of the pathway of NO-induced apoptosis, like inactivation or desensitization of soluble guanylate cyclase. But as shown previously ischemic cardiomyocytes are also protected against other apoptotic stimuli, like oxygen radicals (11). This indicates the induction of general anti-apoptotic mechanisms.
- The development of the ischemia-induced protection is accompanied by characteristic changes in the regulation of apoptosis-related marker genes in the hearts and in cardiomyocytes. Anti-apoptotic genes of the Bcl-2- and IAP-family are increased after ischemia. Anti-apoptotic family members of Bcl-2 all share sequence homologies within four regions (BH regions) and act at distinct points in the apoptotic pathway: They inhibit function of pro-apoptotic family members by heterodimerization (29) and prevent the release of apoptogenic factors from mitochondria (24). Thus the action of the Bcl-2 members converge on the mitochondrial death pathway and act prior to caspase activation (30). Therefore, the upregulation of a set of these genes under ischemic conditions and the increased mitochondrial localization of Bcl-2 under these conditions indicates the enlargement of the anti-apoptotic defense at mitochondria of the cardiomyocytes. The induction of the caspase inhibitor IAP1 may then serve as an additional safeguard for the cells or it may exert its anti-apoptotic effects in receptor-mediated apoptosis which does not necessarily involve mitochondrial pathways. A similar induction of anti-apoptotic genes has been shown recently by Depre and co-workers in an ischemic swine model (6). But evidence for anti-apoptotic actions in these animals has been missing. In future studies, involvement of these genes in anti-apoptotic responses of ischemic cardiomyocytes has to be investigated and inducers of these anti-apoptotic genes have to be defined in order to enlarge the range of protection, also under normoxic conditions.

Although induction of anti-apoptotic genes under ischemic conditions has been shown in previous studies, this study reveals for the first time an ischemia-induced protection against apoptosis in the intact heart. This endogenous mode of ischemia-induced protection is similar to preconditioning. But there are also apparent differences between these two modes of protection. According to its original definition, in ischemic preconditioning a brief ischemic episode provides protection against spontaneously developing cellular damage during subsequent prolonged ischemia (17). This protection may result in part from an attenuation of apoptosis after prolonged ischemia/reperfusion (19). In the present study we show that prolonged, non-lethal ischemic con-

ditions provide protection against exogenous noxious stimuli during reperfusion.

In conclusion, the present study has characterized a novel mode of ischemia-induced cardioprotection, which is directed against NO-induced apoptosis in the reperfused myocardium and which is a property of the cardiomyocytes themselves. Protection against apoptosis is accompanied by an anti-apoptotic balance in the expression and location of apoptosis-related proteins. Elucidation of inducers of the anti-apoptotic gene program and of the causal role of these genes in this novel mode of endogenous protection may lead to new approaches for salvage of ischemic-reperfused myocardium.

Acknowledgments The authors wish to thank Daniela Schreiber and Yolanda Puigfel for their excellent technical assistance. This work was partially supported by BIOMED Concerted Action BMH1-PL95/1254 from the European Union.

References

1. Anversa R, Cheng W, Liu Y, Leri A, Redaelli G, Kajstura J (1998) Apoptosis and myocardial infarction. Basic Res Cardiol 93 (Suppl 3): 8–12
2. Arstall MA, Sawyer DB, Fukazawa R, Kelly RA (1999) Cytokine-mediated apoptosis in cardiac myocytes: the role of inducible nitric oxide synthase induction and peroxynitrite generation. Circ Res 85 (9): 829–840
3. Bergmeyer HU, Bernt E (1974) Lactatdehydrogenase. In: Bergmeyer HU (ed) Methoden der enzymatischen Analyse. Weinheim, Verlag Chemie, pp 607–612
4. Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilising the principle of protein-dye-binding. Anal Biochem 72: 248–254
5. Chang J, Wasser JS, Cornelussen RN, Knowlton AA (2001) Activation of heat-shock factor by stretch-activated channels in rat hearts. Circulation 104 (2): 209–214
6. Depre C, Tomlinson JE, Kudej RK, Gaussin V, Thompson E, Kim SJ, Vatner DE, Topper JN, Vatner SF (2001) Gene program for cardiac cell survival induced by transient ischemia in conscious pigs. Proc Natl Acad Sci USA 98 (16): 9336–9341
7. Desagher S, Martinou JC (2000) Mitochondria as the central control point of apoptosis. Trends Cell Biol 10 (9): 369–377
8. Deveraux QL, Roy N, Stennicke HR, Van Arsdale T, Zhou Q, Srinivasula SM, Alnemri ES, Salvesen GS, Reed JC (1998) IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases. EMBO J 17 (8): 2215–2223
9. Fliss H (1998) Accelerated apoptosis in reperfused myocardium: friend of foe? Basic Res Cardiol 93: 90–93
10. Ing DJ, Zang J, Dzau VJ, Webster KA, Bishopric NH (1999) Modulation of cytokine-induced cardiac myocyte apoptosis by nitric oxide, Bak, and Bcl-x. Circ Res 84 (1): 21–33
11. Inserte J, Taimor G, Hofstaetter B, Garcia-Dorado D, Piper HM (2000) Influence of simulated ischemia on apoptosis induction by oxidative stress in adult cardiomyocytes of rats. Am J Physiol Heart Circ Physiol 278 (1): H94–H99
12. Jia L, Macey MG, Yin Y, Newland AC, Kelsey SM (1999) Subcellular distribution and redistribution of Bcl-2 family proteins in human leukemia cells undergoing apoptosis. Blood 93 (7): 2353–2359
13. Kankaanranta H, Rydell E, Petersson AS, Holm P, Moilanen E, Corell T, Karup G, Vuorinen P, Pedersen SB, Wennmalm A, Metsa-Ketela T (1996) Nitric oxide-donating properties of mesoionic 3-aryl substituted oxatriazole-5-imine derivatives. Br J Pharmacol 117 (3): 401–406
14. Lamprecht JW, Stein P, Heinz F, Weisser H (1974) Creatinphosphat. In: Bergmeyer HU (ed) Methoden der enzymatischen Analyse. Weinheim, Verlag Chemie, pp 1825–1829
15. Matsushita K, Umezawa A, Iwanaga S, Oda T, Okita H, Kimura K, Shimada M, Tanaka M, Sano M, Ogawa S, Hata J (1999) The EAT/mcl-1 gene, an inhibitor of apoptosis, is up-regulated in the early stage of acute myocardial infarction. Biochim Biophys Acta 1472 (3): 471–478
16. Misao J, Hayakawa Y, Ohno M, Kato S, Fujiwara T, Fujiwara H (1996) Expression of bcl-2 protein, an inhibitor of aoptosis, and bax, an accelerator of apoptosis, in ventricular myocytes of human hearts with myocardial infarction. Circulation 94: 1506–1512
17. Murry CE, Jennings RB, Reimer KA (1996) Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. Circulation 74: 1124–1136
18. Olivetti G, Abbi R, Quaini F, Kajstura J, Cheng W, Nitahara JA, Quaini E, Di Loreto C, Beltrami CA, Krajewski S, Reed JC, Anversa P (1997) Apoptosis in the failing human heart. N Engl J Med 336 (16): 1131–1141
19. Piot CA, Padmanaban D, Ursell PC, Sievers RE, Wolfe CL (1997) Ischemic preconditioning decreases apoptosis in rat hearts in vivo. Circulation 96: 1598–1604
20. Piper HM, Probst I, Hütter JF, Spiekermann PG (1982) Culturing of calcium stable adult cardiac myocytes. J Mol Cell Cardiol 14: 397–412
21. Piper HM, Noll T, Siegmund B (1994) Mitochondrial function in the oxygen depleted and reoxygenated myocardial cell. Cardiovasc Res 28 (1): 1–15
22. Suzuki H, Wildhirt SM, Dudek RR, NarayanKS, Bailey AH, Bing RJ (1996) Induction of apoptosis in myocardial infarction and its possible relationship to nitric oxide synthase in macrophages. Tissue & Cell 28: 89–97
23. Taimor G, Hofstaetter B, Piper HM (2000) Apoptosis induction by nitric oxide in adult cardiomyocytes via cGMP-signaling and its impairment after simulated ischemia. Cardiovasc Res 45: 588–594
24. Tsujimoto Y (1998) Role of Bcl-2 family proteins in apoptosis: apoptosomes or mitochondria? Genes Cells 3 (11): 697–707
25. Tsujimoto Y (1997) Apoptosis and necrosis: intracellular ATP level as a determinant for cell death modes. Cell Death and Differ 4: 429–434
26. Wildhirt SM, Dudek RR, Suzuki H, Pinto V, Narayan KS, Bing RJ (1995) Immunohistochemistry in the identification of nitric oxide synthase isoenzymes in myocardial infarction. Cardiovasc Res 29: 526–531

▶

27. Wildhirt SM, Suzuki H, Horstman D, Weismuller S, Dudek RR, Akiyama K, Reichart B (1997) Selective modulation of inducible nitric oxide synthase isozyme in myocardial infarction. Circulation 96 (5): 1616–1623
28. Yamamura K, Tani M, Hasegawa H, Gen W (2001) Very low dose of the Na(+)/Ca(2+) exchange inhibitor, KB-R7943, protects ischemic reperfused aged Fischer 344 rat hearts: considerable strain difference in the sensitivity to KB-R7943. Cardiovasc Res 52 (3): 397–406
29. Yang E, Zha J, Jockel J, Boise LH, Thompson CB, Korsmeyer SJ (1995) Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death. Cell 80 (2): 285–391
30. Yasuhara N, Sahara S, Kamada S, Eguchi Y, Tsujimoto Y (1997) Evidence against a functional site for Bcl-2 downstream of caspase cascade in preventing apoptosis. Oncogene 15 (16): 1921–1928

2. Mecanismos de muerte celular durante la isquemia y reperfusión miocárdica:

L-arginine limits myocardial cell death secondary to hypoxia-reoxygenation by a cGMP-dependent mechanism. L Agulló, D Garcia-Dorado, J Inserte, A Paniagua, P Pyrhonen, J Llevadot, J Soler-Soler. *Am J Physiol*. 1999;276:H1574-1580.

Urodilatin limits acute reperfusion injury in the isolated rat heart. J Inserte, D Garcia-Dorado, L Agulló, A Paniagua, J Soler-Soler. *Cardiovasc Res*. 2000;45:251-259.

***Role of the reverse mode of the Na^+/Ca^{2+} exchanger in reoxygenation-induced cardiomyocyte injury*.** C Schäfer, Y Ladilov, J Inserte, M Schäfer, S Haffner, D Garcia-Dorado, HM Piper. Cardiovasc Res. 2001;51:241-250.

Effect of inhibition of Na^+/Ca^{2+} exchanger at the time of myocardial reperfusion on hypercontracture and cell death. J Inserte, D Garcia-Dorado, M Ruiz-Meana, F Padilla, JA Barrabés, P Pina, L Agulló, HM Piper, J Soler-Soler. Cardiovasc Res. 2002;55:739-748.

L-Arginine limits myocardial cell death secondary to hypoxia-reoxygenation by a cGMP-dependent mechanism

LUIS AGULLÓ, DAVID GARCÍA-DORADO, JAVIER INSERTE, AMAYA PANIAGUA, PASI PYRHONEN, JOAN LLEVADOT, AND JORDI SOLER-SOLER
Servicio de Cardiología, Hospital General Universitario Vall d'Hebron, 08035 Barcelona, Spain

Agulló, Luis, David García-Dorado, Javier Inserte, Amaya Paniagua, Pasi Pyrhonen, Joan Llevadot, and Jordi Soler-Soler. L-Arginine limits myocardial cell death secondary to hypoxia-reoxygenation by a cGMP-dependent mechanism. *Am. J. Physiol.* 276 (*Heart Circ. Physiol.* 45): H1574–H1580, 1999.—The objective of this study was to investigate the effect of L-arginine supplementation on myocardial cell death secondary to hypoxia-reoxygenation. Isolated rat hearts ($n = 51$) subjected to 40 min of hypoxia and 90 min of reoxygenation received 3 mM L-arginine and/or 1 μM 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ; a selective inhibitor of soluble guanylyl cyclase) throughout the experiment or during the equilibration, hypoxia, or reoxygenation periods. The incorporation of L-[^{3}H]arginine into myocytes during energy deprivation was investigated in isolated adult rat myocytes. The addition of L-arginine to the perfusate throughout the experiment resulted in higher cGMP release ($P < 0.05$), reduced lactate dehydrogenase release ($P < 0.05$), and increased pressure-rate product ($P < 0.05$) during reoxygenation. These effects were reproduced when L-arginine was added only during equilibration, but addition of L-arginine during hypoxia or reoxygenation had no effect. Addition of ODQ either throughout the experiment or only during reoxygenation reversed the beneficial effects of L-arginine. L-[^{3}H]arginine was not significantly incorporated into isolated myocytes subjected to energy deprivation. We conclude that L-arginine supplementation protects the myocardium against reoxygenation injury by cGMP-mediated actions. To be effective during reoxygenation, L-arginine must be added before anoxia.

isolated rat heart; reperfusion; contractile function; nitric oxide; guanylyl cyclase inhibition; myocytes; uptake

NITRIC OXIDE (NO) is a free radical endogenously produced by a variety of mammalian cells and shown to be a ubiquitous signal transduction molecule. NO plays an important role in blood pressure regulation, vascular tone, neural signaling, and immunological function. It is synthesized by constitutive (I and III) and inducible (II) NO synthases (NOS), enzymes that use L-arginine and molecular oxygen as substrates.

It has been suggested that alterations in NO homeostasis may play a role in the genesis of myocardial injury secondary to ischemia-reperfusion. NO is a highly reactive molecule and may result in the formation of harmful free radicals when it reacts with O_2^- (14, 26). In some studies, myocardial NO synthesis has been found to be enhanced during ischemia-reperfusion (40, 6), and NOS inhibitors have been found to decrease functional impairment of tissue after ischemia-reperfusion (7, 23, 37, 38).

However, other studies suggest a decrease in endothelium-derived relaxing factor-mediated effects in reperfused myocardium (9, 33) and a protective effect of NO (2, 3, 8, 9, 20, 24, 36). Administration of NO donors or of the NOS substrate L-arginine has been shown in most (although not all) studies to improve functional recovery of reperfused myocardium (2, 3, 8, 9, 33). This beneficial effect has been explained as a consequence of the inhibiting actions of NO on vascular constriction and neutrophil and platelet adherence (2, 36) and to attenuation of the deleterious free radical actions of O_2^- (20). On the other hand, NOS itself can be an important site of O_2^- synthesis when substrate availability is limited (13, 14). A decrease in NO concentration in the capillary blood has been recently documented in rabbit skeletal muscle at the end of ischemia and during reperfusion, concomitantly with a symmetrical increase in O_2^- concentration (16). Both changes were attenuated by administration of L-arginine, suggesting that insufficient substrate availability during ischemia-reperfusion may result in a switch of constitutive NOS enzymes to synthesize O_2^- (16).

In addition to reducing the concentration of O_2^- and increasing the quench of oxygen-derived free radicals by NO, L-arginine supplementation may increase cGMP synthesis. In contrast to the large number of studies analyzing the effects of NO on ischemia-reperfusion injury, data on the role of cGMP in the genesis of these effects are scant, probably because selective inhibitors of NO-dependent cGMP synthesis have not been available until very recently. The potentially favorable cGMP-mediated actions of NO on vascular tone and neutrophil and platelet adhesion are well recognized (4, 21). However, the effects of increased cGMP on cardiomyocytes have received much less attention. cGMP has been found to reduce myofilament responsiveness to Ca^{2+} via activation of a cGMP-dependent protein kinase (25, 29). During reoxygenation, restoration of cell energy in the presence of elevated cytosolic Ca^{2+} concentration may result in hypercontracture and cell death, and interventions inducing a transient reduction of myofilament responsiveness to Ca^{2+} during the initial phase of reoxygenation or reperfusion may prevent hypercontracture and limit myocardial cell death (10, 31). Increasing cytosolic cGMP concentration by NO-independent stimulation of particulate guanylyl cyclase was shown recently to prevent reoxygenation-induced hypercontracture (15).

The objective of this study was to test the hypothesis that L-arginine supplementation limits cell death caused

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

by hypoxia-reoxygenation through cGMP-mediated actions of NO. For this purpose, a novel and specific inhibitor of the soluble guanylyl cyclase was used.

MATERIALS AND METHODS

Isolated, Perfused Heart

The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health, and experimental procedures were approved by the Research Commission on Ethics of the Hospital Vall d'Hebron, Barcelona, Spain.

Adult male Sprague-Dawley rats, weighing 300–350 g, were anesthetized with an intraperitoneal injection of thiopental sodium (200 mg/kg). The hearts were excised and immediately arrested in ice-cold saline solution. The aorta was quickly cannulated, and the heart was perfused with a Krebs-Henseleit bicarbonate buffer (KHB) at 37°C using a nonrecirculating Langendorff apparatus at a constant perfusion pressure of 60 mmHg. The composition of KHB was as follows (in mM): 140 NaCl, 24 $NaHCO_3$, 2.7 KCl, 0.4 KH_2PO_4, 1 $MgSO_4$, 1.8 $CaCl_2$, and 5 glucose. KHB was filtered through a 0.45-μm cellulose filter to remove any particulate matter and continuously gassed with 95% O_2-5% CO_2.

Left ventricular pressure was monitored by means of a water-filled latex balloon inserted through the left atrium and into the left ventricle. The balloon was fixed in the tip of a Cordis 5-F catheter (Cordis, Miami, FL) and connected to a pressure transducer (43600 F, Baxter). At the beginning of the experiment the left ventricular end-diastolic pressure (LVEDP) was set between 8 and 12 mmHg by adjusting the filling of the balloon. The signal obtained was digitized and recorded continuously on hard disk with the aid of ad hoc developed software. Left ventricular developed pressure (LVDP) was calculated as the difference between left ventricular peak systolic pressure and LVEDP.

Experimental Protocol and Groups of Treatment

Five hearts were perfused for 160 min under normoxic conditions (normoxic controls). Hearts to be submitted to hypoxia-reoxygenation ($n = 51$) were previously stabilized for 30 min, followed by 40 min of hypoxic perfusion and 90 min of reoxygenation. Hypoxic perfusion was performed with the same buffer without glucose and gassed with 95% Ar-5% CO_2. In a first series of experiments hearts were allocated to one of four groups receiving the following treatments: none (control, $n = 12$); 3 mM L-arginine (Sigma Chemical, St. Louis, MO) ($n = 12$); 3 mM L-arginine plus 1 μM 1*H*-[1,2,4]oxadiazolo-[4,3,-*a*]quinoxalin-1-one (ODQ; Alexis, San Diego, CA), a selective inhibitor of soluble guanylyl cyclase (12) ($n = 5$); and 1 μM ODQ ($n = 5$). In all groups, drugs were added to the KHB 10 min before hypoxia and maintained throughout the rest of the experiment. Before dilution in buffer L-arginine was neutralized with hydrochloric acid and ODQ was dissolved in dimethyl sulfoxide. Final concentrations of dimethyl sulfoxide in the KHB (0.025%) had no effect on any of the parameters measured. To determine at what moment of the hypoxia-reoxygenation protocol L-arginine exerts its protective actions, a second series of experiments were performed in which hearts received L-arginine only during the equilibration period ($n = 7$), the hypoxic period ($n = 4$), or the reoxygenation period ($n = 2$). Finally, another group of hearts received 3 mM L-arginine throughout the experiment plus ODQ during the last 5 min of hypoxia and all through the reoxygenation period ($n = 4$).

Lactate Dehydrogenase and cGMP Release

The coronary effluent was collected at different times to measure lactate dehydrogenase (LDH) activity and cGMP. LDH was determined in samples collected during the reoxygenation at 1, 2, 3, 4, 6, 8, 10, 12, 15, and 20 min and then every 10 min and spectrophotometrically assayed as described previously (17). Results of LDH release were expressed as units of LDH activity released during the first 30 min of reoxygenation per gram of dry weight. To measure cGMP, samples (9 ml) of the coronary effluent were collected at 25 min of the equilibration period, at 5 and 35 min of the hypoxic period, and at 2, 15, 30, and 90 min of the reoxygenation period and rapidly frozen in liquid N_2. In normoxic controls the coronary effluent was collected at 30, 60, 120, and 160 min of perfusion. Samples were boiled for 10 min and centrifuged (1,250*g* × 10 min), and the supernatant was lyophilized. cGMP was determined in concentrated samples by radioimmunoassay using acetylated [^{3}H]cGMP as previously described (1).

Uptake of L-Arginine in Isolated Cardiomyocytes

Ventricular cardiomyocytes were isolated from adult Sprague-Dawley rat hearts as previously described (27). Whole hearts were retrogradely perfused for 20 min with a buffer containing (in mM) 110 NaCl, 2.6 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 25 $NaHCO_3$, and 11 glucose (pH 7.4) and 0.03% collagenase. Dissociated tissue was filtered and centrifuged, and the pellet was subjected to a progressive normalization of Ca^{2+} levels to 1 mM. Rod-shaped cells were selected by gravity sedimentation and plated in medium 199 (Sigma Chemical) with 4% fetal calf serum in preincubated Falcon dishes.

Cultures were washed twice with HEPES-buffered saline (in mM: 140 NaCl, 3.6 KCl, 1.2 $MgSO_4$, 1 $CaCl_2$, and 20 HEPES, pH 7.4) and incubated in the same buffer plus 5 mM glucose (control dishes) or plus 4 mM sodium cyanide (dishes with energy deprivation) for 1 h. L-[^{3}H]arginine (50 μM) then was added to the cell cultures. The reaction of L-arginine incorporation was terminated at different times by rapid washing with cold HEPES-buffered saline and addition of 0.3 M perchloric acid. L-[^{3}H]arginine in the intracellular extracts was quantified by liquid scintillation spectrometry. Uptake of L-[^{3}H]arginine was linear for up to 5 min and was expressed as picomoles of total L-arginine incorporated per minute and per milligram of protein in this period of time.

Data Analysis and Statistics

Statistical analysis was performed by using commercially available software (Instat, GraphPad Software). Differences between groups were assessed by means of one-way analysis of variance. Individual comparisons between groups were performed by using the Student-Newman-Keuls test. A critical *P* value of 0.05 was used. Values are expressed as means ± SE.

RESULTS

Normoxic Hearts

Hemodynamic parameters were measured in hearts submitted to 160 min of perfusion with a normoxic solution. LVEDP, LVDP, heart rate, and pressure-rate product did not change significantly throughout the perfusion, whereas coronary flow showed a significant decrease (from 9.3 to 7.1 ml/min) (Table 1). cGMP release to the perfusion medium showed a nonsignifi-

Table 1. *Hemodynamic parameters and coronary flow in normoxic perfused hearts*

	10 min	160 min
LVEDP, mmHg	8.4 ± 0.6	7.6 ± 0.8
LVDP, mmHg	85.3 ± 9.26	77.8 ± 7.8
HR, beats/min	284.3 ± 11.4	278.6 ± 8.5
HR × LVDP, mmHg·beats·min^{-1}·10^{-3}	24.2 ± 0.8	21.7 ± 1.0
Flow, ml/min	9.3 ± 0.3	7.1 ± 0.3*

Values are means ± SE. LVDP, left ventricular developed pressure; LVEDP, left ventricular end-diastolic pressure; HR, heart rate; HR × LVDP, pressure-rate product. *$P < 0.05$.

cant trend toward decrease during perfusion (at 160 min of perfusion cGMP release was 82 ± 11% of that observed at the end of the equilibration period). There was no measurable LDH release during continuous normoxic perfusion.

Administration of L-Arginine to Hearts Subjected to Hypoxia-Reoxygenation

Contractile function. In a first series of experiments hearts received L-arginine, ODQ, both, or neither during the whole experimental protocol (equilibration, hypoxia, and reoxygenation periods). At the end of the equilibration period, LVEDP, LVDP, and heart rate were 9.0 ± 0.8 mmHg, 83 ± 5 mmHg, and 279 ± 14 beats/min, respectively, without differences between groups. During hypoxia, LVEDP increased steeply, with a peak of 184 mmHg 3.4 min after the onset of hypoxic perfusion. Reoxygenation induced a second increase in LVEDP, with a peak of 140 mmHg 2.2 min after its onset (Fig. 1). Changes in LVEDP were virtually identical in the four groups. Pressure-rate product (LVDP × heart rate) fell to zero in all four groups because of total abolition of LVDP at the beginning of the hypoxic period, with virtually no recovery during reoxygenation in the control group (Fig. 2). However, in hearts receiving L-arginine pressure-rate product showed a significant recovery (Fig. 2) that was abolished when ODQ was included together with L-arginine. Pressure recordings from hearts receiving ODQ alone were not different from those in the control group.

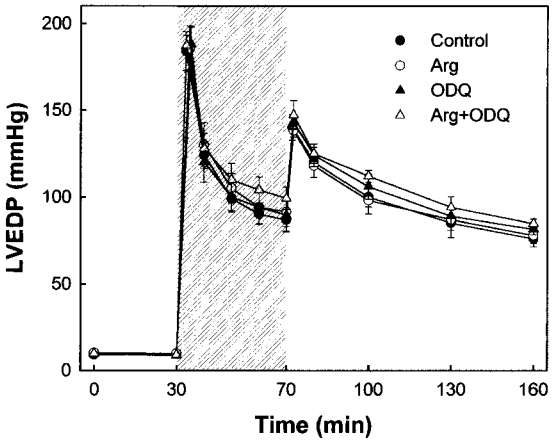

Fig. 1. Changes in left ventricular end-diastolic pressure (LVEDP) during hypoxia (shaded area) and reoxygenation in control hearts and in hearts receiving L-arginine (Arg), 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ), or both. There were no differences between groups.

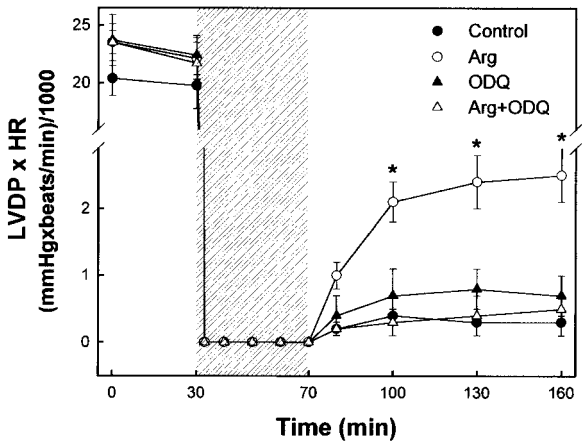

Fig. 2. Changes in pressure-rate product during hypoxia (shaded area) and reoxygenation in hearts receiving Arg, ODQ, or both. Arg improved functional recovery, and this effect was abolished by ODQ. LVDP, left ventricular developed pressure; HR, heart rate. *$P < 0.05$.

Coronary flow. Coronary flow increased during the first minute of hypoxic perfusion and then decreased to values below those obtained before hypoxia (Fig. 3). Reoxygenation did not induce any significant change in coronary flow. No intergroup differences were observed.

LDH release. There was no measurable LDH release during equilibration and hypoxic perfusion. During reoxygenation LDH activity in the effluent followed a characteristic pattern with an early peak 2 min after the onset of reoxygenation, followed by a rapid decay (Fig. 4). LDH release during the first 30 min of reoxygenation was reduced in hearts receiving L-arginine compared with controls, and this effect was reversed by ODQ treatment (Fig. 4, *inset*). ODQ alone had no effect.

cGMP release. In control hearts cGMP release was 350 ± 17 fmol/min at the end of the equilibration period. Hypoxia induced a marked and progressive decrease in cGMP release into the perfusion medium, and at the end of the hypoxic period cGMP release was

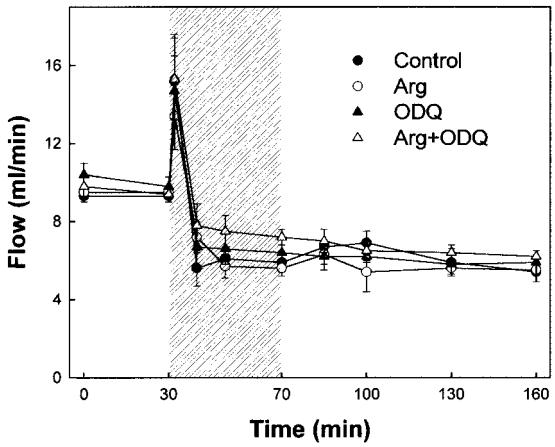

Fig. 3. Changes in coronary flow during hypoxia (shaded area) and reoxygenation in control hearts and in hearts receiving Arg, ODQ, or both. There were no differences between groups.

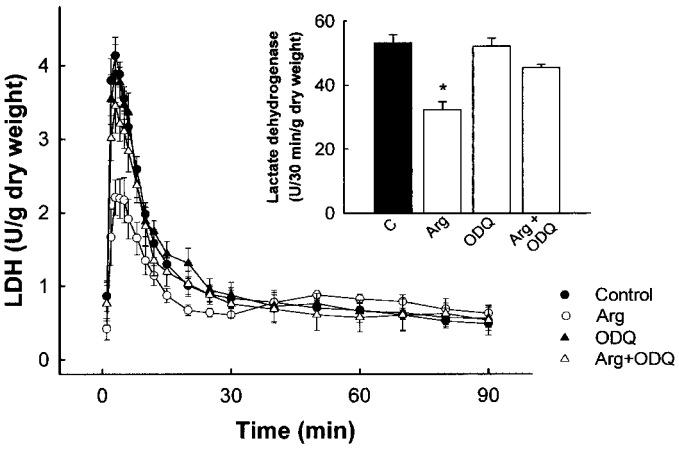

Fig. 4. Lactate dehydrogenase (LDH) release over 90 min after reoxygenation in hearts receiving Arg, ODQ, or both. Arg reduced peak release occurring between 2 and 3 min of reoxygenation, and ODQ abolished this effect. *Inset*, accumulative LDH release during first 30 min of reoxygenation. C, control. $*P < 0.05$.

~30% of the value measured before hypoxia (Fig. 5). The decrease in cGMP was not modified by reoxygenation and persisted until the end of the experiment.

L-Arginine supplementation increased cGMP release during hypoxia. At the end of the hypoxic period cGMP concentration in the coronary effluent was approximately two times greater in hearts receiving L-arginine than in controls ($P < 0.05$), and this difference persisted during reoxygenation (Fig. 5). ODQ completely abolished the effects of L-arginine on cGMP release (Fig. 5).

Critical Timing of L-Arginine Supplementation

In a second series of experiments L-arginine was added to the perfusate during only one of the three phases of the perfusion protocol. When added during hypoxia or reoxygenation L-arginine had no effect on functional recovery (Fig. 6*A*) or LDH release (Fig. 6*B*) during reoxygenation. However, addition of L-arginine during the 10 min before hypoxia afforded the same protective effect on recovery of pressure-rate product and LDH release than when it was added all throughout the experiment (Fig. 6, *A* and *B*). This protective effect was associated with a significant increase of cGMP release at the end of hypoxia (Fig. 6*C*). Addition of L-arginine to the perfusate only during hypoxia had no effect on cGMP release.

Finally, in a series of experiments, hearts were supplemented with L-arginine throughout the experiment and treated with ODQ only for the last 5 min of hypoxia and during reoxygenation. ODQ added during this period was able to inhibit 80 ± 12% ($P < 0.05$) of the functional recovery and 82 ± 8% ($P < 0.05$) of the reduction in LDH release evoked by L-arginine (in

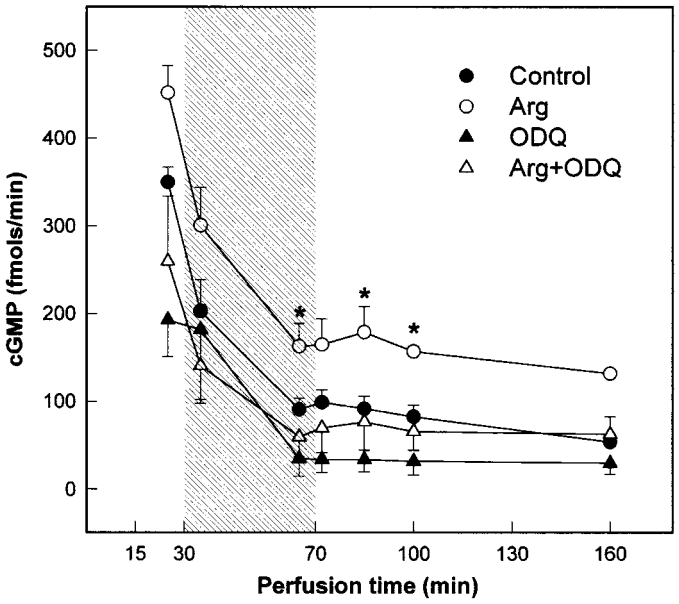

Fig. 5. cGMP release into coronary perfusion media during hypoxia (shaded area) and reoxygenation in hearts receiving Arg, ODQ, or both. Hypoxia induced a marked reduction in cGMP release in all groups, but this reduction was significantly attenuated by Arg. Effect of Arg on cGMP release was abolished by ODQ. $*P < 0.05$.

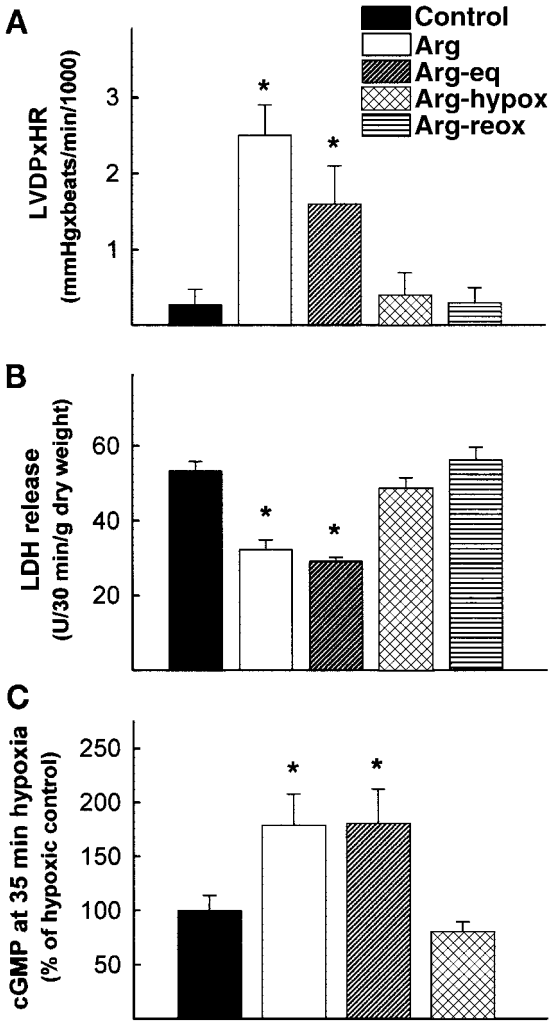

Fig. 6. Pressure-rate product at end of reoxygenation (*A*), LDH release during first 30 min of reoxygenation (*B*), and cGMP release at end of hypoxia (*C*) in hearts receiving L-arginine throughout all perfusion time (Arg), during last 10 min of equilibration (Arg-eq), during hypoxia (Arg-hypox), or during reoxygenation (Arg-reox). L-Arginine was effective only when added during equilibration. $*P < 0.05$.

comparison with 91 ± 9 and 63 ± 5% of inhibition by ODQ treatment throughout the experiment of functional recovery and LDH reduction, respectively).

Uptake of L-Arginine in Isolated Cardiomyocytes

During normoxic conditions, uptake of L-arginine by isolated cardiomyocytes was 43.9 ± 6.3 pmol·min^{-1}·mg protein^{-1}. L-Arginine uptake was markedly reduced in isolated myocytes submitted to energy deprivation (7.8 ± 6.9 pmol·min^{-1}·mg protein^{-1}, $P < 0.05$ with respect to normoxic controls).

DISCUSSION

This study demonstrates that L-arginine supplementation reduces myocardial cell death and improves functional recovery in myocardium submitted to hypoxia-reoxygenation. These beneficial effects are associated with attenuation of the reduction of cGMP synthesis during hypoxia-reoxygenation, as assessed by cGMP release to the coronary circulation, and are abolished when soluble guanylyl cyclase is inhibited by ODQ. L-Arginine supplementation during the equilibration period before hypoxia is both necessary and sufficient to attenuate the reduction of cGMP release and limit myocardial cell death, whereas addition of ODQ during the reoxygenation period is sufficient to abolish the beneficial effects of L-arginine. Supplementation of L-arginine during hypoxia and reperfusion has no effect on cGMP release or cell death. The beneficial effects of L-arginine are independent of any change in coronary flow. These results indicate that the beneficial effects of L-arginine are cGMP mediated, and they are in agreement with previous observations showing that cGMP has a protective effect against reoxygenation-induced hypercontracture.

Previous studies demonstrated that L-arginine supplementation may attenuate endothelial dysfunction (36), reduce edema (16) and necrosis (36), and improve functional recovery in reperfused skeletal (16) and heart (2, 9, 21) muscle. However, the mechanism of these beneficial effects has not been completely elucidated. L-Arginine can increase NO synthesis by NOS (19), may prevent switch of NOS activity to O_2^- production (13, 14), and has been described to release NO by a NOS-independent mechanism (22). All these actions of L-arginine supplementation could contribute to reduce O_2^- concentration and to limit O_2^--dependent tissue damage thanks to the strong scavenging action of NO toward peroxyl radicals, OH, and oxoferryl hemoproteins (20) overproduced during myocardial reperfusion and reoxygenation (11, 35). In fact, previous studies using methylene blue (a less specific inhibitor of soluble guanylyl cyclase) suggested that the beneficial effects of L-arginine were mainly mediated by the antioxidant effects of NO and only to a little extent by cGMP-mediated actions (20). The results of the present study do not support this hypothesis, and they clearly indicate that the protective effects of L-arginine supplementation on reoxygenation injury as assessed by enzyme release and contractile recovery are mainly mediated by increased cGMP availability during reperfusion. However, the present results do not exclude the possible existence of other beneficial effects of L-arginine related to cGMP-independent actions.

Maintenance of cGMP concentration during ischemia-reperfusion by L-arginine supplementation may reduce necrosis by decreasing neutrophil accumulation in reperfused myocardium (36) and/or by improving coronary flow (2, 16). Reduced neutrophil accumulation can be excluded in our crystalloid-perfused model, and the lack of any effect of L-arginine on coronary flow indicates that its beneficial effect is not mediated by flow preservation. The results of the present study are consistent with the beneficial effect of L-arginine being caused by a cGMP-mediated protection against reoxygenation-induced hypercontracture. cGMP inhibits contraction in cardiomyocytes by mechanisms not completely understood and probably involving modulation of both Ca^{2+} availability (34) and myofilament sensitivity to Ca^{2+} (25, 29). Increased cGMP concentration at the time of reoxygenation by stimulation of NO-independent guanylyl cyclase has been shown to prevent hypercontracture in isolated cardiomyocytes (15), and exposure to the cGMP analog 8-bromoguanosine 3′,5′-cyclic monophosphate has been found to improve relaxation in posthypoxic cardiomyocytes (28). This effect was similar to that produced by transient contractile blockade with 2,3-butanedione monoxime, a drug that directly inhibits actin-myosin interaction (10, 31). Because recovery of cell energy and hypercontracture occurs during the first seconds of restoration of oxygen supply (32), contractile blockade must be fully established at the onset of reoxygenation. Stimulation of particulate guanylyl cyclase (15), or administration of 2,3-butanedione monoxime (10, 31), thus had to be

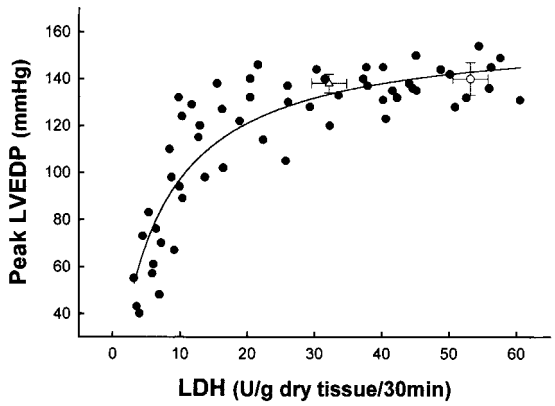

Fig. 7. Regression analysis of peak LVEDP and LDH release during first 30 min of reoxygenation in large series of isolated rat hearts (n = 58) submitted to 40 min of hypoxia (●). Relation between the 2 variables is clearly nonlinear but followed a hyperbolic model (r = 0.882, $P < 0.0001$), and LDH release of one-third of maximal values is associated with maximal values of peak LVEDP. ○ and △, mean values in control and L-arginine groups, respectively, in present study (n = 12 in each group). Vertical and horizontal bars indicate SE for peak LVEDP and LDH release. Although reoxygenation-induced LDH release, indicating sarcolemmal disruption and cell death secondary to hypercontracture, is clearly reduced by L-arginine, this change is not associated with a reduction in peak LVEDP because it occurs along horizontal segment of regression curve.

started during the last few minutes of hypoxia to be effective. In the present study, the failure of L-arginine added only at the time of reoxygenation could be explained by the time required for incorporation of L-arginine into the pool of L-arginine acting as NOS substrate within the cells. On the other hand, uptake of L-arginine by the cells involves an electrogenic transport system that seems to be impaired during hypoxia (Ref. 39 and present study). In fact, addition of L-arginine to the hypoxic buffer had no effect on cGMP release. Thus, to exert its beneficial effect during reoxygenation, L-arginine must be administered before hypoxia.

It could be expected that a protective effect of L-arginine against hypercontracture would result in a detectable reduction of the reoxygenation-induced rise in LVEDP. However, in the present study LVEDP was not modified by L-arginine. This apparent discrepancy can be explained by a nonlinear relationship between the number of cells undergoing hypercontracture and the magnitude of the increase in LVEDP. This nonlinear relationship is suggested by previous unpublished observations in the very same model used in this study (Fig. 7). Regression analysis shows that hypercontracture of a relatively small proportion of cardiomyocytes, as indicated by release of a small fraction of total LDH content during reoxygenation, suffices to produce a maximal rise in LVEDP.

The present results are in agreement with previous studies investigating the effect of NO donors on myocardial injury secondary to experimental ischemia. With a few exceptions (5), NO donors, including *S*-nitroso-*N*-acetylpenicillamine, sodium nitroprusside, and 3-morpholinosydnonimine, have been consistently found to exert a beneficial effect on cell death (30) and functional recovery (3, 8). Our data help to explain these beneficial effects through cGMP-mediated actions.

In other studies, inhibition of NO synthesis before ischemia has been found to improve functional recovery during reperfusion in the isolated, perfused heart (7, 23, 37). The mechanism of this protective effect has not been established, and stimulation of adenosine-mediated preconditioning (37), reduction of hydroxyl free radical (23) or peroxynitrite (38) generation, and preservation of glucose transport and glycolysis (7), with delayed onset of rigor- contracture, have been proposed to play a role. It seems, however, that the protective effect of NOS inhibitors were independent of a reduction in cGMP concentration (7). The beneficial effects of NOS inhibitors on ischemia-reperfused myocardium are not in conflict with our working hypothesis of a cGMP-mediated beneficial effect of L-arginine. Inhibition of NOS during ischemia-reperfusion can result in a beneficial reduction of O_2^- and $ONOO^-$ production (13) that could outweigh the potentially harmful effects of reduced NO synthesis. In fact, these unfavorable effects should be small if, as suggested by this study, cGMP synthesis is already markedly reduced during ischemia-reperfusion.

Study Limitations

In this study, a model of hypoxia-reoxygenation was used. Although this model may be less relevant than ischemia-reperfusion to most clinical situations, it allows better control of drug delivery during energy deprivation and continuous measurement of cGMP release. The actual cellular cGMP concentrations were not measured in this study. However, changes in cGMP release have been shown to be a reliable index of changes in myocardial cGMP synthesis in previous studies (3, 18) and can be continuously monitored in the same heart. The lack of effect of L-arginine on coronary flow in the present study could be caused by the existence of a maximal vasodilatation in the crystalloid-perfused heart. Our results thus do not exclude the possibility of additional vascular related effects of L-arginine during myocardial reperfusion in other models.

Implications

The demonstration of a protective effect of cGMP on ischemia-reperfusion injury opens new possibilities of pharmacological prevention of reperfusion injury. In fact, L-arginine concentrations required to observe beneficial effects are similar to those obtained in plasma after intravenous infusion (16). Although the potential value of L-arginine in the prevention of reoxygenation/reperfusion injury is limited by the need to administer it before energy deprivation, this limitation could be circumvented by substances directly stimulating cGMP synthesis. Currently available substances are able to effectively increase cGMP concentration by NO-independent stimulation of particulate guanylyl cyclase without the side effects of increased NO concentration. The potential therapeutic value of these substances on ischemia-reperfusion injury needs to be investigated.

The authors acknowledge the excellent technical work of Yolanda Puigfel and the contribution of Dr. Marisol Ruiz-Meana and Dr. Juan Cinca in reviewing the manuscript.

This work was partially supported by the BIOMED Concerted Action BMH1-PL95/1254 from the European Union and by a grant from the Fondo de Investigación de la Seguridad Social (FIS 97/0948).

Address for reprint requests and other correspondence: D. García-Dorado, Servicio de Cardiología, Hospital General Universitario Vall d'Hebron, Paseo Vall d'Hebron 119–129, 08035 Barcelona, Spain (E-mail: dgdorado@hg.vhebron.es).

Received 25 September 1998; accepted in final form 22 January 1999.

REFERENCES

1. **Agulló, L., and A. García.** Characterization of noradrenaline-stimulated cyclic GMP formation in brain astrocytes in culture. *Biochem. J*. 288: 619–624, 1992.
2. **Amrani, M., C. C. Gray, R. T. Smolenski, A. T. Goodwin, A. London, and M. H. Yacoub.** The effect of L-arginine on myocardial recovery after cardioplegic arrest and ischemia under moderate and deep hypothermia. *Circulation* 96, *Suppl*. II: 274–279, 1997.
3. **Brunner, F.** Interaction of nitric oxide and endothelin-1 in ischemia/reperfusion injury of rat heart. *J. Mol. Cell Cardiol*. 29: 2363–2374, 1997.
4. **Busse, R., and I. Fleming.** Regulation and functional consequences of endothelial nitric oxide formation. *Ann. Med*. 27: 331–340, 1995.

5. **Cope, J. T., D. Banks, and O. A. Binns.** Sodium nitroprusside exacerbates myocardial ischemia-reperfusion injury. *Ann. Thorac. Surg.* 64: 1656–1659, 1997.
6. **Depré, C., L. Fiérain, and L. Hue.** Activation of nitric oxide synthase by ischemia in the perfused heart. *Cardiovasc. Res.* 33: 82–87, 1997.
7. **Depré, C., J. L. Vanoverschelde, J. F. Goudemant, I. Mottet, and L. Hue.** Protection against ischemic injury by non-vasoactive concentrations of nitric oxide synthase inhibitors in the perfused rabbit heart. *Circulation* 92: 1911–1918, 1995.
8. **Draper, N. J., and A. M. Shah.** Beneficial effects of a nitric oxide donor on recovery of contractile function following brief hypoxia in isolated rat heart. *J. Mol. Cell Cardiol.* 29: 1195–1205, 1997.
9. **Engelman, D. T., M. Watanabe, R. M. Engelman, J. A. Rousou, J. E. Flack III, D. W. Deaton, and D. K. Das.** Constitutive nitric oxide release is impaired after ischemia and reperfusion. *J. Thorac. Cardiovasc. Surg.* 110: 1047–1053, 1995.
10. **García-Dorado, D., P. Théroux, J. M. Duran, J. S. Solares, J. Alonso, E. Sanz, R. Munoz, J. Elizaga, J. Botas, F. Fernandez-Avilés, J. Soriano, and E. Esteban.** Selective inhibition of the contractile apparatus. *Circulation* 85: 1160–1174, 1992.
11. **Garlick, P. B., M. J. Davies, D. J. Hearse, and T. F. Slater.** Direct detection of free radicals in the reperfused rat heart using electron spin resonance spectroscopy. *Circ. Res.* 61: 757–760, 1987.
12. **Garthwaite, J., E. Southam, C. L. Boulton, E. B. Nielsen, K. Schmidt, and B. Mayer.** Potent and selective inhibition of nitric oxide-sensitive guanylyl cyclase by 1*H*-[1,2,4]oxadiazolo[4,3,-*a*]quinoxalin-1-one. *Mol. Pharmacol.* 48: 184–188, 1995.
13. **Griffith, O. W., and D. J. Stuehr.** Nitric oxide synthases: properties and catalytic mechanism. *Annu. Rev. Physiol.* 57: 707–736, 1995.
14. **Gross, S. S., and M. S. Wolin.** Nitric oxide: pathophysiological mechanisms. *Annu. Rev. Physiol.* 57: 737–769, 1995.
15. **Hempel, A., M. Friedrich, K. D. Schlüter, W. G. Forssmann, M. Kuhn, and H. M. Piper.** ANP protects against reoxygenation-induced hypercontracture in adult cardiomyocytes. *Am. J. Physiol.* 273 (*Heart Circ. Physiol.* 42): H244–H249, 1997.
16. **Huk, I., J. Nanobashvili, C. Neumayer, A. Punz, M. Mueller, K. Afkhampour, M. Mittlboeck, U. Losert, P. Polterauer, E. Roth, S. Patton, and T. Malinski.** L-Arginine treatment alters the kinetics of nitric oxide and superoxide release and reduces ischemia/reperfusion injury in skeletal muscle. *Circulation* 96: 667–675, 1997.
17. **Inserte, J., D. García-Dorado, M. Ruiz-Meana, J. Solares, and J. Soler.** The role of Na^+-H^+ exchange occurring during hypoxia in the genesis of reoxygenation-induced myocardial oedema. *J. Mol. Cell Cardiol.* 29: 1167–1175, 1997.
18. **Kelm, M., S. Schäfer, R. Dahmann, B. Dolu, S. Perings, U. K. M. Decking, J. Schrader, and B. E. Strauer.** Nitric oxide induced contractile dysfunction is related to a reduction in myocardial energy generation. *Cardiovasc. Res.* 36: 185–194, 1987.
19. **Knowles, R. G., M. Palacios, R. M. J. Palmer, and S. Moncada.** Formation of nitric oxide from L-arginine in the central nervous system: a transduction mechanism for stimulation of the soluble guanylate cyclase. *Proc. Natl. Acad. Sci. USA* 86: 5159–5162, 1989.
20. **Maulik, N., D. T. Engelman, M. Watanabe, R. M. Engelman, J. A. Rousou, J. E. Flack III, D. W. Deaton, N. V. Gorbunov, N. M. Elsayed, V. E. Kagan, and D. K. Das.** Nitric oxide/carbon monoxide. A molecular switch for myocardial preservation during ischemia. *Circulation* 94, *Suppl*. II: II-398–II-406, 1996.
21. **Moro, M. A., R. J. Russell, S. Cellek, I. Lizasoain, Y. Su, V. M. Darley-Usmar, M. W. Radomski, and S. Moncada.** cGMP mediates the vascular and platelet actions of nitric oxide: confirmation using an inhibitor of the soluble guanylyl cyclase. *Proc. Natl. Acad. Sci. USA* 93: 1480–1485, 1996.
22. **Nagase, S., K. Takemura, A. Ueda, A. Hirayama, K. Aoyagi, M. Kondoh, and A. Koyama.** A novel nonenzymatic pathway for the generation of nitric oxide by the reaction of hydrogen peroxide and D- or L-arginine. *Biochem. Biophys. Res. Commun* 233: 150–153, 1997.
23. **Naseem, S. A., M. C. Kontos, P. S. Rao, R. L. Jesse, M. L. Hess, and R. C. Kukreja.** Sustained inhibition of nitric oxide by N^G-nitro-L-arginine improves myocardial function following ischemia/reperfusion in isolated perfused rat heart. *J. Mol. Cell Cardiol.* 27: 419–426, 1995.
24. **Pabla, R., and M. J. Curtis.** Effect of endogenous nitric oxide on cardiac systolic and diastolic function during ischemia and reperfusion in the rat isolated perfused heart. *J. Mol. Cell Cardiol.* 28: 2111–2121, 1996.
25. **Pfitzer, G., J. C. Rüegg, V. Flockerzi, and F. Hofmann.** cGMP-dependent protein kinase decreases calcium sensitivity of skinned cardiac fibers. *FEBS Lett.* 149: 171–175, 1982.
26. **Pryor, W. A., and G. L. Squadrito.** The chemistry of peroxynitrite: a product from the reaction of nitric oxide with superoxide. *Am. J. Physiol.* 268 (*Lung Cell. Mol. Physiol.* 12): L699–L722, 1995.
27. **Ruiz-Meana, M., D. García-Dorado, M. A. González, J. A. Barrabés, and J. Soler-Soler.** Effect of osmotic stress on sarcolemmal integrity of isolated cardiomyocytes following transient metabolic inhibition. *Cardiovasc. Res.* 30: 64–69, 1995.
28. **Shah, A. M., M. J. Lewis, and A. H. Henderson.** Effects of 8-bromo-cyclic GMP on contraction and on ionotropic response of ferret cardiac muscle. *J. Mol. Cell Cardiol.* 23: 55–64, 1991.
29. **Shah, A. M., H. A. Spurgeon, S. J. Sollot, A. Talo, and E. G. Lakatta.** 8-Bromo-cGMP reduces the myofilament response to Ca^{2+} in intact cardiac myocytes. *Circ. Res.* 74: 970–978, 1994.
30. **Siegfried, M. R., J. Erhardt, T. Rider, X. Ma, and A. M. Lefer.** Cardioprotection and attenuation of endothelial dysfunction by organic nitric oxide donors in myocardial ischemia-reperfusion. *J. Pharmacol. Exp. Ther.* 260: 668–675, 1992.
31. **Siegmund, B., T. Klietz, P. Schwartz, and H. M. Piper.** Temporary contractile blockade prevents hypercontracture in anoxic-reoxygenated cardiomyocytes. *Am. J. Physiol.* 260 (*Heart Circ. Physiol.* 29): H426–H435, 1991.
32. **Siegmund, B. A., A. Koop, T. Klietz, P. Schwartz, and H. M. Piper.** Sarcolemmal integrity and metabolic competence of cardiac myocytes under anoxia-reoxygenation. *Am. J. Physiol.* 258 (*Heart Circ. Physiol.* 27): H285–H291, 1990.
33. **Tsao, P. S., N. Aoki, D. J. Lefer, G. Johnson, and A. M. Lefer.** Time course of endothelial dysfunction and myocardial injury during myocardial ischemia and reperfusion in the cat. *Circulation* 82: 1402–1412, 1990.
34. **Wahler, G. M., N. J. Rusch, and N. Sperelakis.** 8-Bromo-cyclic GMP inhibits the calcium channel current in embryonic chick ventricular myocytes. *Can. J. Physiol. Pharmacol.* 68: 531–534, 1989.
35. **Wang, P., and J. L. Zweier.** Measurement of nitric oxide and peroxynitrite generation in the postischemic heart. *J. Biol. Chem.* 271: 29223–29230, 1996.
36. **Weyrich, A. S., X. Ma, and A. M. Lefer.** The role of L-arginine in ameliorating reperfusion injury after myocardial ischemia in the cat. *Circulation* 86: 279–288, 1992.
37. **Woolfson, R. G., V. C. Patel, G. H. Neild, and D. M. Yellon.** Inhibition of nitric oxide synthesis reduces infarct size by an adenosine-dependent mechanism. *Circulation* 91: 1545–1551, 1995.
38. **Yasmin, W., K. D. Strynadka, and R. Schulz.** Generation of peroxynitrite contributes to ischemia-reperfusion injury in isolated rat hearts. *Cardiovasc. Res.* 33: 422–432, 1997.
39. **Zharikov, S. I., H. Herrera, and E. R. Block.** Role of membrane potential in hypoxic inhibition of L-arginine uptake by lung endothelial cells. *Am. J. Physiol.* 272 (*Lung Cell. Mol. Physiol.* 16): L78–L84, 1997.
40. **Zweier, J. L., P. Wang, and P. Kuppusamy.** Direct measurement of nitric oxide generation in the ischemic heart using electron paramagnetic resonance spectroscopy. *J. Biol. Chem.* 270: 304–307, 1995.

ELSEVIER

Cardiovascular Research 45 (2000) 351–359

Cardiovascular Research

www.elsevier.com/locate/cardiores
www.elsevier.nl/locate/cardiores

Urodilatin limits acute reperfusion injury in the isolated rat heart

Javier Inserte, David Garcia-Dorado*, Luis Agulló, Amaya Paniagua, Jordi Soler-Soler

Servicio de Cardiología, Hospital General Universitari Vall d'Hebron, Passeig Vall d'Hebron, 119–129, 08035 Barcelona, Spain

Received 2 June 1999; accepted 12 October 1999

Abstract

Objectives: Hypercontracture is an important mechanism of myocyte death during reperfusion. cGMP modulates the sensitivity of contractile myofilaments to Ca^{2+}, and increasing cGMP concentration during the last minutes of anoxia prevents reoxygenation-induced hypercontracture in isolated cardiomyocytes. The purpose of this study was to determine whether stimulation of particulate guanylyl cyclase with the natriuretic peptide urodilatin, given at the time of reperfusion, reduces myocardial necrosis in the rat heart submitted to transient ischemia. **Methods:** Isolated rat hearts ($n=38$) were submitted to either 40 or 60 min of no-flow ischemia and 2 h of reperfusion, and were allocated to receive or not receive 0.05 μM urodilatin during the first 15 min of reperfusion or non-reperfusion treatment. **Results:** A marked reduction in myocardial cGMP concentration was observed in control hearts during reperfusion after 40 or 60 min of ischemia. Urodilatin significantly attenuated cGMP depletion during initial reperfusion, markedly improved contractile recovery after 40 min of ischemia ($P<0.0309$), and reduced reperfusion-induced increase in left ventricular end-diastolic pressure ($P=0.0139$), LDH release ($P=0.0263$), and contraction band necrosis ($P=0.0179$) after 60 min of ischemia. The beneficial effect of urodilatin was reproduced by the membrane permeable cGMP analog 8-Bromo-cGMP. **Conclusions:** These results indicate that reduced cGMP concentration may impair myocyte survival during reperfusion. Stimulation of particulate guanylyl cyclase may appear as a new strategy to prevent immediate lethal reperfusion injury.

Keywords: Ischemia; Natriuretic peptide; Necrosis; Reperfusion; Second messengers

1. Introduction

Early reperfusion may prevent necrosis of ischemic myocardium. However, when reperfusion is delayed it is associated with the appearance of areas of contraction band necrosis composed of hypercontracted cardiomyocytes [1,2]. Myocyte hypercontracture is the consequence of excessive contractile activity due to re-energization in the presence of elevated cytosolic Ca^{2+} concentration [3–6]. In in situ myocytes, hypercontracture causes sarcolemmal disruption and cell death [7,8]. Transient contractile blockade with 2,3-butanedione monoxime (BDM) during the time required for recovery of Ca^{2+} control prevents hypercontracture [9,10], and reduces final infarct size [1,11]. However, the potential therapeutic value of BDM in patients with acute myocardial infarction receiving reperfusion therapy is limited by the need for selective intracoronary delivery of the blocker, and its toxicity.

Previous studies have shown that treatments increasing cGMP concentration during reoxygenation and cGMP analogs may prevent reoxygenation-induced hypercontracture in isolated cardiomyocytes [12]. In the isolated rat heart submitted to transient hypoxia, cGMP synthesis and release into the coronary circulation are reduced during oxygen deprivation and remain reduced during reoxygenation [13]. In this model, L-arginine supplementation before hypoxia increases cGMP concentration during reoxygenation, reduces enzyme release, and improves functional recovery, and all these effects are abolished by 1*H*-[1,2,4]oxadiazolo[4,3,-*a*]quinoxalin-1-one (ODQ), a selective inhibitor of soluble guanylyl cyclase. Since hypercontracture occurs during the first few seconds after reoxygenation, treatments increasing cGMP need to be present during the last minutes of anoxia in order to prevent it [12,13]. However, in myocardium submitted to transient ischemia, reperfusion-induced hypercontracture occurs only after disappearance of intracellular acidosis and its

*Corresponding author. +34-93-4894038; fax: +34-93-4894032.
E-mail address: dgdorado@hg.vhebron.es (D. Garcia-Dorado)

Time for primary review 31 days.

0008-6363/00/$ – see front matter
PII: S0008-6363(99)00371-5

negative inotropic effect [6]. This time window could allow treatments increasing cGMP to exert their protective effect against hypercontracture when administered at the time of reperfusion. On the other hand, the protective effect of cGMP against reoxygenation-induced hypercontracture is observed at concentrations that have little effect on systolic shortening of normoxic myocytes [12]. Thus, drugs modulating cGMP appear as potentially useful in the limitation of lethal reperfusion injury occurring in clinical conditions.

Urodilatin is a member of the natriuretic peptide family normally present in urine [14] but not detected in plasma [15] that stimulates particulate guanylyl cyclase in many cell types including myocytes [12,16,17]. Urodilatin has a half-life much longer than that of atrial natriuretic peptide (ANP), has low toxicity and may be safely administered intravenously to patients [18]. Our hypothesis was that urodilatin administered at the time of reperfusion may limit myocardial necrosis secondary to ischemia/reperfusion. Urodilatin was added during the initial phase of reperfusion in isolated rat hearts submitted to transient global ischemia of two different durations, and cGMP release, functional recovery, and myocardial necrosis were measured.

2. Materials and methods

2.1. Isolated perfused rat heart

The care and use of animals conformed 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 the experimental procedures were approved by the Research Commission on Ethics of the Hospital of Vall d'Hebron. Adult male Sprague–Dawley rats, weighing 300–350 g, were deeply anaesthetized with intraperitoneal injection of thiopental sodium (150 mg kg^{-1}). The hearts were removed from the thorax and immediately arrested in ice-cold saline solution. The aorta was quickly cannulated and the heart was retrogradely perfused with a Krebs–Henseleit bicarbonate buffer (KHB) at 37°C using a non-recirculating Langendorff apparatus, at a constant pressure of 60 mm Hg. The composition of KHB was as follows (in mM): NaCl, 140; $NaHCO_3$, 24; KCl, 2.7; KH_2PO_4, 0.4; $MgSO_4$, 1; $CaCl_2$, 1.8; and, glucose, 11. KHB was filtered under vacuum through a 0.45-μm cellulose filter before use to remove any particulate matter, and continuously gassed with 95% O_2 and 5% CO_2.

Left ventricular pressure was monitored by means of a water-filled latex balloon placed in the left ventricle and connected to a pressure transducer 43600 F (Baxter, The Netherlands) through a Cordis 5F catheter (Cordis, Miami, FL). The left ventricular end-diastolic pressure (LVEDP) was set between 6 and 8 mm Hg by adjusting the filling of the balloon. The signal obtained was digitized and recorded continuously on a hard disk with the aid of an ad hoc developed software. The variables measured included heart rate (HR), LVEDP and left ventricular developed pressure (LVdevP), calculated as the difference between left ventricular peak systolic pressure and LVEDP. Coronary flow was measured by timed collection of effluent at regular intervals using a calibrated tube, and expressed in ml min^{-1}.

2.2. Experimental protocol and groups of treatment

2.2.1. Normoxic studies

The effects of urodilatin on contractility and coronary flow under normoxic conditions were studied in 13 hearts perfused for 180 min. Following a 30-min stabilization period, hearts were exposed to 15 min of perfusion with urodilatin ($n=4$) followed by 135 min of perfusion without the drug. Five hearts perfused for 180 min without urodilatin were used as normoxic controls.

The correlation between cGMP released into the coronary effluent and myocardial cGMP content was studied in eight hearts. After 30 min of stabilization, hearts were perfused for 3 min with urodilatin at different concentrations (0, 0.005, 0.05 or 1 μM), and immediately frozen by dropping the hearts into liquid nitrogen for posterior measurement of myocardial cGMP content. cGMP release was measured in samples from the coronary effluent collected immediately before, and at 3 min into, urodilatin perfusion.

2.2.2. Ischemia-reperfusion studies

In a first set of experiments, after 30 min of normoxic perfusion, hearts were subjected to global ischemia for 40 min by clamping the aortic in-flow line, and reperfused for 120 min. Hearts were allocated to one of two groups, receiving, respectively, 0.05 μM urodilatin during the first 15 min of reperfusion ($n=8$) or no drug (control group, $n=10$). In a second set of experiments, hearts were allocated to the same two groups of treatment, but ischemia was extended to 60 min ($n=6$ per group).

A third set of experiments hearts were performed to investigate the effect of 8-Bromo-cGMP. In these experiments hearts ($n=4$ per group) were submitted to either 40 or 60 min of ischemia and perfused with 100 μM 8-Bromo-cGMP under hypoxic conditions for 2 min starting 5 min before reperfusion and, under normoxic conditions, during the first 15 min of reperfusion. The brief hypoxic infusion prior to reperfusion containing 8-Bromo-cGMP was performed to ensure an effective intracellular concentration of drug at the onset of reperfusion. Previous studies (unpubl. observations) have shown that this requires administering the drug at least 5 min before reperfusion. The infusion however was stopped after 2 min to minimize catabolite washout and modify as minimally as possible the reperfusion conditions. The composition of

the hypoxic buffer was identical to that used for normoxic reperfusion except for the replacement of bicarbonate by HEPES 20 mM and for the absence of glucose. Its pH was adjusted to 6.3 to match the extracellular pH previously reported in the ischemic rat heart [19] and was bubbled with 100% N_2. Finally, a fourth set of experiments was used to investigate the effect of urodilatin on contractile function during the initial minutes of reperfusion. These hearts were submitted to 40 min of ischemia and reperfused in the presence of urodilatin. After the first 7 min of reflow urodilatin was withdrawn from the perfusate for 3 min, and added again at between 10 and 15 min of reperfusion.

2.3. Lactate dehydrogenase release

Lactate dehydrogenase (LDH) activity was spectrophotometrically measured in samples collected from the coronary effluent at different times throughout the reperfusion period, as previously described [13].

2.4. cGMP release

Samples (9 ml) from the coronary effluent were collected at different time points throughout the perfusion period, and rapidly frozen in liquid nitrogen. Samples were boiled for 10 min, centrifuged at 1250*g* for 10 min and the supernatant lyophilized. cGMP was determined in concentrated samples by radioimmunoassay using acetylated [^{3}H]-cGMP, as previously described [20].

2.5. Myocardial cGMP content

cGMP concentration was measured in eight normoxic hearts in which the correlation between myocardial cGMP concentration and release was analysed, and in an additional series of 24 hearts submitted to either 40 or 60 min of ischemia and to 10 or 120 min of reperfusion with or without urodilatin according to a 2×2×2 equilibrated design.

Frozen hearts were pulverized under liquid nitrogen. The powdered tissue was homogenized in cold trichloroacetic acid at 7.5% (weight/volume). After centrifugation at 14 000*g* for 15 min at 4°C, the supernatant was collected and washed five times with seven volumes of water-saturated diethylether. The residual ether was removed by placing tubes in a bath at 50°C for 30 min. cGMP was measured in the extracts using the radioimmunoassay method described above and results were expressed as fmols of cGMP per mg of protein. Total protein content from the powdered heart was determined according to Bradford [21].

2.6. Histological analysis

A 3-mm-thick, cross-sectional, midventricular slice was embedded in paraffin, and 4-μm sections were obtained (Leika RM2145 microtome, Leika, Germany) and stained with Masson's trichrome. The presence of contraction band necrosis was assessed as previously described [11] and its extent was quantified morphometrically. Serial microphotographs of adjacent optical fields (×400, Olympus IMT-2, Olympus Optical, Japan) were obtained according to four perpendicular lines irradiating from the center of the left ventricular cavity with one of the lines crossing the right ventricular cavity at its middle point. Microphotographs were digitized (Olympus DP10 camera, Olympus Optical, Japan) for subsequent analysis (Micro Image™, Olympus Optical, Japan). Microphotographs including ventricular cavities or blank extracardiac space were excluded. Microphotographs were classified into one of three scores: 0, no contraction band necrosis; 1, contraction band necrosis involving less than 50% of the photographed area; 2, contraction band necrosis involving more than 50% of the photographed area. The average score was calculated for each heart.

2.7. Data analysis and statistics

Statistical analysis was performed by using commercially available software (Instat, GraphPad Software). Differences between groups were assessed by means of one-way analysis of the variance. Individual comparisons between groups were performed by using the Student–Newman Keuls test. A critical P value of 0.05 was used. Values are expressed as mean±S.E.M.

3. Results

3.1. Effects of urodilatin in normoxic hearts

3.1.1. Myocardial function

LVEDP and HR were stable throughout the 180 min of normoxic perfusion. A slight, non-statistically significant decrease in LVdevP and flow values was observed by the end of the experimental perfusion period, confirming the stability of the preparation. There were no significant differences between groups (Table 1).

3.1.2. cGMP release

In normoxic hearts, cGMP release into the coronary effluent showed a non-significant decrease throughout the perfusion period (from 293±29 fmol min^{-1} at 30 min of perfusion to 283±28 fmol min^{-1} after 180 min of perfusion). Addition of urodilatin to the perfusion buffer for 15 min increased cGMP release since the first minute, reaching a maximum of 2710±102 fmol min^{-1} after 10 min of perfusion with urodilatin.

Table 1
Hemodynamic parameters and coronary flow in normoxic perfused hearts[a]

	LVEDP (mm Hg)		LVdevP (mm Hg)		HR (beats min^{-1})		Flow (ml min^{-1})	
	30 min	190 min	30 min	180 min	30 min	180 min	30 min	180 min
Control	7.0±0.6	8.3±1.4	113.7±13.0	83.3±13.2	282±14	270±16	9.2±22.1	7.4±0.7
Urodilatin	6.8±0.5	7.2±0.6	103.4±4.7	83.2±6.0	298±9	283±9	10.7±1.0	9.0±1.1

[a] Values are mean±S.E.M. Hr, heart rate; LVEDP, left ventricular end-diastolic pressure; LVdevP, left ventricular developed pressure.

3.1.3. Correlation between cGMP release and myocardial cGMP content

In hearts exposed for 3 min to different concentrations of urodilatin, myocardial cGMP content ranged between 48 and 260 fmols/mg protein, and total cGMP release into the coronary effluent during the stimulation period ranged between 276 and 3856 fmol min^{-1}. There was an excellent correlation between both variables (r^2=0.96).

3.2. Effects of urodilatin during reperfusion

3.2.1. Myocardial function

In control hearts subjected to 40 min of ischemia, LVEDP and LVdevP were, respectively, 7.8±0.6 and 112.1±9.9 mm Hg at the end of the equilibration period. At this time HR was 270±15 beats min^{-1} and coronary flow 11.2±0.4 ml min^{-1}. No-flow ischemia resulted in cessation of left ventricular contractile activity [Fig. 1(A)], and in a steep increase in LVEDP with a peak of 53.4±3.3 mm Hg 20 min after the onset of the ischemic period [Fig. 1(B)]. Reperfusion induced a further increase in LVEDP with a peak of 78.2±6.0 mm Hg 3 min after its onset. LVdevP recovered to 37% of its initial value after 30 min of reperfusion and 38% after 120 min [Fig. 1(A)]. Coronary flow during reperfusion did not reach pre-ischemic values. After 30 min of reperfusion it was 5.3±0.5 ml min^{-1} and at the end of reperfusion 4.4±0.4 ml min^{-1} (P<0.0001 with respect to pre-ischemic values).

Addition of urodilatin to the perfusion buffer during the first 15 min of reperfusion was associated with a markedly improved contractile recovery of LVdevP (67% after 30 min of reperfusion and 55% after 120 min [Fig. 1(A)] but only after cessation of urodilatin infusion). The rapid increase in LVdevP after withdrawal of urodilatin suggested a negative inotropic effect that was confirmed in an additional series of hearts submitted to consecutive addition–withdrawal of the drug during the initial minutes of reflow (Fig. 2). These studies disclosed a marked effect of the urodilatin during initial reperfusion, as demonstrated by a 50% reduction in LVdevP, in sharp contrast with the absence of contractile effects in normoxic hearts not previously submitted to ischemia.

Hearts subjected to 60 min of ischemia showed identical behaviour to those submitted to 40 min of occlusion during the equilibration period, as well as during the first 40 min of ischemia [Fig. 3(A) and (B)]. The time and magnitude of LVEDP rise during ischemia were identical in hearts submitted to both durations of ischemia. In control hearts, reperfusion was followed by an increase in LVEDP with a peak of 128.9±7.0 mm Hg 3 min after its onset. LVdevP recovered to 8% of its initial value after 30 min of reperfusion and 10% after 120 min. Addition of urodilatin to the perfusion buffer during the first 15 min of reperfusion did not produce significant changes in the recovery of LVdevP, HR or coronary flow compared to control hearts, but reduced significantly the peak of LVEDP during early reperfusion (105.6±3.5 mm Hg, P=0.0139).

3.2.2. LDH release

No measurable LDH activity was detected in the coronary effluent of hearts submitted to 40 min of ischemia

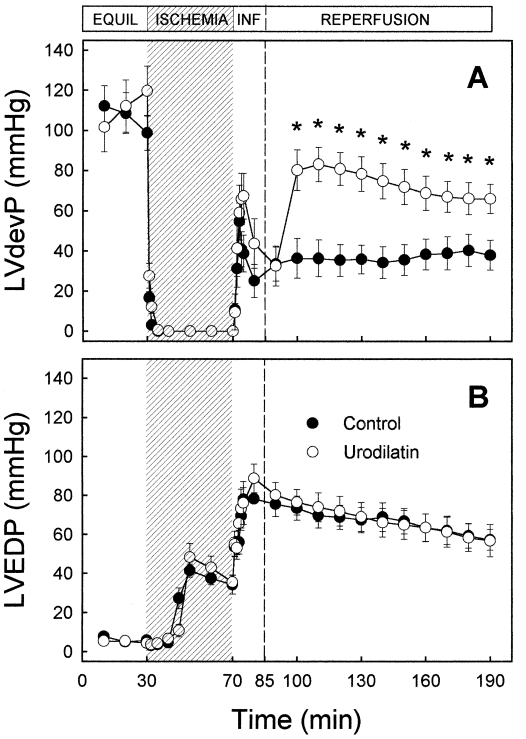

Fig. 1. Changes in left ventricular developed pressure (LVdevP) and left ventricular end-diastolic pressure (LVEDP) during equilibration (EQUIL), 40 min of ischemia (dashed area) and 120 min of reperfusion in control hearts (Control) and in hearts receiving 0.05 μM urodilatin (Urodilatin) during the first 15 min of reperfusion. The 15 min infusion period is denoted by INF. Data are presented as mean±S.E.M. *, P<0.05 vs. control group; †, P<0.05 urodilatin group vs. control group.

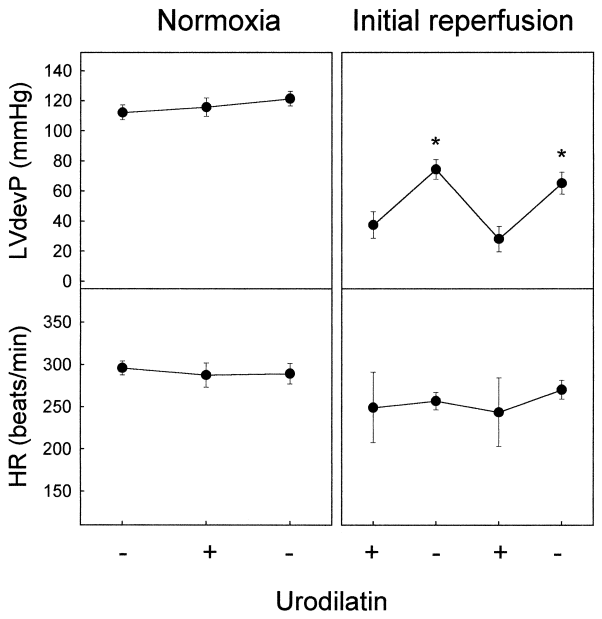

Fig. 2. Changes in left ventricular developed pressure (LVdevP) and heart rate (HR) in hearts submitted to consecutive addition (+) withdrawal (−) of 0.05 μM urodilatin during normoxia without previous ischemia or during the first 30 min of reperfusion after 40 min of ischemia. Data are presented as mean±S.E.M. *, $P<0.05$ vs. control group.

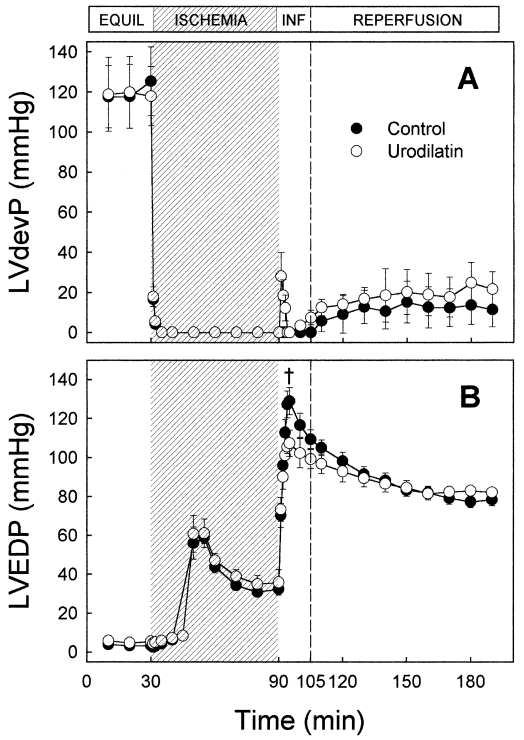

Fig. 3. Changes in left ventricular developed pressure (LVdevP) and left ventricular end-diastolic pressure (LVEDP) during equilibration (EQUIL), 60 min of ischemia (dashed area) and 120 min of reperfusion in control hearts (Control) and in hearts receiving 0.05 μmol/l urodilatin (Urodilatin) during the first 15 min of reperfusion. The 15 min infusion period is denoted by INF. Data are presented as mean±S.E.M. *, $P<0.05$ vs. control group; †, $P<0.05$ URO 0.05 μmol/l group vs. control group.

independently of the presence or absence of urodilatin. However, in hearts subjected to 60 min of ischemia reperfusion was associated with important LDH release with an early peak 4 min after the onset of reoxygenation followed by a rapid decay. Addition of urodilatin during the first 15 min of reperfusion reduced significantly the total LDH release during the reperfusion period compared to the control group (164.3±9.7 U/g dry weight/120 min vs. 131.5±8.0 U/g dry weight/120 min, $P=0.0263$) without modifying its time course (Fig. 4).

3.2.3. cGMP release

In hearts subjected to 40 min of ischemia, cGMP release at the end of the stabilization period was 197±18 fmol min^{-1}. After 10 min of reperfusion, cGMP release reached 32±11% of its initial value in the control group and 94±22% in hearts receiving urodilatin ($P<0.001$ with respect to controls). After cessation of urodilatin infusion, cGMP release decreased progressively [Fig. 5(A)]. In hearts submitted to 60 min of ischemia the time course and magnitude of cGMP release were similar to those observed in the corresponding groups submitted to 40 min of ischemia [Fig. 5(B)].

3.2.4. Myocardial cGMP concentration

cGMP content was severely reduced in myocardium reperfused after 40 or 60 min of ischemia (Fig. 6). Addition of urodilatin to the perfusate during the first 15 min of reflow prevented this reduction in hearts reperfused after 40 min of ischemia, and markedly attenuated it in hearts reperfused after 60 min of ischemia. These effects were lost after 120 min of reperfusion (0.2±0.1 fmols/mg protein in the control group and 1.7±1.5 fmols/mg protein in the urodilatin group subjected to 40 min of ischemia and 1.91±1.81 fmols/mg protein in the control group and 0.2±0.1 fmols/mg protein in the urodilatin group subjected to 60 min of ischemia).

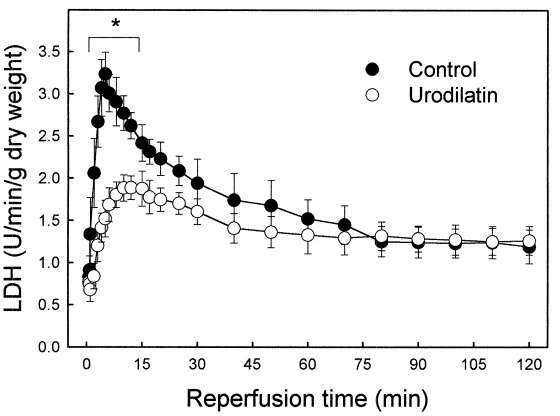

Fig. 4. LDH release during reperfusion following 60 min of ischemia in control hearts (Control) and in hearts receiving 0.05 μmol/l urodilatin (Urodilatin) during the first 15 min of reperfusion. Data are presented as mean±S.E.M. *, $P<0.05$ vs. control group.

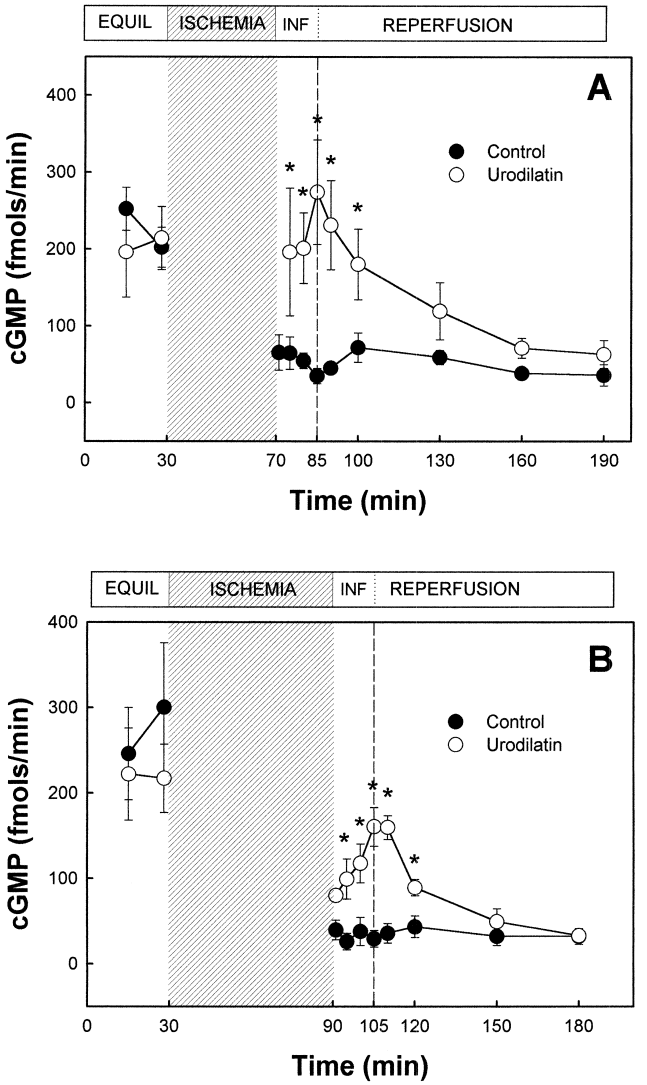

Fig. 5. cGMP release into the coronary effluent during equilibration (EQUIL) and reperfusion in control hearts (Control), and in hearts receiving 0.05 μmol/l urodilatin (Urodilatin) during the first 15 min of reperfusion. The 15 min of infusion period is denoted by INF. Panels A and B correspond, respectively, to hearts submitted to 40 min and 60 min of ischemia. The dashed area corresponds to the ischemic period. Data are presented as mean±S.E.M. *, $P<0.05$ vs. control group.

3.2.5. Effect of 8-Bromo-cGMP

Hearts receiving 8-Bromo-cGMP, a soluble analog of cGMP, during initial reperfusion after 40 min of ischemia showed a significantly better functional recovery than those not receiving it (Table 2). Hearts receiving 8-Bromo-cGMP after 60 min of ischemia did not differ from their controls in their functional behaviour, although a trend towards a better postischemic recovery of LVdevP was observed (Table 2), but showed a marked reduction of reperfusion-induced LDH release compared to the control group (111.4±24.7 U/g dry weight/120 min vs. 182.6±19.1 U/g dry weight/120 min, $P=0.0365$).

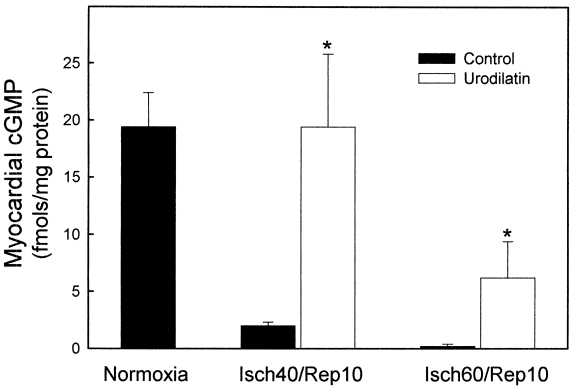

Fig. 6. Myocardial cGMP after 30 min of equilibration (Normoxia) and after 10 min of reperfusion in control hearts (Control), and in hearts receiving 0.05 μmol/l urodilatin (Urodilatin) during the first minutes of reperfusion following either 40 min of ischemia (Isch40/Rep10) or 60 min of ischemia (Isch60/Rep10). Data are presented as mean±S.E.M. *, $P<0.05$ vs. 10 min reperfusion control group.

3.2.6. Histology

No contraction band necrosis was observed in hearts subjected to 3 h of normoxic perfusion. Hearts submitted to 40 min of ischemia did not show areas of contraction band necrosis. After 60 min of ischemia all hearts showed extensive areas of contraction band necrosis. Treatment with urodilatin reduced significantly the presence of contraction band necrosis (mean score (0–2) of 1.17±0.17 vs. 1.83±0.17 in the control group, $P=0.0179$). A similar reduction in the extent of contraction band necrosis was observed in hearts receiving 8-Bromo-cGMP (1.05±0.21, $P=0.0214$).

4. Discussion

This study shows that, in the isolated rat heart, myocardial cGMP is severely depleted during reperfusion following prolonged ischemia, and that stimulation of particulate guanylyl cyclase with urodilatin during the first 15 min of reperfusion attenuates this depletion, markedly improves functional recovery, and protects cardiomyocytes against hypercontracture and necrosis induced by reperfusion after 60 min of ischemia. The effects on functional recovery were evident after 40 min of ischemia, a duration that resulted in minimal necrosis in both treated and control hearts. At the concentration used, urodilatin did not modify coronary flow either during normoxic conditions or during reperfusion and lacks effects on myocardial contraction in normoxic, control hearts, but had a marked, rapidly reversible negative inotropic effect on reperfused myocardium. These results indicate that reduced cGMP levels may have a detrimental influence on cell survival during myocardial reperfusion, and identify stimulation of membrane-bound guanylyl cyclase as a potentially useful

Table 2
Hemodynamic parameters from hearts subjected to ischemia and receiving or not receiving 8-Bromo-cGMP during initial reperfusion[a]

	Pre-ischemia		Ischemia		Reperfusion	
	LVEDP	LVdevP	Max LVEDP	Max LVEDP	LVdevP 30 min	LVdevp 120 min
Ischemia 40 min						
Control	7.8±0.4	108.2±10.5	56.7±5.3	78.6±6.5	32.4±6.2	34.5±7.6
8-Bromo-cGMP	6.7±0.6	112.4±11.3	52.3±6.1	72.8±8.3	73.1±8.1[b]	65.4±5.9[b]
Ischemia 60 min						
Control	6.2±0.4	110.6±9.3	58.4±8.1	121.8±13.5	10.9±7.5	15.3±9.6
8-Bromo-cGMP	7.1±0.3	108.9±8.9	60.4±4.9	110.4±8.7	12.5±6.4	20.9±7.7

[a] Values are mean±S.E.M. and are expressed in mm Hg. LVEDP, left ventricular end-diastolic pressure; LVdevP, left ventricular developed pressure; Max LVEDP, maximal value for left ventricular end diastolic pressure during the corresponding period.
[b] $P<0.05$ vs. control group.

pharmacological approach to enhance myocardial salvage during coronary reperfusion.

4.1. cGMP in reperfused myocardium

Although many studies have investigated the changes in NO synthesis and availability in reperfused myocardium [22–24], little is known about the modifications in cGMP concentration induced by ischemia/reperfusion. Previous studies have described either a reduction [25,26] or an absence of changes [27] in myocardial cGMP content in reperfused myocardium. In a recent study on the isolated rat heart [13] we reported a marked reduction in cGMP release into the coronary circulation during hypoxia and reoxygenation. The results of the present study demonstrate a marked reduction in myocardial cGMP concentration and coronary cGMP release in reperfused myocardium. Moreover, the response of myocardial cGMP to stimulation with urodilatin is markedly reduced in the reperfused myocardium: myocardial cGMP increased from 19.4±3.0 fmols/mg protein to 107.2±14.4 fmols/mg protein in response to urodilatin in normoxic hearts, but only to 19.4±6.4 fmols/mg protein in response to urodilatin during the initial 10 min of reperfusion following 40 min of ischemia. This blunted response to urodilatin was even more pronounced in myocardium reperfused after 60 min of ischemia, and suggests that ischemia/reperfusion may damage the enzymatic system controlling cGMP concentration.

The effect of manoeuvres increasing the activity of soluble guanylyl cyclase (such as the addition of NO donors or L-arginine supplementation) on myocardial injury secondary to ischemia/reperfusion or hypoxia/reoxygenation has been investigated in many studies, most of them with positive results [22–24]. This protective effect can be abolished by selective inhibitors of guanylyl cyclase [13]. However, NO is a free radical with potentially toxic actions at high concentrations that could counterbalance the beneficial effects of increased cGMP synthesis [28]. An alternative approach to increasing cGMP synthesis is stimulation of particulate guanylyl cyclase. In a recent study, addition of urodilatin to the incubation media during the last 15 min of anoxia and the first 15 min of reoxygenation prevented reoxygenation-induced hypercontracture in isolated cardiomyocytes [12], and the soluble cGMP analog 8-Bromo-cGMP mimicked this effect. The present study supports and extends these previous observations showing the beneficial effect of increasing cGMP in reoxygenated or reperfused myocardium, and demonstrates the feasibility of achieving this effect by the addition of urodilatin only at the time of reperfusion.

4.2. Protection mechanism

That the protective effect of urodilatin against myocardial necrosis secondary to ischemia/reperfusion is mediated through its effect on myocardial cGMP is supported by the ability of the soluble cGMP analog 8-Bromo-cGMP to reproduce it. This effect of cGMP against reperfusion injury can be explained by its ability to desensitize contractile myofilaments to Ca^{2+} during the initial minutes of reperfusion. Desensitization by BDM during the initial phase of reoxygenation or reperfusion prevents hypercontracture and limits myocardial necrosis in different models [1,9–11]. In this study urodilatin lacked contractile effects in normoxic hearts, but had a marked negative inotropic effect during the first minutes of reperfusion, a situation in which Ca^{2+} sensitivity of myofilaments and contractility are already depressed (stunning).

Previous studies demonstrate that under normoxic conditions cGMP may desensitize myofilaments to Ca^{2+} without altering Ca^{2+} kinetics. The molecular mechanism of this effect has not been established. It has been described that cGMP may directly reduce the relative myofilament response to Ca^{2+}, probably via a cGMP-dependent protein kinase [29]. More recently, atrial natriuretic peptide has been shown to indirectly decrease myofilament Ca^{2+} sensitivity via cytosolic acidification secondary to modulation of the sarcolemmal Na^{+}–H^{+} exchanger and/or other sarcolemmal transport systems by

cGMP-dependent protein kinase [30,31]. In reperfused myocardium intracellular acidosis is rapidly corrected upon restoration of coronary blood flow by the combined actions of the Na^+–H^+ exchanger and the Na^+–HCO_3^- symporter [32], and prolongation of acidosis during the initial phase of reperfusion has been shown to have a protective effect against hypercontracture in isolated myocytes [33,34] and in intact animals [35]. However, there is ample evidence from whole-cell voltage clamp studies that cGMP may have an inhibitory effect on L-type Ca^{2+} channels [36] and the possibility has not been excluded that changes in cGMP concentration may modulate Ca^{2+} kinetics in reperfused myocytes. Either improved Ca^{2+} kinetics or enhanced and/or prolonged acidosis could explain the striking beneficial effect of urodilatin at 0.05 μM on contractile recovery after 40 min of ischemia.

4.3. Methodological considerations

In the present study two different durations of ischemia (40 and 60 min) were used. The 40-min experiments allowed us to detect the beneficial effect of urodilatin against post-ischemic dysfunction in the absence of significant necrosis, as assessed by LDH release and histology, while the 60-min experiments allowed us to detect the protective effect of urodilatin against necrosis. The failure of urodilatin to significantly improve the extremely severe contractile dysfunction of myocardium surviving 60 min of ischemia may be reflected in the limited efficiency of the drug against stunning.

Changes in cGMP release was used as an index of myocardial cGMP content. Certainly, factors other than myocardial cGMP concentration can influence release of cGMP into the coronary circulation. However cGMP has been successfully used by previous authors to assess the effect of treatments stimulating cGMP synthesis [37,38], and in the present study an excellent correlation was observed between both variables at least under normoxic conditions. In addition, the results of cGMP measured in the coronary effluent were fully correlated with the analysis of myocardial cGMP content at a single time-point early during reperfusion.

4.4. Implications

The isolated perfused rat heart model presents many differences with in vivo situations. In fact, urodilatin has important non-cardiac effects when administered to intact animals [18,39,40], that are not considered in this model. However, the present results identify a new strategy to prevent immediate lethal reperfusion injury of myocardium based on stimulation of particulate guanylyl cyclase. The observation that urodilatin may protect reperfused myocardium from hypercontracture and necrosis when applied at the reflow time at concentrations lacking any detectable effect on normoxic myocardium not previously submitted to ischemia has potential therapeutic relevance, since urodilatin has been safely administered to normal volunteers [40,41] and to patients with heart failure [18]. Further research is clearly needed to define its therapeutic value during in vivo myocardial reperfusion.

Acknowledgements

The authors wish to thank Yolanda Puigfel for her excellent technical assistance and Dr. Marisol Ruiz-Meana and Dr. Juan Cinca for their contribution in reviewing the manuscript. Urodilatin was kindly provided by Prof. Dr. Wolf-Georg Forssmann and PD Dr. Markus Meyer from the Niedersächsisches Institute für Peptid-Forschung, Hannover, Germany. This work was partially supported by the European Union (BMH1-PL95/1254), and by the Spanish Ministry of Health (FIS 97/0948).

References

[1] Schlack W, Uebing A, Schafer M et al. Regional contractile blockade at the onset of reperfusion reduces infarct size in the dog heart. Pflugers Arch 1994;428:134–141.
[2] Miyazaki S, Fujiwara H, Onodera T et al. Quantitative analysis of contraction band and coagulation necrosis after ischemia and reperfusion in the porcine heart. Circulation 1987;75:1074–1082.
[3] Siegmund B, Schlüter K-D, Piper HM. Calcium and the oxygen paradox. Cardiovasc Res 1993;27:1778–1783.
[4] Stern MD, Chien AM, Capogrossi MC, Pelto DJ, Lakatta EG. Direct observation of the 'oxygen paradox' in single rat ventricular myocytes. Circ Res 1985;56:899–903.
[5] Van der Heide RS, Angelo JP, Altschuld RA, Ganote CE. Energy dependence of contraction band formation in perfused hearts and isolated adult cardiomyocytes. Am J Pathol 1986;125:55–68.
[6] Piper HM, Garcia-Dorado D, Ovize M. A fresh look at reperfusion injury. Cardiovasc Res 1998;38:291–300.
[7] Hearse DJ, Humphrey SM, Chain EB. Abrupt reoxygenation of the anoxic potassium-arrested perfused rat heart: A study of myocardial enzyme release. J Mol Cell Cardiol 1973;5:395–407.
[8] Ganote CE. Contraction band necrosis and irreversible myocardial injury. J Mol Cell Cardiol 1983;15:67–73.
[9] Siegmund B, Klietz T, Schwartz P, Piper HM. Temporally contractile blockade prevents hypercontracture in anoxic-reoxygenated cardiomyocytes. Am J Physiol 1991;260:H426–H435.
[10] Schlüter KD, Schwartz P, Siegmund B, Piper HM. Prevention of the oxygen paradox in hypoxic-reoxygenated hearts. Am J Physiol 1991;261:H416–H423.
[11] Garcia-Dorado D, Théroux P, Duran JM et al. Selective inhibition of the contractile apparatus: A new approach to modification of infarct size, infarct composition, and infarct geometry during coronary artery occlusion and reperfusion. Circulation 1992;85:1160–1174.
[12] Hempel A, Friedrich M, Schlüter KD et al. ANP protects against reoxygenation-induced hypercontracture in adult cardiomyocytes. Am J Physiol 1997;42:H244–249.
[13] Agulló L, García-Dorado D, Inserte J et al. L-Arginine limits myocardial cell death secondary to hypoxia-reoxygenation by a cGMP-dependent mechanism. Am J Physiol 1999;276:H1574–1580.
[14] Schulz-Knappe P, Forssmann K, Herbst F et al. Isolation and structural analysis of 'urodilatin', a new peptide of the cardiodilatin-

(ANP)-family, extracted from human urine. Klin Wochenschr 1988;66:752–759.
[15] Drummer C, Fiedler F, König A, Gerzer R. Urodilatin, a kidney-derived natriuretic factor, is excreted with a circadian rhythm and is stimulated by saline infusion in man. J Am Soc Nephrol 1991;2:1109–1113.
[16] Kiemer AK, Vollmar AM. Effects of different natriuretic peptides on nitric oxide synthesis in macrophages. Endocrinology 1997;138:4282–4290.
[17] Saxenhofer H, Fitzgibbon WR, Paul RV. Urodilatin: Binding properties and stimulation of cGMP generation in rat kidney cells. Am J Physiol 1993;264:F267–73.
[18] Elsner D, Muders F, Müntze A et al. Efficacy of prolonged infusion of urodilatin [ANP-(95-126)] in patients with congestive heart failure. Am Heart J 1995;129:766–773.
[19] Pike MM, Luo CS, Clark D et al. NMR measurements of Na^+ and cellular energy in ischemic rat heart: Role of Na^+–H^+ exchange. Am J Physiol 1993;265:H2017–H2026.
[20] Agulló L, García A. Characterization of noradrenaline-stimulated cyclic GMP formation in brain astrocytes in culture. Biochem J 1992;288:619–624.
[21] Bradford MM. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein–dye binding. Anal Biochem 1978;72:248–254.
[22] Amrani M, Gray CC, Smolenski RT et al. The effect of L-arginine on myocardial recovery after cardioplegic arrest and ischemia under moderate and deep hypothermia. Circulation 1997;96:274–279.
[23] Brunner F. Interaction of nitric oxide and endothelin-1 in ischemia/reperfusion injury of rat heart. J Mol Cell Cardiol 1997;29:2363–2374.
[24] Engelman DT, Watanabe M, Engelman RM et al. Constitutive nitric oxide release is impaired after ischemia and reperfusion. J Thorac Cardiovasc Surg 1995;110:1047–1052.
[25] Nesher R, Robinson WF, Gibb L, Bishop SP, Kruger FA. Cyclic nucleotide levels in the perfused rat heart subjected to hypoxia. Experientia 1977;33:215–217.
[26] Yamaguchi F, Nasa Y, Yabe K et al. Activation of cardiac muscarinic receptor and ischemic preconditioning effects in in situ rat heart. Heart Vessels 1997;12:74–83.
[27] Maulik N, Engelman DT, Watanabe M et al. Nitric oxide/carbon monoxide. A molecular switch for myocardial preservation during ischemia. Circulation 1996;94:398–406.
[28] Gross SS, Wolin MS. Nitric oxide: Pathophysiological mechanisms. Annu Rev Physiol 1995;57:737–769.
[29] Shah AM, Spurgeon HA, Sollott SJ, Talo A, Lakatta EG. 8-Bromo-cGMP reduces the myofilament response to Ca^{2+} in intact cardiac myocytes. Circ Res 1994;74:970–978.
[30] Ito N, Bartunek J, Spitzer KW, Lorell BH. Effects of the nitric oxide donor sodium nitroprusside on intracellular pH and contraction in hypertrophied myocytes. Circulation 1997;95:2303–2311.
[31] Tajima M, Bartunek J, Weinberg EO, Ito N, Lorell BH. Atrial natriuretic peptide has different effects on contractility and intracellular pH in normal and hypertrophied myocytes from pressure-overloaded hearts. Circulation 1998;98:2760–2764.
[32] Vanderberg JI, Metcalfe JC, Grace AA. Mechanisms of pHi recovery after global ischemia in the reperfused heart. Circ Res 1993;72:993–1003.
[33] Ladilov YV, Siegmund B, Piper HM. Protection of reoxygenated cardiomyocytes against hypercontracture by inhibition of Na^+/H^+ exchange. Am J Physiol 1995;268:H1531–H1539.
[34] Lemasters JJ, Bond JM, Chacon E et al. The pH paradox in ischemia-reperfusion injury to cardiac myocytes. EXS 1996;76:99–114.
[35] Kaplan SH, Yang H, Gilliam DE et al. Hypercapnic acidosis and dimethyl amiloride reduce reperfusion induced cell death in ischaemic ventricular myocardium. Cardiovasc Res 1995;29:231–238.
[36] Sperelakis N. Regulation of calcium slow channels of heart by cyclic nucleotides and effects of ischemia. Adv Pharmacol 1994;31:1–24.
[37] Brunner F. Interaction of nitric oxide and endothelin-1 in ischemia/reperfusion injury of rat heart. J Mol Cell Cardiol 1997;29:2363–2374.
[38] Kelm M, Schäfer S, Dahmann R et al. Nitric oxide induced contractile dysfunction is related to a reduction in myocardial energy generation. Cardiovasc Res 1987;36:185–194.
[39] Crozier IG, Nicholls MG, Ikram H et al. Haemodynamic effects of atrial peptide infusion in heart failure. Lancet 1986;2:1242–1245.
[40] Cody RJ, Atlas SA, Laragh JH et al. Atrial natriuretic factor in normal subjects and heart failure subjects. Plasma levels and renal, hormonal and hemodynamic responses to peptide infusion. J Clin Invest 1986;78:1362–1374.
[41] Kentsch M, Ludwig D, Drummer C, Gerzer R, Müller-Esch G. Haemodynamic and renal effects of urodilatin bolus injections in patients with congestive heart failure. Eur J Clin Invest 1992;22:662–669.

ELSEVIER

Cardiovascular Research 51 (2001) 241–250

Cardiovascular Research

www.elsevier.com/locate/cardiores

Role of the reverse mode of the Na^+/Ca^{2+} exchanger in reoxygenation-induced cardiomyocyte injury

C. Schäfer[a], Y. Ladilov[a], J. Inserte[b], M. Schäfer[a], S. Haffner[a], D. Garcia-Dorado[b], H.M. Piper[a,*]

[a]*Physiologisches Institut, Justus-Liebig-Universität, Aulweg 129 D-35392 Giessen, Germany*
[b]*Hospital General Universitari, Vall d'Hebron, Barcelona, Spain*

Received 13 December 2000; accepted 15 March 2001

Abstract

Objective: We have recently shown that spontaneous Ca^{2+} oscillations elicit irreversible hypercontracture of cardiomyocytes during reoxygenation. The aim of this study was to investigate whether influx of exterior Ca^{2+} through the reverse mode of the Na^+/Ca^{2+} exchanger (NCE) contributes to the development of these oscillations and, therefore, to reoxygenation-induced hypercontracture. **Methods:** Isolated cardiomyocytes and hearts from rats were used as models. Cardiomyocytes were exposed to 60 min simulated ischemia (pH_o 6.4) and 10 min reoxygenation (pH_o 7.4). During reoxygenation cardiomyocytes were superfused with medium containing 1 mmol/l Ca^{2+} (control), with nominally Ca^{2+}-free medium or with medium containing 10 μmol/l KB-R 7943 (KB), a selective inhibitor of the reverse mode of the NCE. **Results:** In reoxygenated cardiomyocytes rapid Ca^{2+} oscillations occurred which were reduced under Ca^{2+}-free conditions or in presence of KB. Hypercontracture was also significantly reduced under Ca^{2+}-free conditions or in presence of KB. After 30 min of normoxic perfusion isolated rat hearts were subjected to 60 min global ischemia and reperfusion. KB (10 μmol/l) was present during the first 10 min of reperfusion. LVEDP, LVdevP and lactate dehydrogenase (LDH) release were measured. Presence of KB reduced post-ischemic LVEDP and improved left ventricular function (LVdevP). In KB treated hearts the reperfusion induced release of LDH was markedly reduced from 81.1±9.9 (control) to 49.3±8.8 U/60 min/g dry weight. **Conclusion:** Our study shows that inhibition of the reverse mode of the NCE, during reperfusion only, protects cardiomyocytes and whole hearts against reperfusion injury.

Keywords: Calcium (cellular); Ischemia; Myocytes; Na/Ca-exchanger; Reperfusion

This article is referred to in the Editorial by S.J. Conway and S.V. Koushik (pages 194–197) in this issue.

1. Introduction

Resupply of oxygen to the myocardium after extended periods of ischemia or hypoxia can rapidly aggravate the already existing injury [1,2]. We showed in previous investigations, using the model of isolated ventricular cardiomyocytes of the rat, that acute reoxygenation injury is based on sudden development of hypercontracture upon reoxygenation [3]. Reoxygenation-induced hypercontracture represents a major cause for acute lethal cell injury in reperfused myocardium [4,5]. Earlier studies of our group showed that during the early phase of reoxygenation oscillations of cytosolic Ca^{2+} occur spontaneously. It has been shown that agents, which inhibit Ca^{2+} uptake in or release from the sarcoplasmatic reticulum (SR) and thereby reduce these oscillations, protect reoxygenated cardiomyocytes against hypercontracture and reduce reperfusion-induced injury in whole myocardium. The oscillations are due to the cycling of a large amount of Ca^{2+} between cytosol and SR. The Ca^{2+} overload is a result of a

Abbreviations: NCE, Na^+/Ca^{2+} exchanger; KB, KB-R 7943; SR, sarcoplasmic reticulum; LVEDP, left ventricular endiastolic pressure; LVdevP, left ventricular developed pressure; LDH, lactate dehydrogenase

*Corresponding author. Tel.: +49-641-9947-241; fax: +49-641-9947-239.

E-mail address: michael.piper@physiology.med.uni-giessen.de (H.M. Piper).

Time for primary review 21 days.

0008-6363/01/$ – see front matter
PII: S0008-6363(01)00282-6

preceding prolonged ischemic period. The oscillations are initiated by the re-supply of energy to the SR Ca^{2+} pump. The frequency of these oscillations with high cytosolic peak Ca^{2+} concentrations correlates with the extent of hypercontracture [6,7]. It is therefore important to understand the factors that modulate Ca^{2+} oscillations in reoxygenated cardiomyocytes.

The aim of the present study was to investigate on the cellular level (i) whether the development of these Ca^{2+} oscillations is influenced by transsarcolemmal influx of extracellular Ca^{2+} during the early phase of reoxygenation, and in case of a positive answer, (ii) whether extracellular Ca^{2+} enters into the cells via the reverse mode of the Na^+/Ca^{2+} exchanger (NCE) and (iii) whether knowledge of this mechanism can lead to a new approach for protection of cardiomyocytes against reoxygenation-induced injury. Experiments were carried out with isolated cardiomyocytes from the ventricular myocardium of adult rats and isolated perfused rat hearts. A novel compound, KB-R 7943 (KB), was used in our study. It inhibits preferentially the reverse mode of NCE and has lower potency for the forward mode of NCE or other ion transport systems, such as the Na^+/H^+ exchanger, L-type Ca^{2+} channels, voltage-gated Na^+ channels, and inward rectifier K^+ channels [8–11]. The inhibitor was applied to cardiomyocytes or hearts solely during the reoxygenation/reperfusion period.

2. Methods

2.1. Experimental models

2.1.1. Isolated cardiomyocytes

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 1985). Ventricular heart muscle cells were isolated from 200–250 g adult male Wistar rats [12], and plated in medium 199 with 4% fetal calf serum on glass cover-slips that had been preincubated overnight with 4% fetal calf serum. Four hours after plating, the cover-slips were washed with medium 199. As a result of the wash, damaged cells were removed, leaving a homogeneous population of rod-shaped quiescent cardiomyocytes (>95%) attached to the cover-slip. From each isolation 2–3 coverslips were used. On each coverslip 4–6 cells were investigated. Only cells exhibiting a rod-shaped morphology and no signs of sarcolemmal blebbing were used for the experiments. These cells were found to have a low resting $[Ca^{2+}]_i$.

2.1.2. Isolated hearts

Hearts from 300–350 g adult male Sprague–Dawley rats were mounted on a Langendorff system. The hearts were perfused with a Krebs–Henseleit bicarbonate buffer at 37°C using a non-circulating Langendorff apparatus, at a constant pressure of 60 mmHg. Left ventricular pressure was monitored by means of a water-filled latex balloon placed in the left ventricle and connected to a pressure transducer 43600 F (Baxter, The Netherlands) through a Cordis 5F catheder (Cordis, Miami, Fl, USA). The left ventricular end-diastolic pressure (LVEDP) was set at 8 mmHg by adjusting the filling of the balloon which was then left at that filling throughout the experiment. The signal obtained was digitised and recorded continuously. The variables measured included LVEDP and LVdevP (left ventricular developed pressure), calculated as the difference between left ventricular peak systolic pressure and LVEDP. Lactate dehydrogenase (LDH) activity was measured in samples collected from the coronary effluent at different times throughout the reperfusion period. LDH activity was assayed spectrophotometrically in 0.5 mmol/l phosphate buffer, 1 mmol/l sodium pyruvate and 0.3 mmol/l NADH.

2.2. Media

The perfusion chamber (1 ml filling volume) placed on the microscope stage was perfused at a flow-rate of 0.5 ml/min with modified, glucose-free normoxic bicarbonate-buffered solution at 37°C containing (mmol/l): NaCl 118.0, KCl 2.6, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 1.0 and $NaHCO_3$ 22.0; the medium was gassed with 5% CO_2–95% O_2, the resulting pH was 7.4. In the anoxic medium the bicarbonate concentration was reduced to 2.2 mmol/l; resulting in a pH of 6.4 when medium was gassed with 5% CO_2–95% N_2. NaCl concentration of the anoxic medium was elevated to 137.8 mmol/l, in order to equalise Na^+ concentrations of anoxic and normoxic media. Medium was made anoxic by autoclaving as described previously [13]. The Na^+-free medium contained (mmol/l): *N*-methylglucamin 125.0, KCl 2.6, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 1.0 and HEPES 25.0. pH was adjusted to 7.4 with HCl. When made anoxic, the medium was autoclaved and gassed with 100% N_2.

2.3. Ca^{2+}, pH and cell length measurements

To measure $[Ca^{2+}]_i$ or $[H^+]_i$, cardiomyocytes were loaded at 35°C with fura-2 or BCECF, respectively. For loading, cells attached to the glass coverslips were incubated for 30 min in medium 199 with the acetoxymethyl ester of fura-2 (2.5 μmol/l) and for 15 min with BCECF (1.25 μmol/l). After the loading, the cells were washed twice with medium 199 for 30 min to allow hydrolysis of the acetoxymethyl esters within the cell. The fluorescence from dye-loaded cells was 20–30 times higher than background fluorescence, i.e. fluorescence from cells not loaded with the dye. The loading protocols used provided the highest yield in fluorescence and minimal dye compartmentation.

The coverslip with the loaded cells was introduced into a gas-tight, temperature-controlled (37°C), transparent perfusion chamber positioned in the light path of an inverted microscope (Diaphot TMD, Nikon). Alternating excitation of the fluorescent dye at wavelengths of 340 and 380 nm for fura-2 and 440 and 490 nm for BCECF was performed with an AR-cation measurement system adapted to the microscope (Spex Industries). Emitted light (500–520 nm for fura-2 and 520–560 nm for BCECF) from an area of 10×10 mm within a single fluorescent cell was collected by the photomultiplier of the system. The light signal was recorded and analysed by an IBM PC/AT-based data analysis system (Model DM3000CM, ISA). Simultaneously to the measurement of fluorescence, the cell's microscopic image was recorded with a video camera and stored on tape. From these recordings, changes of the cell length were determined later. In the case of hypercontracted cells, the cell dimension along its previous longitudinal axis was determined.

2.4. In vivo calibration of BCECF and fura-2

Calibration of the BCECF ratio signal was performed, as previously described by Koop and Piper [14], with 10 μg/ml nigericin, a K^+/H^+ ionophore, and incubation media with various pH values. The fura-2 signal was calibrated according to the method described by Li et al. [15]. For this purpose, the cells were exposed to 5 μmol/l ionomycin in modified Tyrode's solution (pH 7.4; composition see below) containing either 3 mmol/l Ca^{2+} or 5 mmol/l bis-(aminoethyl)-glycolether)-*N,N,N′,N′*-tetra-acetic acid (EGTA) to obtain the maximum (R_{max}) and the minimum (R_{min}) ratio of fluorescence, respectively. To prevent morphological alterations during calibration, cells were ATP-depleted with 1 mmol/l KCN. The free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) was calculated according to Grynkiewicz et al. [16] with use of pH-dependent K_d values for fura-2, determined in intact cardiomyocytes by constructing calibration curves. For the first 10 min of reoxygenation the integral of the fura-2 ratio was determined as area between the actual trace of the fura-2 ratio and the ratio of 0.5, which is the normoxic value of fura-2 ratio.

2.5. Experimental protocols

2.5.1. Protocol 1: Na^+ withdrawal in isolated cardiomyocytes under normoxic conditions

To estimate the capacity of KB-R 7943 (10 μmol/l) to inhibit the reverse mode of NCE, the following protocol was applied. Under normoxic conditions, cardiomyocytes were pretreated for 30 min with 150 nmol/l thapsigargin, to inhibit the Ca^{2+} ATPase of the sarcoplasmatic reticulum (SR), 5 min with 4 μmol/l ryanodine, to inhibit Ca^{2+} release channels of the SR, 5 min with 4 μmol/l HOE 642, to inhibit the sarcolemmal Na^+/H^+ exchanger, and 2 min with 300 μmol/l ouabain, to inhibit the Na^+/K^+ ATPase. Then the extracellular Na^+ was withdrawn in the presence of these substances. The osmolarity of Na^+-free medium was corrected by the appropriate addition of *N*-methylglucamin. The rate of cytosolic Ca^{2+} accumulation was monitored with the fluorescence indicator fura-2.

2.5.2. Protocol 2: Simulated ischemia–reperfusion in isolated cardiomyocytes

The protocol of simulated ischemia–reperfusion in single cardiomyocytes was established in our previous studies [6,7,10,17]. This protocol consists of 80 min of anoxia at pH_o 6.4 and 10 min of reoxygenation at pH_o 7.4. This protocol has been shown to produce rigor contracture, cytosolic Ca^{2+} overload and acidosis during anoxia and Ca^{2+} oscillation, pH_i recovery and irreversible hypercontracture during reoxygenation. Because of the sarcolemmal integrity changes in ion homeostasis during reoxygenation can be estimated and compared with the degree of hypercontracture.

Three groups of experiments were performed. In the control group, the standard protocol of anoxia and reoxygenation was performed. The reoxygenation buffer contained the vehicle DMSO (dilution 1/1000). In the second group, reoxygenation was performed in presence of 10 μmol/l KB-R 7943 (solved in DMSO). In the third group, cells were reoxygenated under nominally Ca^{2+}-free conditions.

2.5.3. Protocol 3: Inhibition of the Na^+/Ca^{2+} exchanger in forward and reverse mode by depletion of cardiomyocytes from internal Na^+ during anoxia and reoxygenation

In these experiments the NCE was inhibited by the depletion of intra- and extracellular Na^+ as described by Siegmund et al. [18]. Na^+ was replaced by *N*-methyl-D-glucamine. The depletion of cardiomyocytes of internal Na^+ was performed by the following protocol. The cells were first incubated with normoxic Tyrode solution for 5 min, thereafter with a Na^+ and additionally Ca^{2+} free incubation buffer. Absence of external Ca^{2+} was chosen to avoid the Na^+ withdrawal contraction. After 20 min of Na^+- and Ca^{2+}-free incubation Ca^{2+} (1 mmol/l) was readmitted. Then the perfusion media was switched to the anoxic Na^+-free medium. The pH_o of the anoxic media was 7.4. After the fura-2 signal had reached the same end-anoxic level as in anoxic experiments in presence of Na^+, the cells were reoxygenated with use of Na^+-free normoxic media. KB-R 7943 (10 μmol/l) was applied with the onset of reoxygenation. Following this protocol, pH_i at the 4th min of reoxygenation was the same as in cells reoxygenated after simulated ischemia without the inhibition of Na^+ dependent transport processes.

2.5.4. Protocol 4: Ischemia–reperfusion in the whole heart

After 30 min of normoxic perfusion, hearts were subjected to global ischemia for 60 min by clamping the aortic in-flow line, and reperfused for 60 min. Hearts were allocated to one of two groups receiving, respectively, 10 μmol/l KB during the first 10 min of reperfusion ($n=6$) or only the vehicle DMSO (dilution 1/1000) (control group, $n=8$). The composition of the Krebs–Henseleit bicarbonate buffer (KH), used for heart perfusion, was as follows (in mmol/l): NaCl 140.0, $NaHCO_3$ 24.0, KCl 2.7, KH_2PO_4 0.4, $MgSO_4$ 1.0, $CaCl_2$ 1.8, and glucose 11.0. KH was continuously gassed with 95% O_2–5% CO_2 which results in a medium pH 7.4.

2.6. Materials

Medium 199 was purchased from Boehringer Mannheim; fetal calf serum from Gibco; acetoxymethyl esters of fura-2 and BCECF from Paesel and Lorey (Frankfurt, Germany); KB-R 7943 was a gift from Kanebo (Osaka, Japan). All other chemicals were from Merck or Sigma and of highest purity available.

2.7. Statistics

Data are given as mean values±S.E.M. from n individual cells investigated in separate experiments. Statistical comparisons were performed by one-way ANOVA and use of the Student–Newman–Keuls test for posthoc analysis. Differences with $P<0.05$ were regarded as statistically significant.

3. Results

3.1. Inhibition of the reverse mode of the NCE by KB-R 7943 in acidified cardiomyocytes

When cardiomyocytes were exposed to 80 min simulated ischemia (anoxia at pH_o 6.4) the pH_i at the beginning of reoxygenation was 6.4. During the reoxygenation period (pH_o 7.4) the pH_i recovered to the pre-anoxic value 7.1. After 4 min of reoxygenation, when the frequency of Ca^{2+} oscillation reached its maximum, the pH_i was 6.8. We therefore tested the inhibitory effect of 10 μmol/l KB on the reverse mode of the NCE at pH_i 6.8. We found that this degree of cytosolic acidosis could be established in normoxic cardiomyocytes by incubating them for 10 min with medium adjusted to pH 6.8. In order to activate the reverse mode of the NCE, cardiomyocytes were superfused with Na^+-free medium in presence of 1 mmol/l Ca^{2+}. Under these conditions Na^+ withdrawal led to a rapid rise of cytosolic Ca^{2+}, as shown by the rise of the fura-2 ratio (fura-2 ratio under control conditions: 0.52±0.06 a.u.; $n=6$; fura-2 ratio after 2 min of Na^+ withdrawal: 2.33±0.04 a.u.; $n=6$; $P<0.05$). A 10-min treatment with 10 μmol/l KB before and during Na^+-free superfusion was sufficient to completely inhibit the reverse mode of NCE (fura-2 ratio after 2 min of Na^+ withdrawal in presence of 10 μmol/l KB: 0.49±0.05 a.u.; $n=6$).

3.2. Influence of KB and Ca^{2+}-free media on cytosolic Ca^{2+} recovery during 10 min reoxygenation

The ratio of fura-2 fluorescence was monitored to evaluate changes in cytosolic Ca^{2+} concentration during simulated ischemia and reoxygenation. During simulated ischemia isolated cardiomyocytes developed Ca^{2+} overload. According to the calibration protocol, the initial ratio of 0.4 (a.u.) in normoxic cells corresponds to a $[Ca^{2+}]_i$ of 72 nmol/l, the end-anoxic fura-2 ratio of 2.2 (a.u.) corresponds to a $[Ca^{2+}]_i$ of 1.9 μmol/l. This level represents severe Ca^{2+} overload. When cells were reoxygenated in medium with pH 7.4, the fura-2 ratio declined to the initial control value within 10 min. Concomitantly with the Ca^{2+} recovery, transient Ca^{2+} oscillations occurred. Fig. 1 shows original recordings of the fura-2 ratio under control conditions and in presence of KB. Under either condition the fura-2 ratio declined within 10 min to the pre-anoxic level. In controls, Ca^{2+} oscillations were much more rapid than in presence of KB. Note, that the envelopes of upper and lower values of the fura-2 ratio recording are similar in both cases. Fig. 2 presents the statistical summary of these upper and lower values for cardiomyocytes reoxygenated under control conditions, in

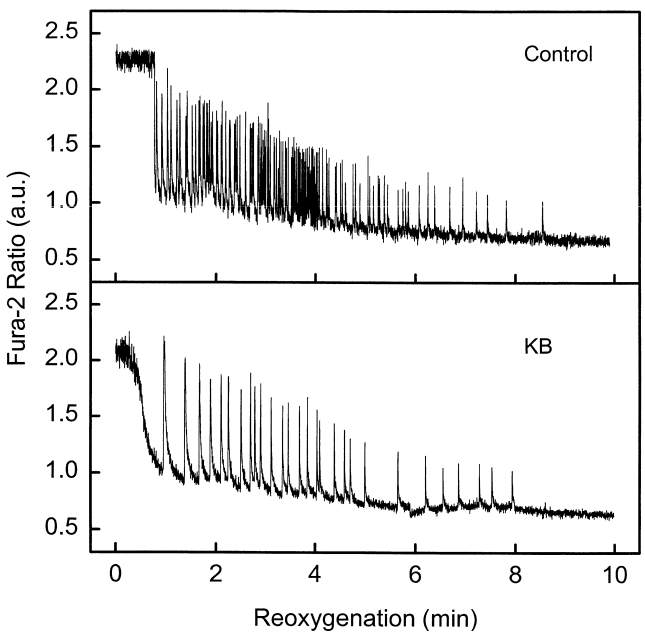

Fig. 1. Time course of fura-2 ratio (original recordings, arbitrary units) under control conditions (top) or in presence of 10 μmol/l KB (below) in cardiomyocytes during the first 10 min of reoxygenation.

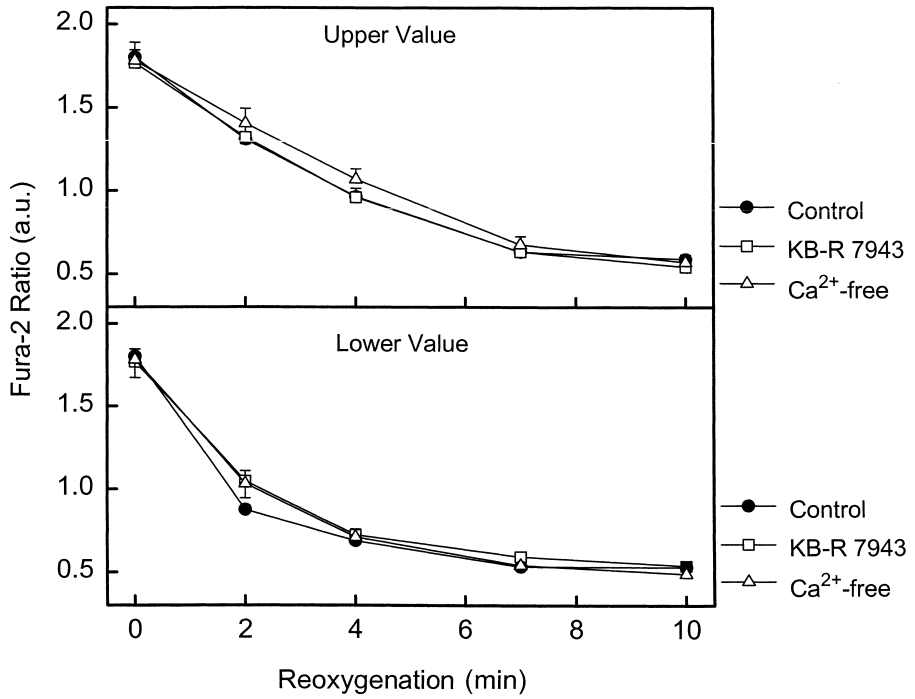

Fig. 2. Upper and lower values of the fura-2 ratio signal (a.u.) under control conditions (closed circle), in presence of KB (open square) or in nominally Ca^{2+}-free (open triangle) media in cardiomyocytes during 10 min of reoxygenation. Data are given as mean±S.E.M.; $n=8$.

presence of KB or in nominally Ca^{2+}-free media. For these parameters significant differences did not occur at any given time. At 10 min reoxygenation upper and lower values coincided since oscillations had ceased at that time. The three experimental conditions nevertheless differed with respect to the frequency of Ca^{2+} oscillations (Fig. 3). Under control conditions the oscillations reached a maximum frequency of about 30 min^{-1} between the 2nd and 4th min of reoxygenation and slowed down thereafter. Under Ca^{2+}-free conditions or in the presence of KB, the

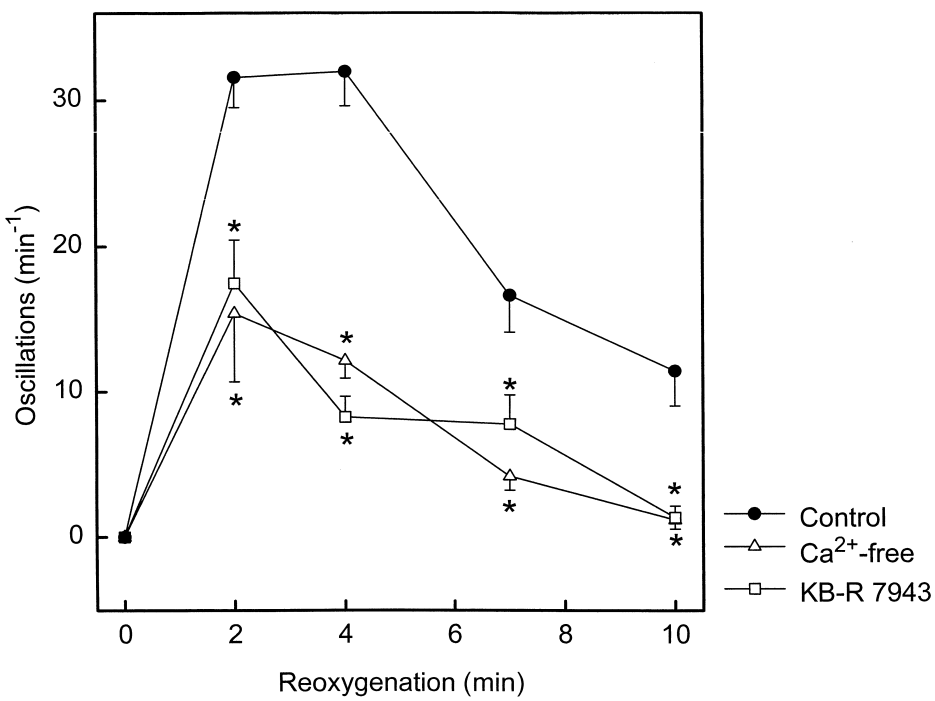

Fig. 3. Oscillation frequency of the fura-2 ratio signal under control conditions (closed circle), in presence of KB (open square) or in nominally Ca^{2+}-free media (open triangle) in cardiomyocytes during the first 10 min of reoxygenation. Data are given as mean±S.E.M. *, $P<0.05$; $n=8$.

oscillations were less frequent at any time. To estimate net changes in the cytosolic Ca^{2+} balance during reoxygenation, the integral of the fura-2 signal was determined. The integral was calculated from the beginning of reoxygenation to complete recovery of the fura-2 signal (10 min of reoxygenation). The integral in the control cells amounted to 1236±116 a.u., in the KB treated cells to 1346±59 a.u. and in the cells reperfused under nominally Ca^{2+}-free conditions to 1332±141 a.u. ($n=8$, no significant differences). These data indicate that the overall rate of Ca^{2+} removal from reoxygenated cells was comparable under all experimental conditions tested, in spite of the differences in Ca^{2+} oscillations.

3.3. Protection against reoxygenation induced hypercontracture

Fig. 4 shows the cell length after 10 min reoxygenation. The changes in cell length are expressed as relative changes compared to the preceding end-ischemic length. Under control conditions the cell length was reduced to 69±2% of the end-ischemic cell length. The inhibition of the reverse mode of NCE with KB and also the superfusion with nominally Ca^{2+}-free media reduced significantly the development of hypercontracture (KB: 80±4%; Ca^{2+}-free: 84±3% of the end-ischemic cell-length; both $P<0.05$ vs. control).

3.4. Recovery of cytosolic pH during reoxygenation

Under normoxic control conditions pH_i of cardiomyocytes was 7.1. Superfusion of cardiomyocytes with anoxic medium at pH_o 6.4 led to a pronounced acidification of the cytosol. After 80 min, the pH_i was 6.4. During reoxygenation the intracellular pH recovered within 10 min from this end-anoxic value to the pre-anoxic control value. As shown in Fig. 5 neither Ca^{2+}-free media nor KB affected the time course of pH_i recovery.

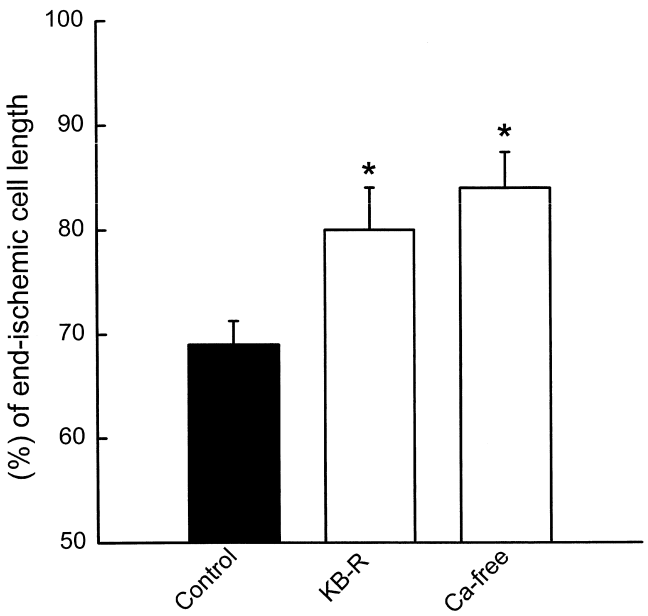

Fig. 4. Cell length (% of end-ischemic) under control conditions, in presence of KB or in nominally Ca^{2+}-free media in cardiomyocytes after the first 10 min of reoxygenation. Data are given as mean±S.E.M. *, $P<0.05$; $n=8$.

3.5. Reoxygenation of Na^+ depleted cells

Apart from the use of KB and extracellular Ca^{2+} removal, the importance of the Na^+/Ca^{2+} exchange during the early phase of reoxygenation was tested with yet another protocol. In this protocol cardiomyocytes were first depleted of their Na^+ contents by incubation in Na^+-and Ca^{2+}-free media, then exposed to anoxic media at pH 7.4 in presence of Ca^{2+} but not Na^+. Once the fura-2 ratio had reached the plateau level of 2.2 a.u., the cells were reoxygenated in media containing Ca^{2+} but not Na^+ with pH 7.4. As shown in Table 1 this treatment led to Ca^{2+} oscillations with a frequency much lower than under Na^+ containing control conditions. Additional presence of KB did not further reduce the oscillation frequency, indicating that KB has no inhibitory effect per se on oscillations. At the 4th minute of reoxygenation, when oscillation frequency was determined, the pH_i was the same in Na^+-depleted cells and cells not depleted from Na^+, i.e. 6.8.

3.6. Reduction of reoxygenation-induced injury by KB treatment in the whole heart

Using the Langendorff-perfused heart model we studied whether KB can also protect the whole heart against acute reperfusion injury. KB, 10 μmol/l, was administered for 10 min with onset of the reperfusion. Effects on LVEDP, LVdevP and LDH release were investigated. Presence of KB significantly reduced the LVEDP (Fig. 6) and improved the left ventricular function given as LVdevP. In Fig. 7 the release of LDH during reperfusion following 60 min of ischemia is presented for control hearts and hearts receiving 10 μmol/l KB. In the control group pronounced enzyme release was observed during the early phase of reperfusion. Presence of KB reduced markedly the reperfusion induced release of LDH, from 81.08±9.9 U/60 min/g dry weight under control conditions to 49.25±8.84 U/60 min/g dry weight ($P<0.05$). In vitro analyses showed that KB does not interfere with the LDH activity assay.

4. Discussion

The aim of this study was to investigate the role of the reverse mode of the NCE in reperfusion injury in isolated cardiomyocytes and in the whole heart. The main findings are the following: Inhibition of reverse mode activation of

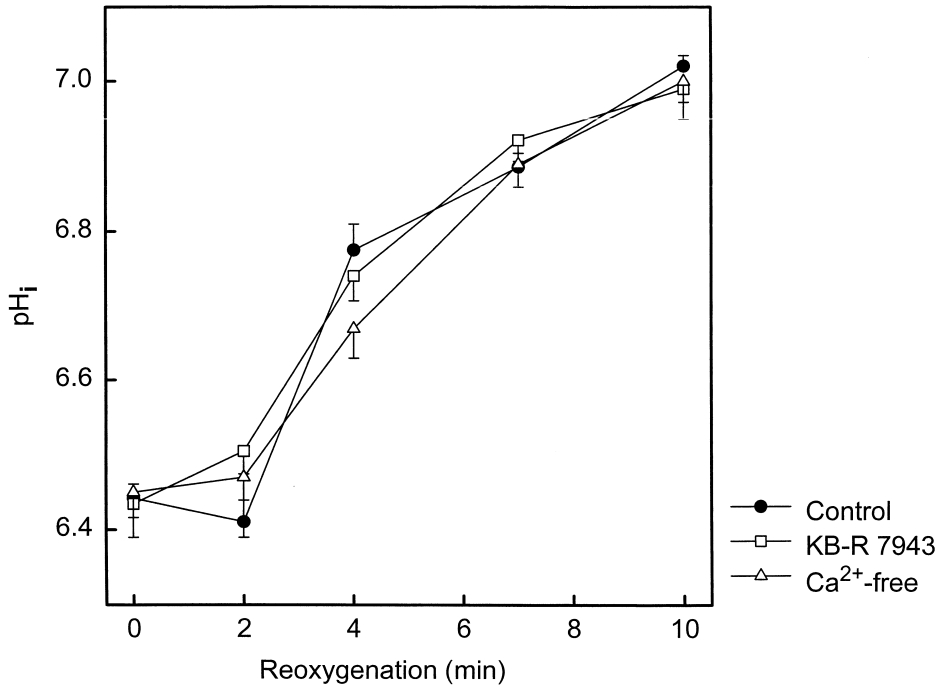

Fig. 5. Cytosolic pH under control conditions (closed circle), in presence of KB (open square) or in nominally Ca^{2+}-free media (open triangle) in cardiomyocytes during the first 10 min of reoxygenation. Data are given as mean±S.E.M. *, $P<0.05$; $n=8$.

the NCE during the early phase of reoxygenation (i) reduces Ca^{2+} oscillations in cardiomyocytes (ii) protects cardiomyocytes against reoxygenation-induced hypercontracture and (iii) protects ischemic–reperfused hearts against contracture and enzyme release.

Isolated cardiomyocytes exposed to conditions of simulated ischemia (pH 6.4) and reoxygenation (pH 7.4) have been characterised in several previous studies [7,17,19]. During simulated ischemia the cardiomyocytes lose their energy reserves and, consecutively, undergo rigor shortening. They develop marked cytosolic overload with H^+, Na^+ and Ca^{2+}. It was shown before that the reverse mode of the NCE can play an important role in the cytosolic accumulation of Ca^{2+} in ischemic cardiomyocytes [10,20–24]. The ischemic disturbance of ion homeostasis can be reversed when the cells are reoxygenated in media with normal extracellular pH [6,7,10]. The transsarcolemmal extrusion of Ca^{2+} from the reoxygenated cells is mediated by a tandem mechanism consisting of the Na^+ pump, creating a normal transsarcolemmal Na^+ gradient, and the NCE, using the Na^+ gradient for the extrusion of Ca^{2+} [20,25]. When acting in this tandem mechanism, the NCE operates in its forward mode, driven by the re-established Na^+ gradient and membrane potential. At some point during the early phase of reoxygenation, therefore, the operation of the NCE changes its directional mode. The present study was based on the hypothesis that during the first few minutes of reoxygenation the equilibrium conditions at the cell membrane still favour the reverse mode of NCE operation and that this influences significantly the outcome of reoxygenation. The results confirm this hypothesis.

Table 1
Effect of KB-R 7943 (10 μmol/l) on reoxygenation-induced Ca oscillations after 4 min of reoxygenation under Na^+-free and Na^+ containing conditions. KB was only given during the reoxygenation period

Experimental conditions	Oscillation frequency (min^{-1})
Na^+-containing media − KB (control)	31.2±2.4; $n=8$
Na^+-containing media + KB	8.3±1.4; $n=8$
Na^+-free media − KB	11.4±0.02; $n=30$
Na^+-free media + KB	10.2±1.2; $n=30$
Nominally Ca^{2+}-free media	12.2±1.2; $n=8$

When the reverse mode of NCE was inhibited during the early phase of reoxygenation, the reoxygenation-induced oscillations of cytosolic Ca^{2+} were markedly reduced. Three different protocols were applied in the cell model to reduce oscillations of cytosolic Ca^{2+} in the early reoxygenation phase, namely application of KB, extracellular Ca^{2+} removal and Na^+ depletion of the cells. All protocols had the same result, i.e. they reduced the oscillatory activity to about one-third of controls. Previous work has demonstrated that these early oscillations of cytosolic Ca^{2+} are due to the uptake and release of Ca^{2+} accumulated during ischemic conditions in the cytosol. They start once resumption of oxidative energy production supplies sufficient amounts of ATP to the SR Ca^{2+} pump [6]. The specific blocker of SR Ca^{2+} release, ryanodine, can suppress these oscillations [6]. The comparison with results of the present study shows that Ca^{2+} influx through the NCE in reverse

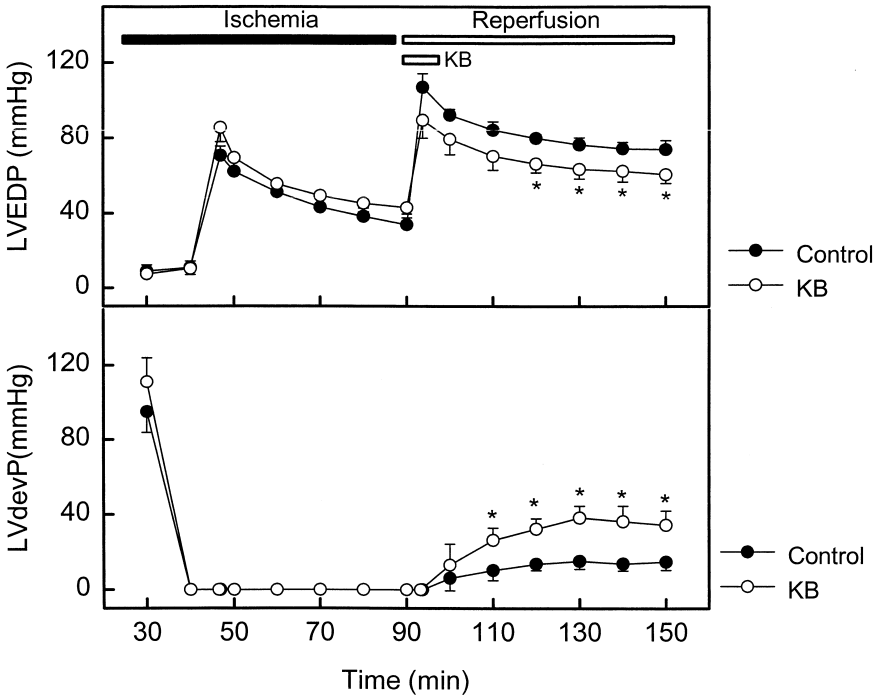

Fig. 6. Changes in LVEDP (top) and LVdevP (below) during equilibration, 60 min ischemia and 120 min reperfusion in control hearts (closed circle) and in hearts receiving 10 μmol/l KB (open circle) in isolated hearts during the first 10 min of reperfusion. Data are given as mean±S.E.M. *, $P<0.05$; $n=6$.

mode operation is responsible only for a part, but an important part, of these SR-dependent Ca^{2+}-oscillations.

The total amount of Ca^{2+} entering the cardiomyocytes through the reverse mode of the NCE during the early phase of reoxygenation seems to be small. This is because, first, the fura-time integral remained unchanged and, second, the time to reach an end of Ca^{2+} oscillations and to re-establish a normal resting level of cytosolic Ca^{2+}

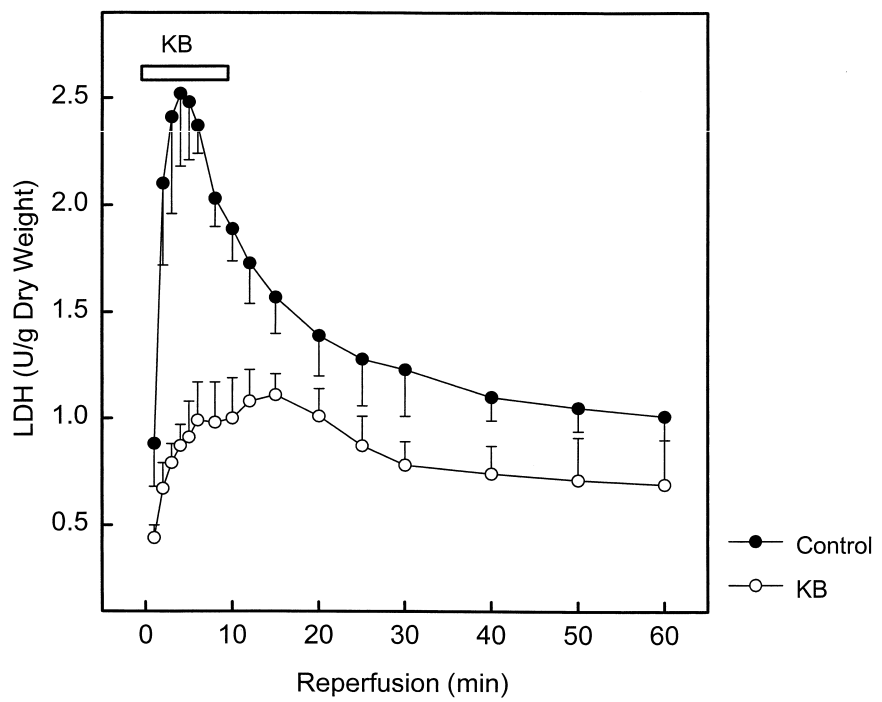

Fig. 7. LDH release during reperfusion following 60 min ischemia in control hearts (closed circle) and in hearts receiving 10 μmol/l KB (open circle) in isolated hearts during the first 10 min of reperfusion. Data are given as mean±S.E.M., $n=6$. $P<0.05$ for all data, control vs. KB, between 1 and 15 min.

concentration was not noticeably changed, when the reverse mode operation was inhibited. The Ca^{2+} influx during this early phase of reoxygenation seems therefore to serve only as trigger for SR Ca^{2+} release, thus accelerating the Ca^{2+} cycling between cytosol and SR. Neither KB nor external Ca^{2+} removal, which both protected reoxygenated cardiomyocytes, had an influence on pH_i recovery during reoxygenation. It was important to analyse the rapidity of pH_i recovery since prolonged intracellular acidosis can also protect reoxygenated cardiomyocytes from too rapid Ca^{2+} oscillations and hypercontracture [7]. In the case of cells subjected to Na^+ depletion a particular incubation protocol made sure that pH_i was identical to the value observed with the other protocols at the time when Ca^{2+} oscillation were determined, i.e. at the 4th min of reoxygenation. We also used the Na^+ depletion protocol to show that KB has no direct effect on Ca^{2+} oscillations as it could not affect the oscillatory activity remaining when the NCE had been blocked.

Previous studies have demonstrated that reoxygenation-induced Ca^{2+} oscillations represent the immediate cause for reoxygenation-induced hypercontracture of cardiomyocytes. Reoxygenation-induced hypercontracture has been demonstrated in vitro and in vivo to contribute substantially to acute lethal reperfusion injury in reperfused myocardium [5,26,27]. In tissue, hypercontracture of adjacent cells causes their disruption and leakage of cytosolic enzymes. Agents interfering with SR Ca^{2+} release, such as the specific blocker ryanodine or the anaesthetic halothane, inhibit Ca^{2+} oscillations and hypercontracture in reoxygenated cardiomyocytes [6]. Halothane has also been shown to protect ischemic reperfused hearts against reperfusion-induced contracture and cell death [28]. The present study has discovered another approach to interfere with this pathomechanism of acute reperfusion injury. On the cellular level KB reduces Ca^{2+} oscillations and hypercontracture. On the organ level it reduces diastolic tension and enzyme release and improves contractile function, indicative of a protection against contracture and severe cell injury.

In conclusion, our study shows that pharmacological inhibition of the reverse mode of the NCE with KB-R 7943, applied solely during reperfusion, can be used as a new therapeutical approach to protect myocardial cells against additional Ca^{2+} uptake and cell injury development during the acute phase of reperfusion. The validity of this concept was confirmed by experiments where the NCE was inhibited by extracellular Ca^{2+} removal or cellular Na^+ depletion.

Acknowledgements

The technical help of D. Schreiber and H. Holzträger is gratefully acknowledged. The study was supported by the European Union through a grant of the Biomed-2 program and grant LA 1159/2-1 of Deutsche Forschungsgemeinschaft.

References

[1] Stern MD, Chien AM, Capogrossi MC et al. Direct observation of the ‘oxygen paradox’ in single rat ventricular myocytes. Circ Res 1985;56:899–903.
[2] Hohl C, Ansel A, Altschuld R et al. Contracture of isolated rat heart cells on anaerobic to aerobic transition. Am J Physiol 1982;242:H1022–1030.
[3] Siegmund B, Koop A, Klietz T et al. Sarcolemmal integrity and metabolic competence of cardiomyocytes under anoxia–reoxygenation. Am J Physiol 1990;258:H285–H291.
[4] Barrabes JA, Garcia-Dorado D, Ruiz-Meana M et al. Myocardial segment shrinkage during coronary reperfusion in situ. Pflügers Arch Eur J Physiol 1996;431:519–526.
[5] Ganote CE. Contraction band necrosis and irreversible myocardial injury. J Mol Cell Cardiol 1983;15:67–73.
[6] Siegmund B, Schlack W, Ladilov YV et al. Halothane protects cardiomyocytes against reoxygenation-induced hypercontracture. Circulation 1997;96:4372–4379.
[7] Ladilov YV, Siegmund B, Piper HM. Protection of reoxygenated cardiomyocytes against hypercontracture by inhibition of Na^+/H^+ exchange. Am J Physiol 1995;268:H1531–H1539.
[8] Iwamoto T, Pan Y, Wakabayashi S et al. Phosphorylation-dependent regulation of cardiac Na^+/Ca^{2+}-exchanger via protein kinase C. J Biol Chem 1996;271:13609–13615.
[9] Watano T, Kimura J, Morita T et al. A novel antagonist, No. 7943, of the Na^+/Ca^{2+}-exchange current in guinea-pig cardiac ventricular cells. Br J Pharmacol 1996;119:555–563.
[10] Ladilov Y, Haffner S, Balser-Schäfer C et al. Cardioprotective effects of KB-R 7943: a novel inhibitor of the reverse mode of Na^+/Ca^{2+} exchanger. Am J Physiol 1999;276:H1868–1876.
[11] Mukai M, Terada H, Sugiyama S et al. Effects of a selective inhibitor of Na^+/Ca^{2+} exchange, KB-R 7943, on reoxygenation-induced injuries in guinea pig papillary muscles. J Cardiovasc Pharmacol 2000;35:121–128.
[12] Piper HM, Probst I, Schwartz P. Culturing of calcium stable adult cardiac myocytes. J Mol Cell Cardiol 1982;14:397–412.
[13] Allshire A, Piper HM, Cuthbertson KSR et al. Cytosolic free calcium in single rat heart cells during anoxia and reoxygenation. Biochem J 1987;244:381–385.
[14] Koop A, Piper HM. Protection of energy status of hypoxic cardiomyocytes by mild acidosis. J Mol Cell Cardiol 1992;24:55–65.
[15] Li Q, Altschuld RA, Stokes BT. Quantitation of intracellular free calcium in single adult cardiomyocytes by fura-2 fluorescence microscopy: calibration of fura-2 ratios. Biochem Biophys Res Commun 1987;147:120–126.
[16] Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 1985;260:3440–3450.
[17] Ladilov YV, Siegmund B, Balser C et al. Simulated ischemia increases the susceptibility of rat cardiomyocytes to hypercontracture. Circ Res 1997;80:69–75.
[18] Siegmund B, Ladilov YV, Piper HM. Importance of sodium for recovery of calcium control in reoxygenated cardiomyocytes. Am J Physiol 1994;267:H506–H513.
[19] Ladilov Y, Balser C, Piper HM. Protection of rat cardiomyocytes against simulated ischemia and reoxygenation by treatment with protein kinase C activator. Circ Res 1998;82:451–457.
[20] Haigney M, Lakatta EG, Stern MD et al. Sodium channel blockade reduces sodium loading and sodium-dependent calcium loading. Circulation 1994;90:391–399.

[21] Murphy E, Perlmann M, London RE et al. Amiloride delays the ischemia-induced rise in cytosolic free calcium. Circ Res 1991;68:1250–1258.
[22] Tani M, Neely JR. Na^+ accumulation increases Ca^{2+} overload and impairs function in anoxic rat hearts. J Mol Cell Cardiol 1990;22:57–72.
[23] Ziegelstein RC, Zweier JL, Mellits ED et al. Dimethylthiourea, an oxygen radical scavenger, protects isolated cardiac myocytes from hypoxic injury by inhibition of Na^+–Ca^{2+} exchange and not by its antioxidant effects. Circ Res 1992;70:804–811.
[24] Ver-Donck L, Borgers M, Verdonck F. Inhibition of sodium and calcium overload pathology in the myocardium: a new cytoprotective principle. Cardiovasc Res 1993;27:349–357.
[25] Siegmund B, Zude R, Piper HM. Recovery of anoxic–reoxygenated cardiomyocytes from severe Ca^{2+}-overload. Am J Physiol 1992;263:H1262–H1269.
[26] Schlack W, Uebing A, Schafer M et al. Regional contractile blockade at the onset of reperfusion reduces infarct size in the dog heart. Pflügers Arch 1994;428:134–141.
[27] Schlüter KD, Schwartz P, Siegmund B et al. Prevention of the oxygen paradox in hypoxic–reoxygenated hearts. Am J Physiol 1991;261:H416–H423.
[28] Schlack W, Hollmann M, Stunneck J et al. Effect of halothane on myocardial reoxygenation injury in isolated rat heart. Br J Anaesth 1996;76:860–867.

ELSEVIER

Cardiovascular Research 55 (2002) 739–748

Cardiovascular Research

www.elsevier.com/locate/cardiores

Effect of inhibition of Na^+/Ca^{2+} exchanger at the time of myocardial reperfusion on hypercontracture and cell death

Javier Inserte[a], David Garcia-Dorado[a,*], Marisol Ruiz-Meana[a], Ferran Padilla[a], José A. Barrabés[a], Pilar Pina[a], Luis Agulló[a], Hans Michael Piper[b], Jordi Soler-Soler[a]

[a]*Servicio de Cardiologia, Hospital Universitari Vall d'Hebron, Passeig Vall d'Hebron 119-129, 08035 Barcelona, Spain*
[b]*Physiologisches Institut Justus-Liebig-Universität, Giessen, Germany*

Received 8 October 2001; accepted 29 April 2002

Abstract

Objective: There is recent evidence that Ca^{2+} influx via reverse mode Na^+/Ca^{2+} exchange (NCX) at the time of reperfusion can contribute to cardiomyocyte hypercontracture. However, forward NCX is essential for normalization of $[Ca^{2+}]_i$ during reperfusion, and its inhibition may be detrimental. This study investigates the effect of NCX inhibition with KB-R7943 at the time of reperfusion on cell viability. **Methods:** The effect of several concentrations of KB-R7943 added at reperfusion was studied in Fura-2 loaded quiescent cardiomyocytes submitted to 40 min of simulated ischemia (NaCN 2 mM, pH 6.4), and in rat hearts submitted to 60 min of ischemia. $[Ca^{2+}]_i$ and cell length were monitored in myocytes, and functional recovery and LDH release in isolated hearts. From these experiments an optimal concentration of KB-R7943 was identified and tested in pigs submitted to 48 min of coronary occlusion and 2 h of reperfusion. **Results:** In myocytes, KB-R7943 at concentrations up to 15 μM reduced $[Ca^{2+}]_i$ rise and the probability of hypercontracture during re-energization ($P<0.01$). Nevertheless, in rat hearts, the effects of KB-R7943 applied during reperfusion after 60 min of ischemia depended on concentration and timing of administration. During the first 5 min of reperfusion, KB-R7943 (0.3–30 μM) induced a dose-dependent reduction in LDH release (half-response concentration 0.29 μM). Beyond 6 min of re-flow, KB-R7943 had no effect on LDH release, except at concentrations ≥15 μM, which increased LDH. KB-R7943 at 5 μM given during the first 10 min of reflow reduced contractile dysfunction ($P=0.011$), LDH release ($P=0.019$) and contraction band necrosis ($P=0.014$) during reperfusion. Intracoronary administration of this concentration during the first 10 min of reperfusion reduced infarct size by 34% ($P=0.033$) in pigs submitted to 48 min of coronary occlusion. **Conclusions:** These results are consistent with the hypothesis that during initial reperfusion NCX activity results in net reverse mode operation contributing to Ca^{2+} overload, hypercontracture and cell death, and that NCX inhibition during this phase is beneficial. Beyond this phase, NCX inhibition may impair forward mode-dependent Ca^{2+} extrusion and be detrimental. These findings may help in the design of therapeutic strategies against lethal reperfusion injury, with NCX as the target.

Keywords: Calcium (cellular); Ischemia; Myocytes; Na/Ca-exchanger; Reperfusion

This article is referred to in the Editorial by S. Anderson (pages 706–707) in this issue.

1. Introduction

Cell death occurring during the first minutes of reperfusion is explained as a consequence of the combined action of mechanical and chemical stress on cardiomyocytes in which previous ischemia has induced sarcolemmal and cytoskeletal fragility [1]. Excessive contractile activation, secondary to increased energy availability together with increased intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), plays a central role among the causes of stress [2,3]. Excessive contractile activation causes hypercontracture, which in myocardium in situ results in sarcolemmal rupture, mas-

*Corresponding author. Tel.: +34-93-489-4038; fax: +34-93-489-4032.
E-mail address: dgdorado@hg.vhebron.es (D. Garcia-Dorado).

Time for primary review 22 days.

0008-6363/02/$ – see front matter
PII: S0008-6363(02)00461-3

sive enzyme release, and a pathologic pattern known as contraction band necrosis [4,5].

The mechanisms responsible for altered Ca^{2+} handling in reperfused cardiomyocytes have not been completely elucidated. $[Ca^{2+}]_i$ rises during prolonged anoxia or ischemia [6,7]. During reoxygenation or reperfusion, Ca^{2+} sequestration by the sarcoplasmic reticulum (SR) tends to rapidly normalize $[Ca^{2+}]_i$ [8]. Ca^{2+} uptake by the SR may be followed by Ca^{2+} release and re-uptake resulting in Ca^{2+} oscillations that contribute to the development of hypercontracture [8]. It has been proposed that Ca^{2+} influx at reperfusion can contribute to reperfusion injury [9–11] at least in part by increasing Ca^{2+} oscillations.

In mammalian cardiomyocytes, sarcolemmal Na^+/Ca^{2+} exchange (NCX) takes place through the NCX1 isoform, with a Ca^{2+}:Na^+ coupling ratio of 1:3 and a net movement of a positive charge. The direction of NCX operation depends on the difference between trans-membrane potential (V_M) and reversal potential (RP), determined by the intra and extracellular concentrations of Na^+ and Ca^{2+}. During diastole RP is close to −60 mV, and positive with respect to resting V_M (close to −80 mV), which results in forward (Ca^{2+} out) NCX. During systolic depolarization, RP is negative with respect to V_M, which results in reverse NCX. The contribution of reverse NCX to Ca^{2+} transients is negligible in normal conditions in most animal species [12]. However, reverse NCX may have an important pathophysiological role in situations in which $[Na^+]_i$ is elevated. The role of reverse NCX in the genesis of cytosolic Ca^{2+} overload during ischemia has been recently established in cardiomyocytes [13].

The highly increased $[Na^+]_i$ observed after re-energization is likely to be the result of three major factors: first, Na^+ gain during prior metabolic inhibition [14]; second, additional Na^+ influx associated with normalization of pH_i through Na^+/H^+ exchange and Na^+/HCO_3^- cotransport; and third, Na^+ entry from adjacent myocytes via gap-junctions [15]. Moreover, Na^+/K^+-ATPase activity may be impaired in myocardium that is reperfused after prolonged ischemia [16]. The concept that Ca^{2+} influx at the time of re-energization may contribute to cell injury secondary to transient anoxia or ischemia is supported by the protective effect observed when extracellular Ca^{2+} concentration is reduced during reperfusion [9,11], and by the fact that high extracellular Na^+ during reperfusion attenuates post-ischemic contractile dysfunction [17]. However, evidence of the long suspected contribution of reverse NCX to Ca^{2+} overload during reperfusion [10,17] has remained elusive until recently [18], due in part to the absence of sufficiently selective inhibitors of this exchanger.

An isothiourea derivative, 2-[2-4[(nitrobenzyloxy)phenyl]ethyl]isothiourea methanesulphonate (KB-R7943; Kanebo, Osaka, Japan), was recently characterized as a fairly selective inhibitor of NCX in cardiomyocytes [19–22]. Under ionic conditions allowing alternate net forward and net reverse NCX transport during the cardiac cycle in response to changes in membrane potential, KB-R7943 inhibits forward and reverse exchange at a virtually identical half-maximal inhibition concentration (IC_{50}) (~1 μM) [21]. However, in cells under ionic conditions allowing a preponderance of NCX reverse operation ($[Na^+]_{out}=0$), the inhibitory ability of KB-R7943 is significantly higher ($IC_{50}=0.32$ μM) than in cells exposed to ionic conditions allowing a preponderance of forward transport via NCX ($[Ca^{2+}]_{out}$~0, $IC_{50}=17$ μM) or in cells exposed to ionic conditions allowing alternate net forward or net reverse NCX transport ($IC_{50}=1$ μM) [19–21]. The mechanism responsible for these differences is not known, but it could be related to a different prevalence of distinct exchange transport states under the different ionic conditions. Inhibition becomes higher as the prevalence of the intracellular $3Na^+$-loaded conformation increases [23].

In a recent study we showed that the presence of 10 μM KB-R7943 in the reoxygenation buffer reduced Ca^{2+} oscillations and hypercontracture in cardiomyocytes previously exposed to hypoxia, and attenuated LDH release in isolated hearts exposed to 60 min of ischemia [18]. The potentially harmful effects of NCX inhibition using forward NCX to extrude Ca^{2+} in reperfused cells, the dependence of these effects on the time of administration, and the effects of NCX inhibition during in vivo reperfusion were not analyzed in that study. These important points are addressed in the present study. The effects of different concentrations of KB-R7943 added at the time of reperfusion were analyzed in cardiomyocytes exposed to simulated ischemia, and in isolated rat hearts submitted to non-flow ischemia. To evaluate the potential relevance of the effects of NCX inhibition to in vivo conditions, selected concentrations of KB-R7943 were administered to pigs during coronary reperfusion.

2. Methods

The experimental procedures conformed with the *Guide for the Care and Use of Laboratory Animals* published by the United States National Institute of Health (NIH Publication No. 85-23, revised 1996), and were approved by the Research Commission on Ethics of the Hospital Vall d'Hebron.

2.1. Studies in isolated cardiomyocytes

Ventricular myocytes were isolated from adult male Sprague–Dawley rats and plated in glass-bottom dishes coated with 199/HEPES medium containing 4% fetal calf serum (Gibco), as previously described [24]. Then 2 h after plating, non-attached cells were discarded by changing the medium (in mM: NaCl 140, KCl 3.6, $MgSO_4$ 1.2, $CaCl_2$ 1, HEPES 20, pH 7.4).

2.1.1. Experimental protocols

The inhibitory effect of KB-R7943 on NCX during conditions allowing a preponderance of reverse transport was studied by monitoring $[Ca^{2+}]_i$ in normoxic myocytes during Na^+ withdrawal ($[Na^+]_{out}=0$; osmolarity corrected by addition of Li^+) for 30 s after functional blockade of the SR with 150 nM thapsigargin and 10 μM ryanodine [18,25].

To analyze the effect of KB-R7943 during simulated reperfusion, cardiomyocytes were first subjected to 40 min of metabolic inhibition (in mM: NaCl 140, KCl 3.6, $MgSO_4$ 1.2, $CaCl_2$ 1, HEPES 20, NaCN 2, 2-deoxyglucose 20, pH 6.4). The inhibitor and 2-deoxyglucose were then removed, 5 mM glucose was added, and pH was normalized at 7.4. KB-R7943 at 0 (control), 5, 10 or 15 μM was added to the simulated reperfusion buffer. The contribution of inhibition of Ca^{2+} oscillations to the effects of inhibition of NCX with KB-R7943 was analyzed in additional experiments in which simulated reperfusion was performed in the presence of either 10 μM KB-R7943 or SR functional blockade, both these conditions, or neither of them. The changes in osmolarity of the medium associated with the addition or removal of metabolic inhibition were not corrected.

2.1.2. Measurement of isolated cell morphology and $[Ca^{2+}]_i$

All experiments were performed at 37 °C on the stage of an inverted microscope [15]. Individual myocytes were imaged throughout the experiment, and changes in cell length and $[Ca^{2+}]_i$ were simultaneously monitored using color-coded 340/380 ratio fluorescence images (QuantiCell900, Visitech, UK) in myocytes loaded with Fura-2 [15]. Cardiomyocyte hypercontracture was defined as a reduction in cell length >10% of the length at the end of the metabolic inhibition period.

2.2. Studies in the isolated perfused rat heart

Hearts from male Sprague–Dawley rats ($n=40$) were perfused with a modified Krebs-Henseleit bicarbonate buffer (in mM: NaCl 140, $NaHCO_3$ 24, KCl 2.7, KH_2PO_4 0.4, $MgSO_4$ 1, $CaCl_2$ 1.8, and glucose 11) at a constant pressure of 60 mmHg [24]. Left ventricle (LV) pressure was monitored during equilibration through the use of a water-filled latex balloon inserted into the LV and inflated to obtain an end-diastolic pressure (LVEDP) between 6 and 8 mmHg [24]. Coronary flow was measured at regular intervals. Lactate dehydrogenase (LDH) activity was measured in samples of coronary effluent by spectrophotometry, as previously described [24].

2.2.1. Experimental protocol

In the ischemia–reperfusion experiments, hearts were subjected to 37 °C non-flow ischemia for 60 min, and reperfused for 60 min. KB-R7943 at concentrations ranging from 0 (control) to 30 μM was added to the perfusion buffer during the first 10 min of reperfusion. In additional experiments ($n=8$), 15 μM KB-R7943 or its vehicle was added only during the first 4 min of reperfusion. The balloon was deflated during ischemia [26] and at the end of the reperfusion period it was filled again with the same amount of water as had been used during the preischemic perfusion.

In a separate series of experiments, the effect of 5 μM KB-R7943 (vs. vehicle) was investigated in 12 hearts in which LV pressure was monitored throughout the experiment by maintaining the LV balloon inflated during ischemia and reperfusion.

2.2.2. Histological analysis

Cross-sectional 4-μm midventricular sections were stained with Masson's trichrome. Contraction band necrosis was morphometrically quantified as previously described [24]. Two perpendicular lines crossing at the center of the LV cavity were drawn for each section. Serial microphotographs of adjacent optical fields (×400) were obtained along these lines, and digitized for subsequent analysis (Olympus DP10 camera and Micro Image™, Olympus Optical, Japan). Each microphotograph was graded using a semiquantitative scoring system from 0 to 3 (0, 1, 2 and 3 correspond to contraction band necrosis involving <1/4, 1/4–1/2, 1/2–3/4 and >3/4 of the photographed frame, respectively) [24], and the average score was calculated for each heart.

2.3. Transient coronary occlusion in the in situ pig heart

A total of 12 Large White pigs weighing 30–40 kg were premedicated with 10 mg/kg azaperone i.m., anesthetized with thiopental 30 mg/kg i.v., followed by continuous infusion, intubated, mechanically ventilated with room air, and monitored as previously described [23]. A midline sternotomy was performed and the left anterior descending coronary artery (LAD) was dissected free at its midpoint and surrounded by an elastic snare. Two pairs of 1-mm diameter ultrasonic crystals were inserted into the inner third of the LV wall in the LAD and circumflex territory, respectively, and LV pressure and LAD coronary blood flow were measured [4,24]. A 2.5F catheter for intracoronary infusion was advanced through a Judkins 7F guiding catheter into the LAD until its tip was placed immediately proximal to the occlusion site [24].

2.3.1. Study protocol

A total of 10 animals were submitted to 48 min of LAD occlusion and randomly allocated to receive an intracoronary infusion of saline alone or saline containing 35 μM KB-R7943 during the first 10 min of reperfusion. In both groups, the infusion rate was continuously adjusted to obtain a final concentration of 5 μM KB-R7943. In

additional experiments, animals were allocated to receive KB-R7943 at 15 μM ($n=4$) or saline ($n=2$). Finally, coronary occlusion was not performed in two pigs and KB-R7943 at a final concentration of 5 μM was infused into the mid LAD for 10 min.

2.3.2. Infarct size measurement

After 2 h of reperfusion the LAD was re-occluded and 5 ml of 10% fluorescein was injected into the left atrium. The heart was excised, cooled at 4 °C, and cut into 5–7-mm slices that were imaged (Olympus Digital Camera C-1400L) under UV light to outline the area at risk. The slices were then incubated at 37 °C for 10 min in 1% triphenyltetrazolium chloride (pH 7.4) and imaged again under white light to outline the area of necrosis. The area at risk and the area of necrosis were measured on the digitized images, and the mass of myocardium at risk and the mass of necrotic myocardium were calculated as described [2,24].

2.4. Statistical analysis

Differences between groups were assessed by means of one-way ANOVA. Individual comparisons between groups were performed by the Student's *t*-test for independent samples. Changes along time were assessed by multiple ANOVA. Significance was set at a *P*-value of 0.05. Results are expressed as mean±S.E.M.

3. Results

3.1. Effect of KB-R7943 on hypercontracture and $[Ca^{2+}]_i$ kinetics in isolated cardiomyocytes

In normoxic myocytes, KB-R7943 inhibited $[Ca^{2+}]_i$ rise induced by Na^+ withdrawal with a calculated IC_{50} of 0.15±0.02 μM (Fig. 1).

After 40 min of metabolic inhibition, re-energization induced hypercontracture in 77.2% of cells within 5 min. The presence of KB-R7943 at 5, 10 or 15 μM in the re-energization media significantly reduced the proportion of cells undergoing hypercontracture (60.9, 41.2 and 35.3%, respectively, $P<0.05$).

During the first minute of reoxygenation, a decrease in $[Ca^{2+}]_i$ was observed in all the treatment groups, with no between-group differences. After this time, $[Ca^{2+}]_i$ increased in the control group but continued to decrease in cells re-energized in the presence of KB-R7943 during the first 5 min of reoxygenation, remaining stable thereafter (Fig. 2). There were no differences in the change in Fura-2 ratio during the first 10 min of re-energization between cells treated with 5, 10 or 15 μM KB-R7943 (1.02±0.10, 0.86±0.08 and 0.92±0.07, respectively).

SR blockade with thapsigargin and ryanodine was not associated with a significant reduction in the number of cells undergoing hypercontracture. Hypercontracture was significantly less frequent in KB-R7943 treated cells, and was virtually absent (one out of 22) in cells receiving both KB-R7943 and SR functional blockade (Fig. 3). The frequency of Ca^{2+} oscillations was lower in cells re-energized in the presence of 10 μM KB-R7943 than in the controls (27.2±2.8 vs. 35.6±2.8 oscillations/min, $P=$

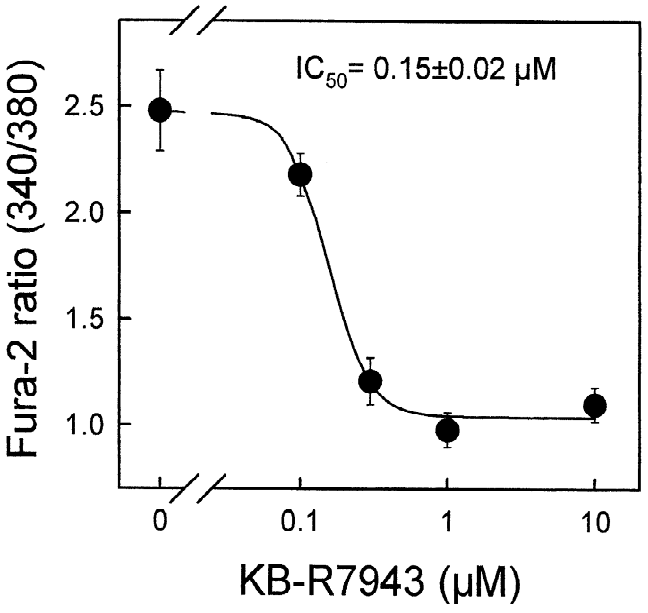

Fig. 1. Inhibition of reverse NCX by KB-R7943 in isolated cardiomyocytes. $[Ca^{2+}]_i$ rise induced by 30 s of Na^+ withdrawal was measured in the presence of different concentrations of KB-R7943. $n=6$–9 cells per group. Data are mean±S.E.M.

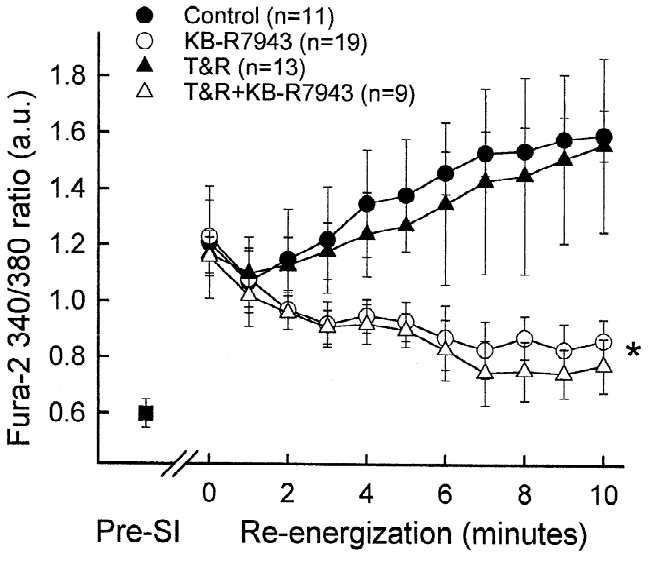

Fig. 2. Effect of 10 μM KB-R7943 and 150 nM thapsigargin plus 10 μM ryanodine (T&R) on diastolic Fura-2 ratio (a.u., arbitrary units) during re-energization of isolated cardiomyocytes after 40 min of simulated ischemia. Pre-SI (■, $n=19$) indicates Fura-2 ratio before simulated ischemia. Data are mean±S.E.M. *$P<0.001$ for KB-R7943 groups in 2×2 factorial ANOVA.

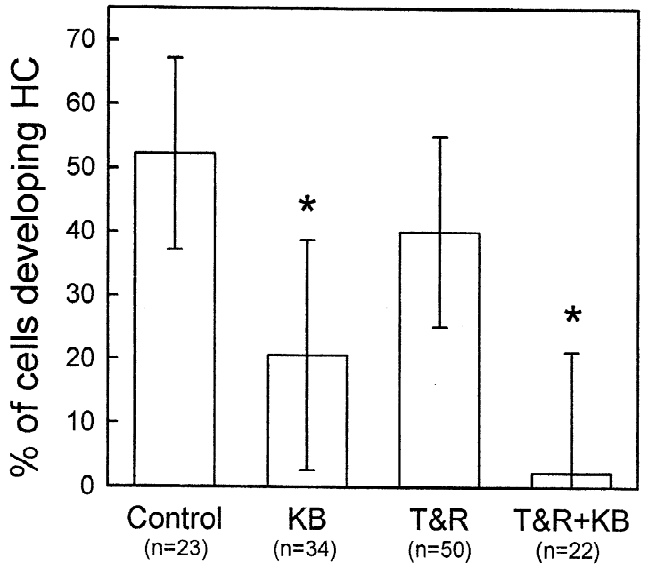

Fig. 3. Effect of inhibition of NCX with 10 μM KB-R7943 (KB) and functional blockade of the sarcoplasmic reticulum with 150 nM thapsigargin plus 10 μM ryanodine (T&R) on cardiomyocyte hypercontracture during re-energization after 40 min of simulated ischemia. Error bars indicate 95% CI. *$P<0.05$ versus control.

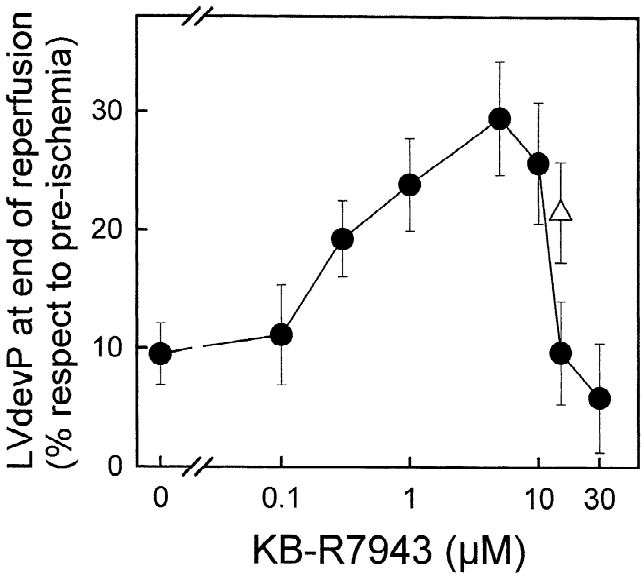

Fig. 4. Effect of addition of KB-R7943 during the initial 4 (△) or 10 min of reperfusion on LVdevP recovery in isolated rat hearts (B). $n=4$ per group. Data are mean±S.E.M.

0.060). There were no Ca^{2+} oscillations in cells in which SR function had been blocked by treatment with thapsigargin plus ryanodine.

3.2. Studies in perfused rat heart

The concentration- and time-dependent effects of KB-R7943 exposure during initial myocardial reperfusion were investigated in the perfused rat heart model.

In control hearts subjected to transient ischemia, LV developed pressure (LVdevP), defined as LV peak systolic pressure−LVEDP measured at 60 min of reperfusion, recovered to 9.5±2.6% of its initial value. LVdevP recovery increased by the addition of KB-R7943 at concentrations of 0.3–10 μM during the first 10 min of reperfusion (to values between 19.3±3.2 and 25.7±6.35.1%, $P<0.01$). This effect was lost at higher concentrations, but was sustained when perfusion with 15 μM KB-R7943 was restricted to the first 4 min of reperfusion (21.5±4.3%, $P=0.0.012$) (Fig. 4).

LDH release during the 60 min of reperfusion decreased by more than 25% with exposure to KB-R7943 at 0.3–10 μM ($P<0.05$). Concentrations of 15 or 30 μM KB-R7943 exacerbated LDH release (212 and 233% of control value, respectively, $P<0.01$). However, 15 μM KB-R7943 was beneficial ($P=0.038$) when added only during the first 4 min of reperfusion (Fig. 5). Separate analysis of the effects of the different concentrations of KB-R7943 during the first 5 min of reperfusion and thereafter (minutes 6–12) disclosed a protective effect on the initial period with a calculated half-response concentration of 0.29±0.09 μM. After this initial period, KB-R7943 had detrimental effect at concentrations ≥15 μM (Fig. 6).

In the series of hearts in which LV pressure was monitored throughout the experiment, perfusion with 5 μM KB-R7943 significantly reduced the LVEDP peak during early reperfusion (126.1±7.6 mmHg in the control group vs. 101.1±3.9 mmHg in 5 μM KB-R7943 group, $P=0.014$, Fig. 7A) and increased the LVdevP measured at 60 min of reperfusion (14.5±3.3% with respect to pre-ischemic values in controls vs. 30.5±4.4% in KB-R7943 group, $P=0.016$, Fig. 7B). Ventricular fibrillation or tachycardia occurred frequently during reperfusion, but resolved spontaneously within the first 5 min of reflow. These

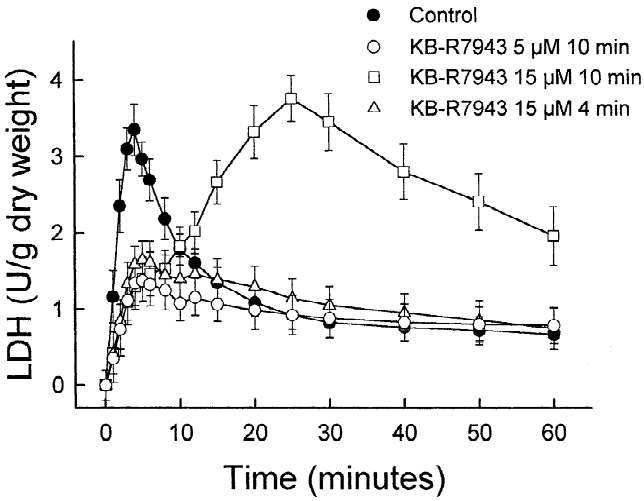

Fig. 5. Effect of addition of KB-R7943 during the initial 4 or 10 min of reperfusion on LDH release in isolated rat hearts. $n=4$ per group. Data are mean±S.E.M.

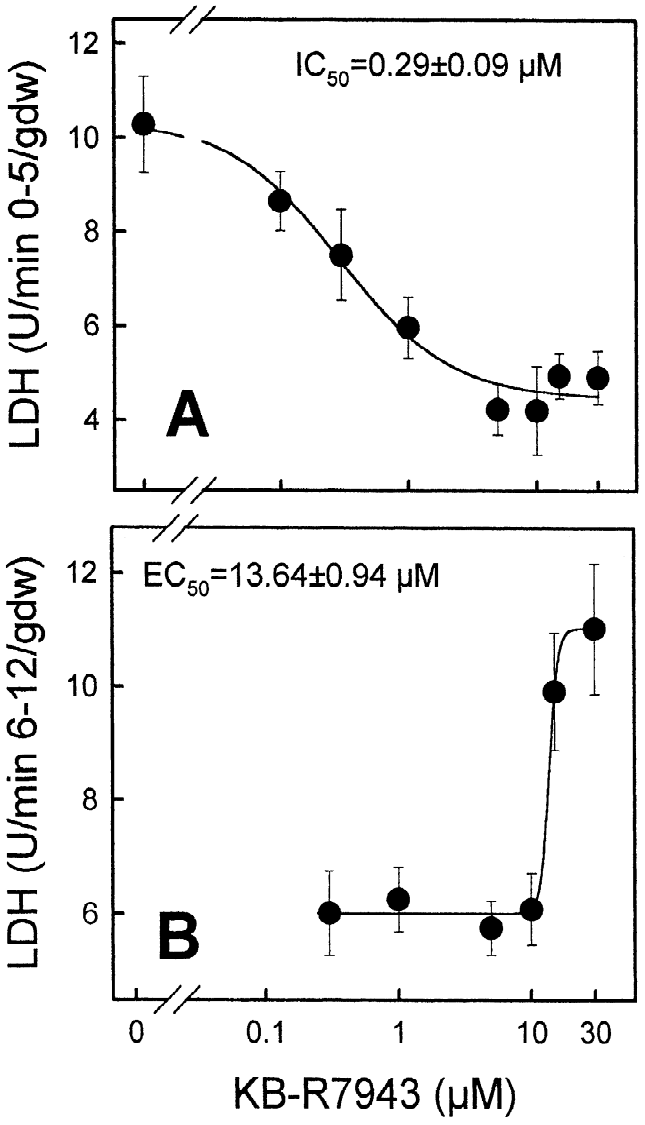

Fig. 6. Effect of KB-R7943 on LDH release during the initial 5 min of reperfusion (panel A) and during subsequent reperfusion (minutes 6–12, panel B) in isolated rat hearts. Control and 0.1 μM KB-R7943 groups were not included in panel B because large LDH release during the initial 5 min made them not comparable with the remaining groups. EC_{50}, concentration of KB-R7943 eliciting half-maximal increase of LDH release; IC_{50}, concentration of KB-R7943 eliciting half-maximal inhibition of LDH release. Data are mean±S.E.M.

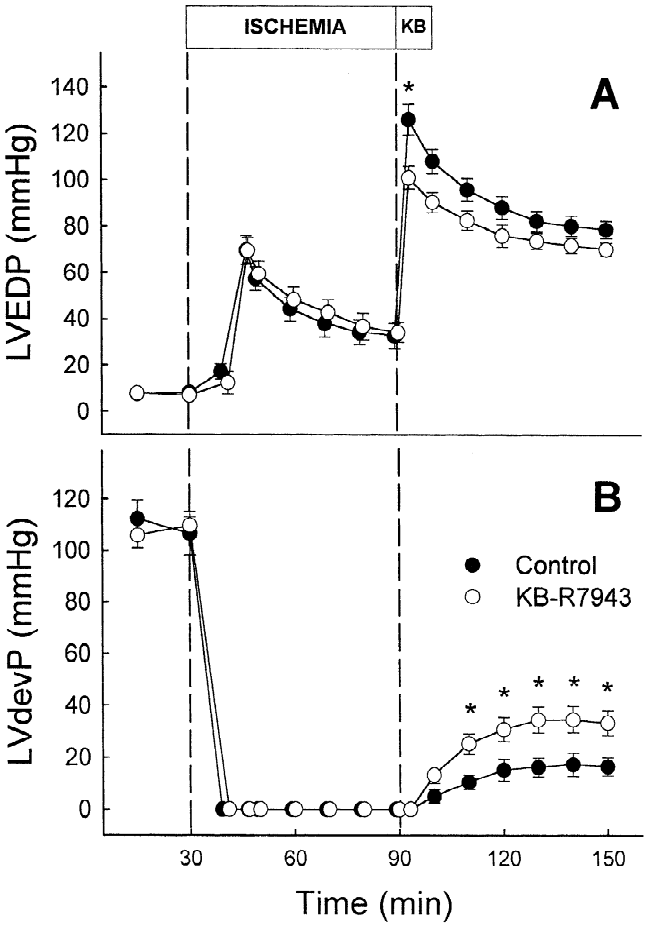

Fig. 7. Changes in left ventricular end-diastolic pressure (LVEDP, panel A) and left ventricular developed pressure (LVdevP, panel B) during ischemia and reperfusion in control hearts (Control, $n=6$) and in hearts receiving 5 μM KB-R7943 during the first 10 min of reperfusion (KB-R7943, $n=6$). The period of drug infusion is denoted by KB. Data are presented as mean±S.E.M. *$P<0.05$ versus control group.

events occurred in 73% of control hearts and 41% of hearts receiving 5 μM KB-R7943 during the first 10 min of reperfusion ($P=0.151$).

In this series of experiments, LDH release during reperfusion was 79.3±9.8 U/gdw per 60 min in the control group and 50.0±5.6 U/gdw per 60 min in the KB-R7943 group ($P=0.016$). Histological analysis revealed extensive areas of contraction band necrosis (mean score (0–3) of 2.17±0.24) in control hearts, and significantly smaller areas of contraction band necrosis in hearts that had received 5 μM KB-R7943 during initial reperfusion (mean score: 1.42±0.15, $P=0.014$).

3.3. Intracoronary KB-R7943 in the pig heart in situ

The potential therapeutic relevance of NCX inhibition during coronary reperfusion was investigated in the pig heart in situ model.

In animals not submitted to coronary occlusion, intracoronary infusion of 5 μM KB-R7943 for 10 min did not induce arrhythmia, changes in heart rate, mean aortic pressure or coronary blood flow, or systolic shortening in the LAD territory.

3.3.1. Transient coronary occlusion

There were no differences between groups in hemodynamic variables throughout the experiment (Table 1). Three controls and one treated animal presented ventricular fibrillation during ischemia, and one animal from each group presented ventricular fibrillation during reperfusion.

3.3.2. Infarct size

There were no significant differences between groups in

Table 1
Haemodynamic and regional contractility data during coronary occlusion and reperfusion in the in situ pig heart

	Basal	Ischemia			Reperfusion		
		15 min	45 mim	5 min	15 min	60 min	120 min
Haemodynamics							
MAP (mmHg)							
Control	76.2±4.5	71.9±3.1	74.6±2.0	73.9±3.2	77.1±2.8	81.1±3.6	83.6±4.6
KB-R7943	77.5±4.3	69.7±3.7	71.6±6.2	71.7±5.5	79.0±8.4	76.1±6.7	81.6±5.2
HR (beats/min)							
Control	79.0±4.1	76.5±4.5	91.5±10.6	97.7±4.7	112.3±9.1	100.4±6.3	96.6±5.8
KB-R7943	76.2±4.7	81.2±5.1	86.4±5.9	103.2±7.2	96.5±5.3	97.5±4.2	92.0±5.9
LVDP (mmHg)							
Control	9.6±1.6	16.0±1.4	13.0±2.2	18.5±3.4	12.7±1.9	12.0±2.0	15.0±2.1
KB-R7943	6.8±1.2	15.4±2.9	15.4±1.7	23.0±2.5	18.4±3.8	16.0±2.2	15.2±1.4
CBF (ml/min per g)							
Control	0.7±0.1	–	–	1.9±0.2	2.7±0.2	3.3±0.2	3.0±0.2
KB-R7943	0.6±0.1	–	–	2.4±0.4	3.3±0.4	3.0±0.3	2.8±0.3
Wall function							
Control myocardium							
EDSL (% of basal)							
Control	100	105±6	104±2	104±3	103±9	98±5	104±5
KB-R7943	100	100±5	98±1	98±1	103±9	94±4	95±5
SS (% of basal)							
Control	100	103±6	107±8	90±5	90±9	102±5	103±3
KB-R7943	100	110±7	109±8	92±11	85±1	92±10	95±4
Area at risk							
EDSL (% of basal)							
Control	100	120±8	119±7	98±12	99±3	98±1	100±1
KB-R7943	100	117±3	118±3	98±2	90±5	92±4	96±4
SS (% of basal)							
Control	100	0±1	0±1	−13±7	4±12	−5±8	−7±7
KB-R7943	100	−2±2	−1±1	−4±20	−1±4	7±6	−1±7

CBF, maximal blood flow related to the mass of the area at risk; EDSL, end-diastolic segment length related to basal value; HR, heart rate; LVDP, left ventricle diastolic pressure; MAP, mean arterial pressure; SS, systolic shortening related to basal value. Values are means±S.E.M.

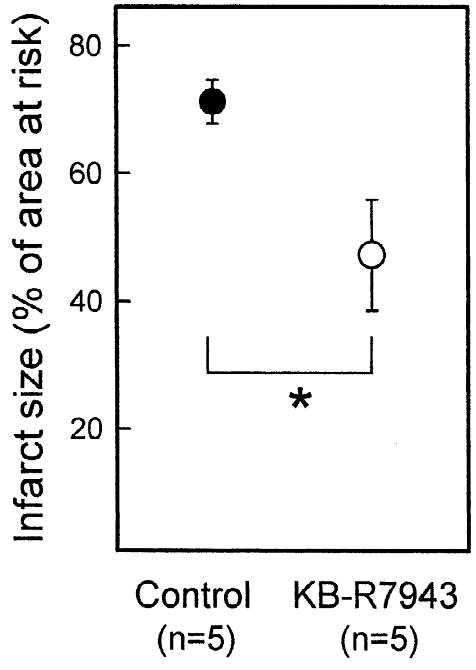

Fig. 8. Infarct size after 48 min of coronary occlusion and 2 h of reperfusion in pigs. Data are mean±S.E.M. *$P<0.05$ versus control group.

the mass of myocardium at risk (10.3±0.9% of ventricular mass in the control group vs. 9.1±0.7% in the KB-R7943 group, $P=0.340$). Necrosis involved 7.4±0.9% of the ventricular mass in controls and 4.4±1.0% in animals receiving 5 μM KB-R7943; infarct size (expressed as percentage of area at risk developing necrosis) was 37.8% smaller in treated animals ($P=0.033$, Fig. 8). At 15 μM, KB-R7943 had no beneficial effect on the extent of the necrosis (6.8±2.0% of ventricular mass, $P=0.233$).

4. Discussion

The present study confirms our previous results indicating that inhibition of NCX with KB-R7943 during reenergization reduces $[Ca^{2+}]_i$ and the probability of hypercontracture in isolated quiescent myocytes submitted to simulated ischemia, and demonstrates that KB-R7943 addition during initial reperfusion limits hypercontracture in rat and pig myocardium, and in vivo. In intact, contracting myocardium, these beneficial effects were observed at low drug concentrations (0.5–10 μM) lacking significant functional effects on normoxic myocardium, but were lost at higher concentrations. These results are

consistent with the hypothesis that reverse NCX contributes to Ca^{2+} mediated cell injury during initial reperfusion.

4.1. Effect of KB-R7943 on $[Ca^{2+}]_i$ oscillations and hypercontracture in quiescent myocytes

The results in isolated cardiomyocytes confirm previous observations suggesting that NCX activity may result in additional Ca^{2+} influx and injury during initial reperfusion [18]. The initial decay in cytosolic Ca^{2+} in the control group has been explained by the higher rate of Ca^{2+} sequestration by the SR as compared to the rate of extracellular Ca^{2+} influx. Accumulation of Ca^{2+} in the SR has been shown to result in repetitive cycles of Ca^{2+} release and re-uptake leading to Ca^{2+} oscillations [8,18], and the late increase in $[Ca^{2+}]_i$ observed in myocytes reperfused under control conditions could reflect a net Ca^{2+} flux from SR to cytoplasm during this period. The sustained reduction in $[Ca^{2+}]_i$ observed in the presence of KB-R7943 can be thus explained as the consequence of reduced extracellular Ca^{2+} influx during initial reperfusion resulting in attenuated SR Ca^{2+} overload, less Ca^{2+} oscillation, and reduced $[Ca^{2+}]_i$. Previous studies have shown that SR functional blockade has a beneficial effect on reoxygenation-induced hypercontracture as a consequence of a reduction in Ca^{2+} oscillations with no significant effect on $[Ca^{2+}]_i$ [8]. In an earlier work we demonstrated that KB-R7943 reduced the frequency of Ca^{2+} oscillations in isolated myocytes submitted to hypoxia/reoxygenation [18].

Together with results from previous studies [8], the present findings suggest that reduced diastolic $[Ca^{2+}]_i$ protects against reperfusion-induced hypercontracture, independently of the potentially protective effect of reduced Ca^{2+} oscillations. The mechanism by which $[Ca^{2+}]_i$ declines in myocytes during the first minute of re-energization even in the presence of thapsigargin and ryanodine is not clear [8]. The role of Ca^{2+} stores other than the SR (e.g. the mitochondria), cannot be ruled out. We speculate that mitochondria contribute to Ca^{2+} buffering during initial reperfusion and that mitochondrial Ca^{2+} overload, resulting in mitochondrial injury and subsequent Ca^{2+} release, contributes to the late increase in $[Ca^{2+}]_i$ (Fig. 2). In this context, reduced influx during initial reoxygenation in the presence of KB-R7943 could attenuate mitochondrial Ca^{2+} overload and prevent the opening of mitochondria transition pore and mitochondrial driven cell death [27]. KB-R7943 might also modify mitochondrial Ca^{2+} overload through a direct effect on the mitochondrial Na^+/Ca^{2+} exchanger, a molecule not identical to the sarcolemmal exchanger NCX1, for which specific inhibitors have been described [28]. However, the relevance of this potential effect would be limited by the poor diffusion of KB-R7943 across cell membranes [23]. So far, there are no available data documenting any effect of the drug on mitochondrial Na^+/Ca^{2+} exchanger in intact cells.

4.2. Concentration-dependent effect of KB-R7943 on reperfused myocardium

In reperfused rat hearts, NCX inhibition with KB-R7943 at concentrations of 0.3–10 μM during the first 10 min of reperfusion improved functional recovery and markedly reduced enzyme release and contraction band necrosis. However, in contrast to what was observed in quiescent cells, KB-R7943 was clearly detrimental at 15 or 30 μM. The increase in late LDH release with high KB-R7943 concentrations cannot be explained as a mere consequence of reduced initial release, as it was prevented by limiting drug infusion to the first 4 min of reperfusion. The contrasting effect of high versus low KB-R7943 concentrations in intact myocardium can be explained by the different effect of the drug in cells presenting continuous reverse NCX operation during initial reperfusion and in cells that survive to this period. In cardiomyocytes that have not yet recovered the trans-sarcolemmal Na^+ gradient and in which there is a net Ca^{2+} influx through reverse mode NCX activity, the potentially detrimental effect of high concentrations of KB-R7943 on forward mode need not be considered, since there is no forward NCX activity. In these cells, even low concentrations of KB-R7943 should have a beneficial effect on Ca^{2+} homeostasis, since KB-R7943 has an IC_{50} close to 0.3 μM under these conditions. This interpretation is consistent with the concentration–response curve of LDH release in isolated hearts in the present study, with a calculated half response concentration of 0.29 μM.

In myocytes surviving initial reperfusion and recovering normal trans-membrane Na^+ gradient the RP returns to normal values, above diastolic V_M, resulting in alternating net forward (diastole) and reverse (systole) NCX operation during the cardiac cycle. Under these conditions KB-R7943 is expected to inhibit both modes of operation similarly, although with a higher IC_{50} than in cells under ionic conditions resulting in continuous net reverse NCX [20]. Since forward NCX is important for recovery of Ca^{2+} homeostasis [6], KB-R7943 could have detrimental effects in this situation. The concentration–response curve for LDH release between minutes 6 and 12 of reflow is consistent with this prediction. The fact that enhanced LDH release only occurs at 15 μM, a concentration substantially larger than the IC_{50} for forward or reverse NCX under normal ('bi-directional'), ionic conditions, suggests that near-complete inhibition of forward NCX is necessary to elicit the adverse effect.

In the present study, the dose and time dependence of the effects of KB-R7943 on reperfused myocardium were analyzed in unloaded hearts. Hearts were maintained unloaded (with LV balloons deflated) to avoid the potentially confounding effects of the extremely high, non-physiological LV pressure reached in the isovolumic heart during reperfusion. Nevertheless, the protective effect of 5 μM KB-R7943 was similar in hearts submitted to ischemia–reperfusion under isovolumic conditions.

Finally, in isolated cardiomyocytes [22] or isolated rat hearts under normal conditions, 15 μM of KB-R7943 had no effect on Ca^{2+} transients or contraction (data not shown). The dissimilar effects of 15 μM on reperfused and normoxic cells can be explained by a higher dependence of reperfused cells on Ca^{2+} extrusion via forward NCX [6]. It is also possible that NCX1 activity is reduced during early reperfusion as a consequence of changes in phosphorylation states, pH, and other derangements [16,22], as has been proven true during prolonged ischemia [13].

4.3. Effects of KB-R7943 during in vivo myocardial reperfusion

The relative importance of NCX in Ca^{2+} homeostasis may vary between species and be larger in humans than in rats [22]. The therapeutic implications of the present study are further increased by the reduction in infarct size induced by inhibition of NCX during initial reperfusion in the pig heart. Importantly, this beneficial effect was observed in the absence of any detectable influence in hemodynamic status or in reperfusion arrhythmias. In previous studies, intracoronary infusion of KB-R7943 at final concentrations up to 15 μM had no effect on coronary blood flow or regional wall function of normally perfused pig myocardium (unpublished data). Furthermore, although this high concentration did not limit infarct size, it was not detrimental.

Overall, the present results are consistent with the hypothesis that reverse NCX may result in additional Ca^{2+} influx and injury during myocardial reperfusion, and they demonstrate that inhibition of NCX during initial reperfusion attenuates reperfusion injury and reduces myocardial infarction without interfering with normal cell function. The development of new inhibitors able to target NCX in cells exhibiting continuous net reverse mode exchange during initial reperfusion at a wide range of concentrations, without affecting forward mode exchange in other cells, could have important therapeutic applications.

Acknowledgements

This work was supported by a CICYT grant (SAF99/0102). We thank Yolanda Puigfel and Lourdes Trobo for their excellent technical work, and Dr Alberto Fernandez for his contribution to the discussion of pharmacologic issues.

References

[1] Piper HM, Garcia-Dorado D, Ovize M. A fresh look at reperfusion injury. Cardiovasc Res 1998;38:291–300.
[2] Garcia-Dorado D, Théroux P, Munoz R et al. Selective inhibition of the contractile apparatus: a new approach to modification of infarct size, infarct composition, and infarct geometry during coronary artery occlusion and reperfusion. Circulation 1992;85:1160–1174.
[3] Van der Heide RS, Angelo JP, Altschuld RA, Ganote CE. Energy dependence of contraction band formation in perfused hearts and isolated adult cardiomyocytes. Am J Pathol 1986;125:55–68.
[4] Barrabés JA, Garcia-Dorado D, Ruiz-Meana M et al. Myocardial segment shrinkage during coronary reperfusion 'in situ'. Relation to hypercontracture and myocardial necrosis. Pflügers Arch 1996;431:519–526.
[5] Ganote CE. Contraction band necrosis and irreversible myocardial injury. J Mol Cell Cardiol 1983;15:67–73.
[6] Siegmund B, Ladilov YV, Piper HM. Importance of sodium for recovery of calcium control in reoxygenated cardiomyocytes. Am J Physiol 1994;267:H506–H513.
[7] Steenbergen C, Murphy E, Levi L, London RE. Elevation of cytosolic free calcium concentration early in myocardial ischemia in reperfused rat heart. Circ Res 1987;60:700–707.
[8] Siegmund B, Schlack W, Ladilov YV, Balser C, Piper HM. Halothane protects cardiomyocytes against reoxygenation-induced hypercontracture. Circulation 1997;96:4372–4379.
[9] Shine KI, Douglas AM. Low calcium reperfusion of ischemic myocardium. J Mol Cell Cardiol 1983;15:251–260.
[10] Tani M, Neely JR. Role of intracellular Na^+ and Ca^{2+} overload and depressed recovery of ventricular function of reperfused ischemic rat hearts: possible involvement of Na^+–H^+ and Na^+–Ca^{2+} exchange. Circ Res 1989;65:1045–1056.
[11] Klein HH, Pich S, Lindert S, Nebendahl K, Warneke G, Kreuzer H. Treatment of reperfusion injury with intracoronary calcium channel antagonists and reduced coronary free calcium concentration in regionally ischemic, reperfused porcine hearts. J Am Coll Cardiol 1989;13:1395–1401.
[12] Blaustein M, Lederer J. Sodium/calcium exchange: its physiological implications. Physiol Rev 1999;79:763–854.
[13] Ladilov Y, Haffner S, Balser-Schäfer C, Maxeiner H, Piper HM. Cardioprotective effects of KB-R7943: a novel inhibitor of the reverse mode of Na^+/Ca^{2+} exchanger. Am J Physiol 1999;276:H1868–H1876.
[14] Pike MM, Kitakaze M, Marban E. ^{23}Na-NMR measurement of intracellular sodium in intact perfused ferret hearts during ischemia and reperfusion. Am J Physiol 1990;259:H1767–H1773.
[15] Ruiz-Meana M, Garcia-Dorado D, Hofstaetter B, Piper HM, Soler-Soler J. Propagation of cardiomyocyte hypercontracture by passage of Na^+ through gap junctions. Circ Res 1999;85:280–287.
[16] Bersohn MM. Sodium pump inhibition in sarcolemma from ischemic hearts. J Mol Cell Cardiol 1995;27:1483–1489.
[17] Kusuoka H, Camilion de Hurtado MC, Marbán E. Role of sodium/calcium exchange in the mechanism of myocardial stunning: protective effect of reperfusion with high sodium. J Am Coll Cardiol 1993;21:240–248.
[18] Schafer C, Ladilov Y, Inserte J et al. Role of the reverse mode of the Na^+/Ca^{2+} exchanger in reoxygenation-induced cardiomyocyte injury. Cardiovasc Res 2001;51:241–250.
[19] Iwamoto T, Watano T, Shigekawa M. A novel isothiourea derivative selectively inhibits the reverse mode of Na^+/Ca^{2+} exchange in cells expressing NCX1. J Biol Chem 1996;271:22391–22397.
[20] Watano T, Kimura J, Morita T, Nakanishi H. A novel antagonist, No. 7943, of the Na^+/Ca^{2+} exchange current in guinea-pig cardiac ventricular cells. Br J Pharmacol 1996;119:555–563.
[21] Kimura J, Watano T, Kawahara M, Sakai E, Yatabe J. Direction-independent block of bi-directional Na^+/Ca^{2+} exchange current by KB-R7943 in guinea-pig cardiac myocytes. Br J Pharmacol 1999;128:969–974.
[22] Shigekawa M, Iwamoto T. Cardiac Na^+-Ca^{2+} exchange. Molecular and pharmacological aspects. Circ Res 2001;88:864–876.
[23] Elias CL, Lukas A, Shurraw S et al. Inhibition of Na^+/Ca^{2+} exchange by KB-R7943: transport mode selectivity and antiarrhythmic consequences. Am J Physiol 2001;281:H1334–H1345.
[24] Garcia-Dorado D, Inserte J, Ruiz-Meana M et al. Gap junction

uncoupler heptanol prevents cell-to-cell progression of hypercontracture and limits necrosis during myocardial reperfusion. Circulation 1997;96:3579–3586.
[25] Satoh H, Ginsburg KS, Qing K, Terada H, Hayashi H, Bers DM. KB-R7943 block of Ca^{2+} influx via Na^{+}/Ca^{2+} exchange does not alter twitches or glycoside inotropy but prevents Ca^{2+} overload in rat ventricular myocytes. Circulation 2000;101:1441–1446.
[26] Humphrey SM, Thomson RW, Gavin JB. The effect of an isovolumic left ventricle on the coronary vascular competence during reflow after global ischemia in the rat heart. Circ Res 1981;49:784–791.
[27] Griffiths E, Halestrap AP. Protection by cyclosporin A of ischemia/reperfusion-induced damage in isolated rat hearts. J Mol Cell Cardiol 1993;25:1461–1469.
[28] Cox DA, Matlib MA. A role for the mitochondrial Na^{+}-Ca^{2+} exchanger in the regulation of oxidative phosphorylation in isolated heart mitochondria. J Biol Chem 1993;268:938–947.

DISCUSIÓN

IV- DISCUSIÓN

1. Contribución de la apoptosis a la muerte por reperfusión

Desde el primer trabajo que documentaba en conejo *in situ* la aparición de apoptosis en el corazón reperfundido [47] son numerosos los artículos que durante los últimos años han confirmado que, junto a la muerte por necrosis, también aparece muerte por apoptosis en el miocardio isquémico y reperfundido. Sin embargo, continúa existiendo una gran controversia a la hora de valorar la contribución relativa de la apoptosis en el total de la muerte celular [111, 112]. El hecho de que la extensión de la necrosis miocárdica pueda determinarse poco después de iniciarse la reperfusión, antes de que la apoptosis tenga lugar, y permanezca constante durante las siguientes 24-72 h [60] va fuertemente en contra de la posibilidad de que la apoptosis juegue un papel predominante en la muerte por reperfusión.

Durante la reperfusión se generan en cantidades anormalmente altas factores con demostrada capacidad para inducir apoptosis en diferentes tipos celulares (radicales libres de oxígeno (RLO), NO, citoquinas). Este estudio plantea la posibilidad de que, si bien estos factores producidos durante la isquemia/reperfusión miocárdica pueden provocar apoptosis, el episodio isquémico también podría actuar alterando el metabolismo celular de tal modo que un proceso regulado a nivel de transcripción génica como es la muerte celular por apoptosis resulte afectado. En consecuencia, los miocitos sometidos a isquemia/reperfusión serían menos susceptibles a la inducción de apoptosis por factores pro-apoptóticos que aquellos miocitos normóxicos expuestos a los mismos factores. Algunos trabajos recientes parecen apoyar esta hipótesis al sugerir que un episodio de isquemia transitoria aumenta la expresión de diferentes genes anti-apoptóticos, lo que indica la activación de mecanismos endógenos de protección frente a la apoptosis. Se ha demostrado aumento en la expresión de bcl-2 en corazones humanos con fallo cardiaco [88] o tras infarto de miocardio [113], de mcl-1 tras una oclusión coronaria en rata [114], e IAP-1 en modelo porcino sometido a isquemia [115], todas ellas proteínas con un papel inhibidor de la apoptosis.

Para confirmar esta hipótesis, en estos estudios se comparó la inducción de apoptosis generada por exposición a fuentes exógenas de RLO y NO (H_2O_2 y SNAP) de cardiomiocitos sometidos a condiciones normóxicas y a protocolos que simulan isquemia/reperfusión. Los experimentos se reprodujeron en un modelo de corazón aislado.

1.1 Inducción de apoptosis por radicales libres de oxígeno

Los cardiomiocitos reperfundidos liberan cantidades anormalmente altas de RLO [19, 96], pudiendo ser su origen tanto endógeno (mitocondria, ácido araquidónico) como exógeno (activación de neutrófilos, oxidación de xantina en células endoteliales).

El H_2O_2 es un agente prooxidante con conocida capacidad para inducir apoptosis en diferentes tipos celulares [116, 117]. Por si solo no es un radical libre pero reacciona con el anión superóxido generando radicales hidroxilo que son altamente reactivos. Con su uso se ha pretendido simular las condiciones de exposición a RLO en las que se encontrarían los miocitos reperfundidos en el corazón intacto.

En este estudio, un protocolo de isquemia simulada/reoxigenación no fue causa suficiente para inducir apoptosis. La incubación con H_2O_2 de miocitos mantenidos en condiciones normóxicas provocó apoptosis de manera dosis dependiente sin incrementar la muerte por necrosis. Sin embargo, los miocitos resultaron menos susceptibles a desarrollar apoptosis inducida por H_2O_2 si previamente habían estado sometidos a isquemia simulada.

Trabajos previos demuestran que durante la fase isquémica disminuyen los componentes de la defensa antioxidante [100,101] dejando a las células en un estado más sensible a los efectos deletéreos de los radicales libres. Uno de los mecanismos antioxidantes afectados es el de la glutation peroxidasa, enzima que cataliza la descomposición de H_2O_2 y peróxidos orgánicos usando para ello glutation en su forma reducida y generando glutation oxidado. Los niveles intracelulares de glutation reducido determinan el funcionamiento de la enzima y las variaciones en el ratio entre su forma reducida y oxidada indica de manera precisa el grado de estrés oxidativo en el que se encuentran las células [118]. En este trabajo se ha observado que durante isquemia simulada/reoxigenación, los miocitos pierden gran parte de su contenido en glutation y, en el pool remanente, disminuye el ratio entre su forma reducida y oxidada indicando un elevado grado de estrés oxidativo. La preincubación de los miocitos con N-acetilcisteína, precursor del glutation, incrementó de un modo dependiente de la dosis el contenido intracelular de la forma reducida del glutation. La exposición de miocitos incubados con diferentes concentraciones de N-acetilcisteína a H_2O_2 demostró que la apoptosis inducida por RLO es claramente dependiente de la capacidad antioxidante de la célula: cuanto mayor son los niveles intracelulares de glutation, menor es la apoptosis asociada a la reoxigenación.

De esta manera, cuando se cuantifica la apoptosis inducida por H_2O_2 en miocitos normóxicos y miocitos sometidos a isquemia simulada/reoxigenación preincubados con la concentración de N-acetilcisteína necesaria para conseguir que los niveles de glutation sean similares en ambos grupos, se observa que estos últimos no desarrollan apoptosis.

Este estudio demuestra que en miocitos aislados un episodio de isquemia genera un efecto protector frente a la muerte por apoptosis inducida por RLO, efecto protector que parcialmente quedaría oculto por la disminución en la capacidad antioxidante celular que tiene lugar durante el periodo isquémico.

1.2 Inducción de apoptosis con NO

Tras observar el efecto protector frente a la apoptosis provocada por H_2O_2 generado por un periodo de isquemia en miocitos aislados, se investigó si es éste un efecto general que se manifiesta frente a diferentes agentes inductores de apoptosis y si se reproduce en un modelo de corazón intacto. Se decidió utilizar NO como inductor de apoptosis por que se ha demostrado que provoca apoptosis en cardiomiocitos [105-107] y son numerosos los trabajos que demuestran aumento en su liberación durante la reperfusión [108]. Estas características lo convierten, tal como sucede con los RLO, en un probable inductor de apoptosis tras un infarto de miocardio. Se usó un conocido generador de NO, SNAP, a concentraciones que, según la literatura, reflejarían niveles de NO observados *in vivo* en el miocardio reperfundido [119].

En corazones aislados, al igual que pasa en miocitos aislados, un protocolo de isquemia/reperfusión no incrementó la tasa de apoptosis. Corroborando trabajos previos [106], un aumento en la concentración intracelular de NO indujo apoptosis en corazones sometidos a normoxia. Sin embargo, no se observó muerte por apoptosis cuando la administración de SNAP se realizó durante la reperfusión que siguió a una isquemia subletal. Este mismo efecto protector frente a la apoptosis inducida por NO se reprodujo en experimentos realizados en miocitos aislados confirmando que el mecanismo responsable de esta protección es intrínseco a este tipo celular.

1.3 Mecanismo de protección.

Los resultados obtenidos en estos estudios demuestran que la isquemia miocárdica genera un efecto protector frente a distintos estímulos inductores de apoptosis, sugiriendo la alteración

durante la depleción energética de algún mecanismo básico en el desencadenamiento de la muerte por apoptosis.

Dado que la apoptosis es un proceso dependiente de energía, se ha sugerido que el mecanismo apoptótico se iniciaría durante el periodo isquémico pero quedaría abortado con la depleción energética [53]. Se podría pensar que el efecto protector observado en estos estudios es debido a la inhibición de la síntesis de ATP. Sin embargo, la cuantificación de fosfocreatina y ATP tras una hora de reoxigenación descarta esta posibilidad ya que se observa que la recuperación del metabolismo celular es anterior a la pérdida del efecto protector frente a la apoptosis inducida por SNAP.

Una de las principales características que diferencian a la muerte por apoptosis de la muerte por necrosis es la existencia de un mecanismo de ejecución altamente regulado a nivel de transcripción génica. Así, su activación parece depender del equilibrio en la expresión de genes que actuarían como pro- o anti-apoptóticos, siendo especialmente crítico el balance entre el gen bcl-2 (anti-apoptótico) y bax (pro-apoptótico) [120]. En este estudio, el tratamiento de corazones normóxicos con SNAP supuso una sobrexpresión de RNAm de bax y una disminución en la expresión de bcl-2, confiriendo al corazón una situación pro-apoptótica que se manifestó con un aumento en la muerte por apoptosis. Cuando los corazones se sometieron a isquemia/reperfusión se pasó a una situación anti-apoptótica al aumentar la expresión de bcl-2 y no producirse cambios en los niveles de bax. Esta situación no se modificó con el tratamiento con SNAP, correlacionándose con el grado de apoptosis observado. Este mismo comportamiento de bax y bcl-2 se reprodujo en miocitos aislados. En este modelo además se amplió el análisis de la expresión de genes anti-apoptóticos. De la familia de genes bcl-2, a parte del propio bcl-2, se obtuvo sobreexpresión de mcl-1 y bcl-xl, sin variaciones en bcl-w. El aumento en el RNAm se acompañó de un aumento en los niveles de proteína Bcl-xl y de Bcl-2 en su fracción unida a la mitocondria que es dónde se le atribuye su función antiapoptótica al impedir la liberación del citocromo c [121]. De los miembros analizados de la familia de genes antiapoptóticos IAP, se encontró aumentado de manera significativa IAP-1, proteína que ejerce su acción inhibiendo directamente la activación de caspasas [122].

Estos resultados indican que la cardioprotección que la isquemia miocárdica ejerce frente a la apoptosis inducida por NO se acompaña de un aumento general en la expresión del programa génico anti-apoptótico.

1.4 Conclusiones.

La tasa de muerte por apoptosis obtenida en miocitos aislados y corazón aislado en estos estudios demuestra que el daño subletal producido en miocitos sometidos a depleción energética no constituye un factor inductor de apoptosis. Por el contrario, las alteraciones bioquímicas que tienen lugar durante un período de isquemia generan en los cardiomiocitos un mecanismo endógeno de protección frente a estímulos apoptóticos. Este mecanismo está probablemente mediado por un balance claramente anti-apoptótico en la expresión y localización de proteínas implicadas en la apoptosis.

La activación e infiltración de neutrófilos que tiene lugar en una fase más tardía de la reperfusión constituye una importante fuente de RLO y NO exógena pudiendo inducir apoptosis en el miocardio que ya ha perdido el efecto protector generado inicialmente por el periodo isquémico (en estos estudios la pérdida del efecto protector sucede a partir de las 2 h de reoxigenación, Fig4B [123]). Esta hipótesis atribuiría a la reacción inflamatoria un papel principal en la apoptosis perinecrótica que se observa en el infarto de miocardio tras varias horas de reperfusión. La constatación de que miocitos aislados sometidos a diferentes tiempos de anoxia presentan muerte por necrosis pero no por apoptosis [124] sugiere la necesidad de algún factor inductor de apoptosis exógeno a los cardiomiocitos que explique el desarrollo de la muerte por apoptosis observada en el miocardio reperfundido *in situ*. Adicionalmente, los trabajos que demuestran una correlación entre activación de neutrófilos y muerte por apoptosis [125] apoyarían esta idea.

Dado que el tamaño del infarto queda prácticamente definido durante el inicio de la reperfusión [60], antes de que desaparezca el efecto protector generado por el episodio isquémico, estos resultados refuerzan la idea de que la apoptosis juega un papel residual en la muerte por isquemia/reperfusión.

2. Prevención farmacológica del daño inmediato por reperfusión.

La muerte celular por necrosis que tiene lugar de manera inmediata a la reperfusión constituye la forma de muerte celular más importante tras una isquemia transitoria [62, 65]. En el miocardio *in situ*, esta muerte precoz se manifiesta histológicamente en forma de necrosis en bandas de contracción [61, 63] siendo la principal causa de su aparición la hipercontractura que sufren los miocitos con la restauración del flujo [8, 54]. La muerte causada por la hipercontractura no tiene

lugar de manera inmediata al restablecimiento del flujo sino tras la corrección del pH intracelular, dado que la acidosis estaría actuando como inhibidor de la contractilidad. Este pequeño margen temporal es el que permitiría poder aplicar tratamientos farmacológicos en el momento de la reperfusión dirigidos a reducir el daño letal por reperfusión.

En esta segunda parte de la tesis se han estudiado dos estrategias con un claro interés terapéutico encaminadas a disminuir la muerte inmediata por reperfusión. Las dos buscan prevenir el desarrollo de la hipercontractura aunque siguen mecanismos distintos para conseguirlo: inhibiendo la sensibilidad de las miofibrillas al Ca^{2+} mediante la estimulación de la síntesis de GMPc y, por otro lado, acelerando la recuperación de la homeostasis del Ca^{2+} al inhibir la entrada de este ión durante la reperfusión.

2.1 Estimulación de la síntesis de GMPc.

2.1.1 GMPc en la isquemia/reperfusión miocárdica.

La síntesis de GMPc está mediada por la activación de la guanilato ciclasa soluble (GCs), donde el NO es su agonista más importante, y por la activación de la guanilato ciclasa de membrana (GCm) mediada por factores natriuréticos atriales. Aunque no de manera unánime, numerosos estudios experimentales dirigidos a aumentar los niveles de NO con donadores de NO o con el sustrato de la NO sintasa, L-arginina, han descrito un efecto beneficioso sobre el daño por reperfusión [126, 127]. El mecanismo de protección sugerido en estos trabajos se ha atribuido por un lado a las propiedades vasodilatadoras del NO [126] y a su acción inhibidora de la adhesión de neutrófilos y plaquetas [127] y, por otro lado, a su capacidad antioxidante que se traduciría en una reducción del estrés oxidativo inducido por la sobreproducción de O_2^- durante la reperfusión [128]. En nuestro estudio, la suplementación del medio de perfusión con L-arginina redujo el grado de hipercontractura y muerte celular y mejoró la recuperación funcional de corazones aislados sometidos a hipoxia/reoxigenación. Sin embargo, a diferencia de los estudios anteriores, los efectos protectores obtenidos fueron principalmente mediados por GMPc puesto que se abolieron con un inhibidor específico de la GCs (ODQ). De todas maneras, aunque no sean predominantes, este estudio no puede descartar la posibilidad de efectos independientes del GMPc mediados por NO. La cuantificación en este estudio del contenido en GMPc demostró que éste disminuye durante la hipoxia y permanece reducido durante el inicio de la reoxigenación. El efecto protector obtenido con la suplementación del

medio de perfusión con L-arginina se correlacionó con una atenuación de la disminución en la concentración de GMPc.

Otra manera de conseguir elevar los niveles de GMPc durante la reperfusión es estimulando directamente la síntesis de GMPc a través de la GCm. Esta alternativa tiene la ventaja de aumentar los niveles de GMPc por una vía independiente del NO con lo que se descartarían posibles acciones no mediadas por GMPc, entre ellas los efectos tóxicos que presenta el NO en grandes cantidades y que podrían contrarrestar los efectos beneficiosos de un aumento en la síntesis de GMPc. Además, dado que la GCm no necesita de un precursor como es la L-arginina, la síntesis más rápida de GMPc permitiría la administración de sus agonistas en el momento de la reperfusión, antes de la desaparición de la acidosis intracelular y de su efecto inhibidor de la contractilidad. En un estudio previo se había descrito que el factor natriurético atrial (ANP) y su análogo, urodilatina, protegían frente a la hipercontractura inducida por reoxigenación en miocitos aislados [72]. La urodilatina es un miembro de la familia de péptidos natriuréticos que se encuentra en orina pero no en plasma y con una vida media mayor a la del ANP. En nuestro estudio, la estimulación de la GCm con urodilatina durante los primeros 15 minutos de la reperfusión atenuó la depleción de GMPc que tiene lugar durante la isquemia/reperfusión en corazones aislados. Este efecto estuvo asociado a una disminución de la hipercontractura, liberación de LDH y de la necrosis en bandas de contracción que se produce durante la reperfusión posterior a 60 minutos de isquemia. En corazones sometidos a 40 minutos de isquemia, duración que no produce necrosis en este modelo, se observó una mejor recuperación funcional. La confirmación de que el efecto protector obtenido tras la administración de urodilatina está mediado por GMPc se obtuvo al reproducir los resultados con el uso de su análogo soluble 8-bromo-GMPc.

2.1.2 Posible mecanismo de protección del GMPc

Se ha descrito en trabajos anteriores que concentraciones elevadas de ANP inhiben la actividad contráctil de miocitos en condiciones normóxicas [129]. Esta inhibición es consecuencia del aumento en la actividad de la GCm dado que altas concentraciones de GMPc inhiben la contractilidad [72]. En nuestro estudio la perfusión con urodilatina no afectó a la contractilidad de corazones normóxicos. Sin embargo, tuvo un efecto inotrópico negativo durante los primeros minutos de la reperfusión. La capacidad del GMPc para producir una desensibilización de los miofilamentos contráctiles al Ca^{2+} durante los primeros minutos de la reperfusión podría explicar su mecanismo de protección. Este efecto recordaría al obtenido con la droga 2,3-butanediona

monoxima (BDM). El completo bloqueo de la contractilidad con BDM durante el inicio de la reperfusión ha demostrado ser eficaz para prevenir la hipercontractura y reducir la necrosis en diferentes modelos experimentales [56, 66]. Trabajos previos han observado que el GMPc reduce la sensibilidad al Ca^{2+} en condiciones normóxicas sin alterar la cinética del Ca^{2+}. Aunque todavía no se ha descrito el mecanismo de este efecto, se sugiere que puede ser por disminución directa de la respuesta de las miofibrillas al Ca^{2+} por acción de la proteína quinasa dependiente de GMPc (PKG) [130]. Recientemente, se ha propuesto que el ANP reduciría la sensibilidad al Ca^{2+} de manera indirecta por modulación del transportador Na^+/H^+ vía PKG, situación que provocaría una prolongación de la acidosis durante el inicio de la reperfusión [129, 131]. La acidosis disminuye la respuesta de las miofibrillas al Ca^{2+} y su prolongación durante el inicio de la reperfusión tiene un efecto protector frente a la hipercontractura [68]. Sin embargo, también hay estudios describiendo un efecto inhibitorio del GMPc sobre los canales de Ca^{2+} tipo L por lo que no se puede descartar que el GMPc también pueda actuar modulando la cinética del Ca^{2+} durante la reperfusión.

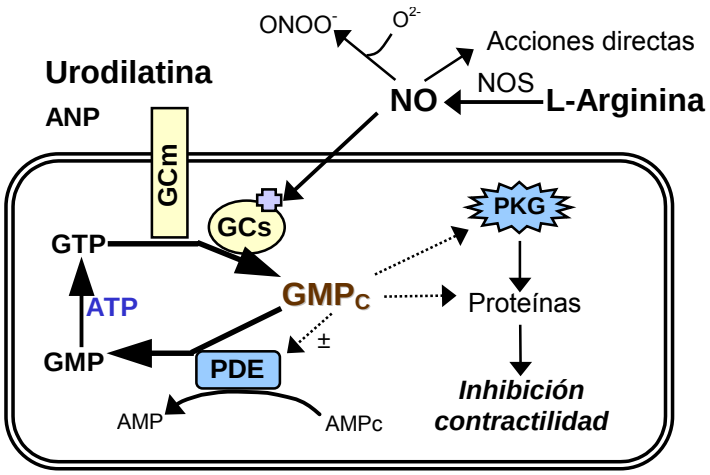

Figura 3. Esquema con las vías de síntesis de GMPc estimuladas con los tratamientos farmacológicos realizados. La administración de L-arginina puede presentar efectos independientes del GMPc. El aumento en la síntesis de GMPc produce inhibición de la contractilidad por mecanismos asociados o no a la activación de la proteína quinasa dependiente de GMPc (PKG). ANP: factor natriurético atrial. GCm: guanilato ciclasa de membrana. GCs: guanilato ciclasa soluble. PDE: fosfodiesterasa.

2.1.3 Implicaciones

El potencial terapéutico de la L-arginina en la prevención del daño por isquemia/reperfusión se muestra limitado por la necesidad de su administración antes del episodio isquémico y por la posible presencia de efectos no deseados independientes del GMPc. Este problema queda resuelto con la estimulación de la GCm usando urodilatina. Los resultados obtenidos muestran una atenuación en el desarrollo de la hipercontractura y muerte por necrosis a concentraciones que no ejercen ningún efecto sobre el corazón no sometido a isquemia y son de gran relevancia clínica ya que la urodilatina se ha podido administrar de manera segura en voluntarios sanos [132] y en pacientes con fallo cardiaco [133].

Estudios posteriores realizados en nuestro laboratorio han acentuado el potencial terapéutico que presenta la elevación de los niveles de GMPc durante la reperfusión al reproducir sus efectos beneficiosos en un modelo de corazón porcino *in situ* sometido a isquemia regional transitoria. En ellos, tanto la administración endovenosa preisquémica de L-arginina como la de urodilatina durante el inicio de la reperfusión produjeron una elevación de los niveles de GMPc hasta valores similares a los preisquémicos, aumento que se acompañó de una reducción significativa en el tamaño del infarto [134, 135]. Estos estudios aportan un dato adicional obtenido en el modelo de corazón de rata aislado que indica que el efecto protector del GMPc se pierde cuando los niveles de GMPc en la reperfusión son de 5-10 veces superiores a los preisquémicos. Las razones por las que una concentración demasiado elevada de GMPc resulta perjudicial no han sido determinadas pero el hecho de que no se reproduzca en miocitos aislados podría sugerir que la toxicidad ocurre a nivel de las células endoteliales microvasculares.

2.2 Contribución del intercambiador Na^+/Ca^{2+} al daño por reperfusión

2.2.1 Estudios previos

Un gran número de estudios apoya la hipótesis de que el daño inducido por la reperfusión es el resultado de la sobrecarga de Ca^{2+} citosólica que se produce durante el periodo isquémico como consecuencia de la activación del modo reverso del NCX (entrada de Ca^{2+}, salida de Na^+) [8]. La reenergización celular que tiene lugar con la reperfusión en presencia de unos niveles elevados de Ca^{2+} citosólico provoca el desarrollo de hipercontractura y la rotura de la membrana celular. Sin embargo, la implicación del NCX en este mecanismo se ha basado en pruebas no concluyentes debido a la falta de inhibidores específicos. Recientemente, la

molécula 2-[2-4[(nitrobenziloxil]isotiourea metanosulfonato (KB-R7943; Kanebo, Osaka, Japón) ha sido caracterizada como un inhibidor altamente selectivo del NCX [136, 137]. En estudios previos realizados en miocitos aislados se había observado que el KB-R7943 ejercía una inhibición más potente sobre el modo reverso (IC_{50} 0.32 μM) que sobre el directo (entrada de Na^+, salida de Ca^{2+}, IC_{50} 17 μM). Sin embargo las condiciones experimentales utilizadas en estos trabajos, definidas como unidireccionales, son condiciones iónicas en las que las concentraciones, tanto intracelulares como extracelulares, de Na^+ y Ca^{2+} sólo permiten que el NCX funcione en un sentido [136, 137] independientemente del potencial de membrana. Posteriormente se ha demostrado que bajo condiciones experimentales que permiten alternar la forma reversa y directa del NCX en respuesta a cambios en el potencial de membrana (condiciones bi-direccionales), tal y como ocurre durante el ciclo cardiaco, el KB-R7943 inhibe los dos modos de transporte con la misma potencia (IC_{50} 1 μM) [138]. Aunque no se conoce el mecanismo que explica estas diferencias en la selectividad dependiendo de las condiciones iónicas, se sugiere que éstas podrían ser debidas a la diferente prevalencia de los distintos estados conformacionales del transportador según las concentraciones de Na^+ y Ca^{2+} intra y extracelulares y a la mayor afinidad de la droga por la conformación que une Ca^{2+} extracelular.

En estudios previos, el KB-R7943 no altera el potencial de reposo ni ningún componente del potencial de acción a las concentraciones que inhibe el NCX [139]. Su aplicación previa a una isquemia transitoria ha reducido de manera significativa la sobrecarga de Ca^{2+} y la muerte celular asociada a hipoxia/reoxigenación en células aisladas y a isquemia/reperfusión en diferentes preparaciones que incluyen músculo papilar, corazón aislado y modelo porcino *in situ.* [75, 139, 140].

En nuestro estudio se sugiere la idea de que durante el inicio de la reperfusión se produce una entrada de Ca^{2+} adicional a la que tiene lugar durante el episodio isquémico que contribuiría al desarrollo de la hipercontractura al impedir una rápida normalización del Ca^{2+} intracelular y aumentar la frecuencia de las oscilaciones de Ca^{2+}. Esta hipótesis se apoya en estudios previos en los que una disminución de la concentración extracelular de Ca^{2+} o un aumento en la de Na^+ durante el inicio de la reperfusión atenúa el daño post-isquémico [141,142]. Por otro lado, durante el inicio de la reperfusión tras una isquemia prolongada, la concentración citosólica de Na^+ y la despolarización celular predicen un funcionamiento del NCX preferentemente en su forma reversa. La concentración citosólica de Na^+ se encuentra elevada debido a cuatro factores principales: 1) los miocitos llegan con una sobrecarga de Na^+ producida durante la fase

isquémica [14]; 2) la rápida normalización de pH está asociada a una entrada adicional de Na^+ a través del intercambiador Na^+/H^+, cotransportador Na^+/HCO_3^- [12]; 3) el Na^+ procedente de células adyacentes puede entrar a través de uniones tipo *gap* [22]; 4) después de una isquemia prolongada la actividad de la bomba Na^+/K^+ queda inhibida [21]. La magnitud de la entrada de Ca^{2+} dependerá del tiempo de recuperación de los niveles fisiológicos de Na^+ y por tanto de la capacidad de recuperación de la Na^+/K^+- ATPasa [24]. Sin embargo, a pesar de estos indicios no existían pruebas directas demostrando de manera inequívoca la entrada adicional de Ca^{2+} durante la reperfusión a través de la forma reversa del NCX debido principalmente a la ausencia hasta hace poco de inhibidores selectivos para este intercambiador.

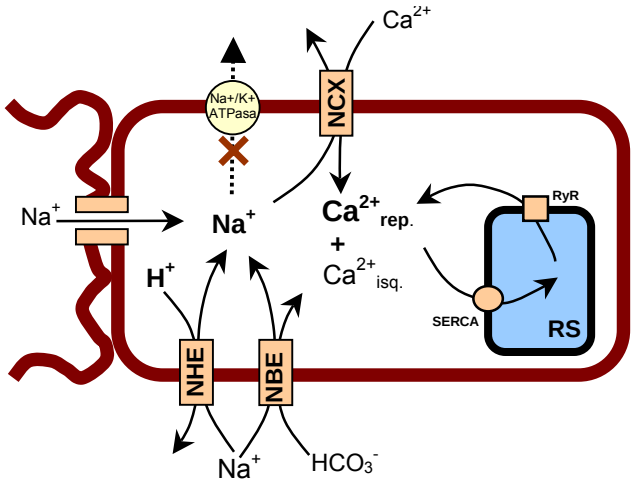

Figura 4. Mecanismos que llevan a la sobrecarga de Na^+ y Ca^{2+} durante la reperfusión. El Na^+ se acumula por su entrada asociada a la corrección del pH (NHE:intercambiador Na^+/H^+, NBE: cotransportador Na^+/HCO_3^-) y a la inhibición de la bomba de Na^+. El Na^+ también puede entrar procendente de miocitos adyacentes a través de uniones tipo gap. El gradiente electroquímico generado determina un funcionamiento en forma reversa del transportador Na^+/Ca^{2+} (NCX) que provoca una entrada adicional de Ca^{2+} durante la reperfusión. RS: retículo sarcoplasmático; RyR: receptor sensible a la rianodina; SERCA: bomba de Ca^{2+} del retículo sarcoplasmático.

2.2.2 Inhibición del intercambiador Na^+/Ca^{2+} durante el inicio de la reperfusión

Este estudio ha investigado la hipótesis de que la forma reversa del NCX produce una entrada adicional de Ca^{2+} durante la reperfusión miocárdica que contribuye al desarrollo de hipercontractura y a la muerte celular que tiene lugar de manera inmediata a la reperfusión. Para demostrarlo se inhibió el NCX con KB-R7943 durante los primeros minutos de la reperfusión en tres modelos experimentales complementarios: cardiomiocitos aislados, corazones de rata aislados y modelo porcino sometido a oclusión coronaria transitoria y reperfusión.

KB-R7943 inhibió la forma reversa del NCX en miocitos aislados con una IC_{50} de 0.15 μM, similar a la obtenida en trabajos anteriores [137]. Cuando las células se sometieron a inhibición metabólica, la re-energización en presencia del KB-R7943 redujo de manera dosis dependiente la proporción de células que desarrolló hipercontractura. La medición de Ca^{2+} intracelular con Fura-2 indicó una disminución en el Ca^{2+} citosólico durante los primeros minutos de la re-energización. Esta reducción fue debida a una menor entrada de Ca^{2+} extracelular a través de la forma reversa del NCX y no a una mayor captación del retículo sarcoplamático puesto que la inhibición de la captación y liberación de Ca^{2+} por parte del retículo sarcoplasmático con tapsigargina y rianodina, no afectó a la cinética del Ca^{2+} citosólico durante la reperfusión.

En miocitos sometidos a isquemia simulada, la reoxigenación en presencia de KB-R7943 redujo de manera significativa la frecuencia de oscilaciones espontáneas de Ca^{2+} que tienen lugar durante la fase inicial de la reoxigenación, disminución que se acompañó de una reducción en el desarrollo de hipercontractura. Estos resultados se reprodujeron con dos protocolos alternativos que previenen el transporte en forma reversa del NCX: eliminando el Ca^{2+} del medio extracelular y deplecionando el Na^{+} intracelular. Por otro lado, la reoxigenación con KB-R7943 no alteró la recuperación del pH intracelular descartándose la posibilidad de un efecto protector por prolongación de la acidosis intracelular [143].

En corazones de rata aislados y perfundidos sometidos a isquemia/reperfusión, la presencia del KB-R7943 a concentraciones entre 0.3 y 10 μM durante los primeros 10 minutos de la reperfusión produjo un aumento en la recuperación funcional, y disminuyó la hipercontractura, la liberación de LDH y la necrosis en bandas de contracción. La IC_{50} calculada para la inhibición en la liberación de LDH fue de 0.3 μM. Sin embargo, la reperfusión con concentraciones mayores a 15 μM tuvo un efecto deletéreo sobre la función y liberación enzimática. Cuando el tiempo de perfusión con KB-R7943 15 μM se redujo a 4 minutos, volvió a recuperarse su efecto beneficioso. La interpretación de estos efectos antagónicos ejercidos por KB-R7943 dependiendo de la concentración y tiempo de administración es la siguiente: durante el inicio de la reperfusión la concentración intracelular de Na^{+} existente y el potencial de membrana predicen un funcionamiento continuo del NCX en modo reverso. La inhibición del transportador en esta situación previene la entrada de Ca^{2+} y por tanto resulta beneficiosa. Sin embargo, aquellas células que sobreviven a los primeros minutos de la reperfusión vuelven a recuperar el gradiente transmembrana de Na^{+} y reactivan el transporte en modo directo, necesario para

normalizar el Ca^{2+} citosólico. Es en este momento cuando el bloqueo del NCX con una concentración superior a 10 μM resulta perjudicial. El efecto beneficioso obtenido reduciendo el tiempo de perfusión a 4 minutos indica que el NCX se encuentra en modo reverso y por tanto no se produce efecto deletéreo con altas concentraciones de KB-R7943 por inhibición de modo directo dado que no hay modo directo que inhibir.

La relevancia terapéutica de la inhibición del NCX durante el inicio de la reperfusión se puso de manifiesto en un modelo porcino sometido a una oclusión coronaria transitoria. En animales control, la administración de KB-R7943 en un rango de concentraciones inferior a 15 μM no tuvo efectos hemodinámicos. La infusión intracoronaria de KB-R7943 5 μM durante los primeros 10 minutos de la reperfusión redujo el tamaño del infarto en un 38%, mientras que el efecto protector se perdió con una concentración de 15 μM.

2.2.3 Implicaciones

Los resultados obtenidos en estos estudios apoyan la hipótesis de que durante los primeros minutos de la reperfusión se produce una entrada neta de Ca^{2+} a través del modo reverso del NCX que contribuye al desarrollo de hipercontractura y a la muerte inmediata por reperfusión. La inhibición del NCX durante el inicio de la reperfusión disminuye el daño por reperfusión y reduce el infarto de miocardio sin interferir en la función de células sanas. Esto se consigue atenuando el desarrollo de hipercontractura por 1) reducción de la entrada de Ca^{2+} durante la reperfusión con lo que se acelera la recuperación de la homeóstasis del Ca^{2+} 2) disminución de las oscilaciones espontáneas de Ca^{2+} mediadas por el retículo sarcoplasmático.

A pesar de la existencia de pruebas atribuyendo al NCX un papel crucial en el desarrollo de la hipercontractura, este no había sido inicialmente considerado una buena diana terapéutica sobre la que actuar debido a la falta de inhibidores selectivos y a su papel como principal vía de salida de Ca^{2+} intracelular. El hecho de que KB-R7943 inhiba diferentes canales iónicos a concentraciones cercanas a las requeridas para inhibir el NCX [136, 137], y la necesidad de reducir su presencia a los minutos iniciales de la reperfusión para asegurar la inhibición de la forma reversa sin afectar la salida de Ca^{2+}, limita su aplicación clínica y la reduce a aquellas condiciones de laboratorio en las que la concentración circulante de la droga se puede controlar con exactitud. Sin embargo, la eficacia que presenta esta droga en el modelo porcino con oclusión coronaria debe servir de estímulo para seguir buscando nuevos inhibidores del NCX. En vista de los resultados obtenidos es lógico pensar que análogos a KB-R7943 o nuevas

moléculas que presenten una mayor selectividad por el modo reverso del NCX tendrán un alto potencial terapéutico. Recientemente han aparecido estudios farmacológicos con una nueva droga, SEA0400, con una mayor selectividad por el NCX que la presentada por KB-R7943. La reperfusión de corazones aislados sometidos a isquemia con este nuevo inhibidor ha confirmado los resultados obtenidos en nuestro estudio acentuando con ello la importancia de la entrada de Ca^{2+} durante la reperfusión en el daño inmediato por reperfusión [144].

CONCLUSIONES

V- CONCLUSIONES

- Los resultados obtenidos en estos estudios demuestran por primera vez que las alteraciones metabólicas que tienen lugar durante un episodio isquémico inducen en los miocitos un estado de protección frente a la apoptosis generada por RLO y NO. El mecanismo por el que se produce esta protección se correlaciona con un aumento general en la expresión de genes con función anti-apoptótica. Estos resultados sugieren que la apoptosis perinecrótica observada tras un infarto agudo de miocardio es consecuencia de factores pro-apoptóticos (RLO, NO, citoquinas) liberados por células distintas a los miocitos durante la reacción inflamatoria (neutrófilos) que desencadenarían apoptosis tras la pérdida del estado protector inducido en miocitos por la isquemia miocárdica. Los resultados presentados apoyan la hipótesis de que la contribución de la apoptosis al total de la muerte celular que tiene lugar durante la reperfusión es poco relevante.

- Por el contrario, la mayor parte de la muerte celular ocurre de manera inmediata a la reperfusión en forma de necrosis debido a la sobrecarga mecánica causada principalmente por la hipercontractura. En estos estudios se describen dos nuevas estrategias que atenúan por mecanismos distintos el desarrollo de la hipercontractura:

1. La isquemia miocárdica provoca una reducción en la concentración de GMPc. La estimulación de la GCm con urodilatina durante el inicio de la reperfusión atenúa la depleción de GMPc, inhibe la contractilidad reduciendo la fuerza contráctil que genera la reenergización celular y, con ello, disminuye la hipercontractura. Los efectos beneficiosos de la urodilatina se pueden reproducir estimulando la GCs con L-arginina pero tiene los inconvenientes frente a la anterior de que precisa ser administrada de manera previa al periodo isquémico y puede presentar efectos independientes del GMPc mediados por NO.

2. Se demuestra por primera vez que durante los primeros minutos de la reperfusión actúa la forma reversa del NCX contribuyendo con una entrada adicional de Ca^{2+} a la sobrecarga de Ca^{2+} citosólico que tiene lugar durante el episodio isquémico. La inhibición del NCX durante el inicio de la reperfusión acelera la recuperación de la homeóstasis de Ca^{2+}, disminuye las oscilaciones, atenúa la hipercontractura y reduce la muerte celular. Sin embargo, una vez

reestablecido el gradiente transmembrana de Na^+, necesario para que el NCX normalice el Ca^{2+} citosólico a través de su modo directo, la inhibición del intercambiador puede resultar deletérea.

En resumen, estos estudios demuestran que la fase temprana de la reperfusión y en particular la atenuación del desarrollo de la hipercontractura representa una importante diana para desarrollar estrategias encaminadas a proteger al miocardio sometido a isquemia/reperfusión. La adaptación de estas estrategias al ámbito clínico podría representar un importante avance en el tratamiento del infarto agudo de miocardio y en la protección miocárdica durante la cirugía cardiaca.

BIBLIOGRAFÍA

BIBLIOGRAFIA

[1] Grande P, Christiansen C, Pedersen A. Influence of acute myocardial infarct size on acute and one-year mortality. *Eur Heart J*. 1983;4:20-5.

[2] Davies MJ, Thomas AC. Plaque fissuring: the cause of acute myocardial infarction, sudden ischaemic death, and crescendo angina. *Br Heart J*. 1985;53:363-73.

[3] Bolli R, Marban E. Molecular and cellular mechanisms of myocardial stunning. *Physiol Rev*. 1999;79:609-34.

[4] Matsumura K, Jeremy RW, Schaper J, Becker LC. Progression of myocardial necrosis during reperfusion of ischemic myocardium. *Circulation*. 1998;97:795-804.

[5] Grines CL, Browne KF, Marco J, Rothbaum D, Stone GW, O'Keefe J, Overlie P,Donohue B, Chelliah N, Timmis GC, et al. A comparison of immediate angioplasty with thrombolytic therapy for acute myocardial infarction. The Primary Angioplasty in Myocardial Infarction Study Group. *N Engl J Med*. 1993;328:673-9.

[6] Zijlstra F, de Boer MJ, Hoorntje JC, Reiffers S, Reiber JH, Suryapranata H. A comparison of immediate coronary angioplasty with intravenous streptokinase in acute myocardial infarction. *N Engl J Med*. 1993;328:680-4.

[7] Vetterlein F, Schrader C, Volkmann R, Neckel M, Ochs M, Schmidt G, Hellige G. Extent of damage in ischemic, nonreperfused, and reperfused myocardium of anesthetized rats. *Am J Physiol.* 2003;285:H755-65.

[8] Piper HM, Garcia-Dorado D, Ovize M. A fresh look at reperfusion injury. *Cardiovasc Res*. 1998;38:291-300.

[9] Berridge MJ, Lipp P, Bootman MD. The versatility and universality of calcium signalling. *Nat Rev Mol Cell Biol*. 2000;1:11-21.

[10] Anderson SE, Dickinson CZ, Liu H, Cala PM. Effects of Na-K-2Cl cotransport inhibition on myocardial Na and Ca during ischemia and reperfusion. *Am J Physiol*. 1996;270:C608-18.

[11] Neely JR, Grotyohann LW. Role of glycolytic products in damage to ischemic myocardium. Dissociation of adenosine triphosphate levels and recovery of function of reperfused ischemic hearts. *Circ Res.* 1984;55:816-24.

[12] Leem CH, Lagadic-Gossmann D, Vaughan-Jones RD. Characterization of intracellular pH regulation in the guinea-pig ventricular myocyte. *J Physiol*. 1999;517:159-80.

[13] Cross HR, Radda GK, Clarke K. The role of Na^+/K^+ ATPase activity during low flow ischemia in preventing myocardial injury: a 31P, 23Na and 87Rb NMR spectroscopic study. *Magn Reson Med*. 1995;34:673-85.

[14] Pike MM, Luo CS, Clark MD, Kirk KA, Kitakaze M, Madden MC, Cragoe EJ Jr, Pohost GM. NMR measurements of Na^+ and cellular energy in ischemic rat heart: role of $Na(^+)$-H^+ exchange. *Am J Physiol*. 1993;265:H2017-26.

[15] Pike MM, Luo CS, Yanagida S, Hageman GR, Anderson PG. 23Na and 31P nuclear magnetic resonance studies of ischemia-induced ventricular fibrillation. Alterations of intracellular Na^+ and cellular energy. *Circ Res*. 1995;77:394-406.

[16] Shivkumar K, Deutsch NA, Lamp ST, Khuu K, Goldhaber JI, Weiss JN. Mechanism of hypoxic K loss in rabbit ventricle. *J Clin Invest*. 1997;100:1782-8.

[17] Blaustein MP, Lederer WJ. Sodium/calcium exchange: its physiological implications. *Physiol Rev*. 1999;79:763-854.

[18] Steenbergen C, Murphy E, Levi L, London RE. Elevation of cytosolic free calcium concentration early in myocardial ischemia in rperfused rat heart. *Circ Res*. 1987;60:700-707.

[19] Garlick PB, Davies MJ, Hearse DJ, Slater TF. Direct detection of free radicals in the reperfused rat heart using electron spin resonance spectroscopy. *Circ Res*. 1987;61:757-60.

[20] Siegmund B, Koop A, Klietz T, Schwartz P, Piper HM. Sarcolemmal integrity and metabolic competence of cardiomyocytes under anoxia-reoxygenation. *Am J Physiol*. 1990;258:H285-91.

[21] Bersohn MM. Sodium pump inhibition in sarcolemma from ischemic hearts. *J Mol Cell Cardiol*. 1995;27:1483-1489.

[22] Ruiz-Meana M, Garcia-Dorado D, Hofstaetter B, Piper HM, Soler-Soler J. Propagation of cardiomyocyte hypercontracture by passage of Na(+) through gap junctions. *Circ Res*. 1999;85:280-7.

[23] Siegmund B, Schlack W, Ladilov YV, Balser C, Piper HM. Halothane protects cardiomyocytes against reoxygenation-induced hypercontracture. *Circulation*. 1997;96:4372-9.

[24] Siegmund B, Ladilov YV, Piper HM. Importance of sodium for recovery of calcium control in reoxygenated cardiomyocytes. *Am J Physiol*. 1994;267:H506-13.

[25] Ladilov YV, Siegmund B, Balser C, Piper HM. Simulated ischemia increases the susceptibility of rat cardiomyocytes to hypercontracture. *Circ Res*. 1997;80:69-75.

[26] Ganote CE, Vander Heide RS. Irreversible injury of isolated adult rat myocytes. Osmotic fragility during metabolic inhibition. *Am J Pathol*. 1988;132:212-22.

[27] Jennings RB, Steenbergen C Jr, Reimer KA. Myocardial ischemia and reperfusion. *Monogr Pathol*. 1995;37:47-80.

[28] Garcia-Dorado D, Oliveras J, Gili J, Sanz E, Perez-Villa F, Barrabes J, Carreras MJ, Solares J, Soler-Soler J. Analysis of myocardial oedema by magnetic resonance imaging early after coronary artery occlusion with or without reperfusion. *Cardiovasc Res*. 1993;27:1462-9.

[29] Inserte J, Garcia-Dorado D, Ruiz-Meana M, Solares J, Soler J. The role of Na^+-H^+ exchange occurring during hypoxia in the genesis of reoxygenation-induced myocardial oedema. *J Mol Cell Cardiol*. 1997;29:1167-75.

[30] Van der Heide RS, Angelo JP, Altschuld RA, Ganote CE. Energy dependence of contraction band formation in perfused jearts and isolated adult cardiomyocytes. *Am J Pathol*. 1986;125:55-68.

[31] Cummings BS, McHowat J, Schnellmann RG. Phospholipase A(2)s in cell injury and death. *J Pharmacol Exp Ther*. 2000;294:793-9.

[32] Daleau P. Lysophosphatidylcholine, a metabolite which accumulates early in myocardium during ischemia, reduces gap junctional coupling in cardiac cells. *J Mol Cell Cardiol*. 1999;31:1391-401.

[33] Yu L, Netticadan T, Xu YJ, Panagia V, Dhalla NS. Mechanisms of lysophosphatidylcholine-induced increase in intracellular calcium in rat cardiomyocytes*. J Pharmacol Exp Ther*. 1998;286:1-8.

[34] Halestrap AP, Kerr PM, Javadov S, Woodfield KY. Elucidating the molecular mechanism of the permeability transition pore and its role in reperfusion injury of the heart. *Biochim Biophys Acta*. 1998;1366:79-94.

[35] Wang KK. Calpain and caspase: can you tell the difference?*Trends Neurosci*. 2000;23:20-6.

[36] Yoshida K, Inui M, Harada K, Saido TC, Sorimachi Y, Ishihara T, Kawashima S, Sobue K. Reperfusion of rat heart after brief ischemia induces proteolysis of calspectin (nonerythroid spectrin or fodrin) by calpain. *Circ Res*. 1995;77:603-10.

[37] Inserte J, Garcia-Dorado D, Ruiz-Meana M, Agulló L, Pina P, Soler-Soler J. Attenuated proteolysis of structural proteins by calpain is an end-effector mechanism of ischemic preconditioning and depends on protein kinase A activation. *Circulation*. En prensa.

[38] Gao WD, Liu Y, Mellgren R, Marban E. Intrinsic myofilament alterations underlying the decreased contractility of stunned myocardium. A consequence of Ca^{2+}-dependent proteolysis? *Circ Res*. 1996;78:455-65.

[39] Halestrap AP. Regulation of mitochondrial metabolism through changes in matrix volume. *Biochem Soc Trans*. 1994;22:522-9.

[40] Griffiths EJ, Halestrap AP. Protection by Cyclosporin A of ischemia/reperfusion-induced damage in isolated rat hearts. *J Mol Cell Cardiol*. 1993;25:1461-9.

[41] Halestrap AP. Calcium-dependent opening of a non-specific pore in the mitochondrial inner membrane is inhibited at pH values below 7. Implications for the protective effect of low pH against chemical and hypoxic cell damage. *Biochem J*. 1991;278:715-9.

[42] Di Lisa F, Menabo R, Canton M, Barile M, Bernardi P. Opening of the mitochondrial permeability transition pore causes depletion of mitochondrial and cytosolic NAD+ and is a causative event in the death of myocytes in postischemic reperfusion of the heart. *J Biol Chem*. 2001;276:2571-5.

[43] Kroemer G, Reed JC. Mitochondrial control of cell death. Nat Med. 2000;6:513-9.

[44] Korsmeyer SJ, Wei MC, Saito M, Weiler S, Oh KJ, Schlesinger PH. Pro-apoptotic cascade activates BID, which oligomerizes BAK or BAX into pores that result in the release of cytochrome c. *Cell Death Differ*. 2000;7:1166-73.

[45] Yang J, Liu X, Bhalla K, Kim CN, Ibrado AM, Cai J, Peng TI, Jones DP, Wang X. Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. *Science*. 1997;275:1129-32.

[46] Cande C, Cecconi F, Dessen P, Kroemer G. Apoptosis-inducing factor (AIF): key to the conserved caspase-independent pathways of cell death?J *Cell Sci*. 2002;115:4727-34.

[47] Gottlieb RA, Burleson KO, Kloner RA, Babior BM, Engler RL. Reperfusion injury induces apoptosis in rabbit cardiomyocytes. *J Clin Invest*. 1994;94:1621-8.

[48] Majno G, Joris I. Apoptosis, oncosis, and necrosis. An overview of cell death. *Am J Pathol*. 1995;146:3-15.

[49] Saraste A, Pulkki K. Morphologic and biochemical hallmarks of apoptosis. *Cardiovasc Res*. 2000;45:528-37.

[50] Krippner A, Matsuno-Yagi A, Gottlieb RA, Babior BM. Loss of function of cytochrome c in Jurkat cells undergoing fas-mediated apoptosis. *J Biol Chem*. 1996;271:21629-36.

[51] Nakagawa T, Yuan J. Cross-talk between two cysteine protease families. Activation of caspase-12 by calpain in apoptosis. *J Cell Biol*. 2000;150:887-94.

[52] Chen M, Won DJ, Krajewski S, Gottlieb RA. Calpain and mitochondria in ischemia/reperfusion injury. *J Biol Chem*. 2002;277:29181-6.

[53] Leist M, Single B, Castoldi AF, Kuhnle S, Nicotera P. Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. *J Exp Med*. 1997;185:1481-6.

[54] Freude B, Masters TN, Robicsek F, Fokin A, Kostin S, Zimmermann R, Ullmann C, Lorenz-Meyer S, Schaper J. Apoptosis is initiated by myocardial ischemia and executed during reperfusion. *J Mol Cell Cardiol*. 2000;32:197-208.

[55] Ganote CE. Contraction band necrosis and irreversible myocardial injury. *J Mol Cell Cardiol*. 1983;15:67-73.

[56] Garcia-Dorado D, Theroux P, Duran JM, Solares J, Alonso J, Sanz E, Munoz R, Elizaga J, Botas J, Fernandez-Aviles F, et al. Selective inhibition of the contractile apparatus. A new

approach to modification of infarct size, infarct composition, and infarct geometry during coronary artery occlusion and reperfusion. *Circulation*. 1992;85:1160-74.

[57] Bartling B, Holtz J, Darmer D. Contribution of myocyte apoptosis to myocardial infarction? *Basic Res Cardiol*. 1998;93:71-84.

[58] Vakeva AP, Agah A, Rollins SA, Matis LA, Li L, Stahl GL. Myocardial infarction and apoptosis after myocardial ischemia and reperfusion: role of the terminal complement components and inhibition by anti-C5 therapy. *Circulation*. 1998;97:2259-67.

[59] Park JL, Lucchesi BR. Mechanisms of myocardial reperfusion injury. *Ann Thorac Surg*. 1999;68:1905-1912.

[60] Garcia-Dorado D, Ruiz-Meana M, Padilla F, Rodriguez-Sinovas A, Mirabet M. Gap junction-mediated intercellular communication in ischemic preconditioning. *Cardiovasc Res*. 2002;55:456-65.

[61] Solares J, Garcia-Dorado D, Oliveras J, Gonzalez MA, Ruiz-Meana M, Barrabes JA, Gonzalez-Bravo C, Soler-Soler J. Contraction band necrosis at the lateral borders of the area at risk in reperfused infarcts. Observations in a pig model of in situ coronary occlusion. *Virchows Arch*. 1995;426:393-9.

[62] Barrabes JA, Garcia-Dorado D, Ruiz-Meana M, Piper HM, Solares J, Gonzalez MA, Oliveras J, Herrejon MP, Soler Soler J. Myocardial segment shrinkage during coronary reperfusion in situ. Relation to hypercontracture and myocardial necrosis. *Pflugers Arch*. 1996;431:519-26.

[63] Miyazaki S, Fujiwara H, Onodera T, Kihara Y, Matsuda M, Wu DJ, Nakamura Y, Kumada T, Sasayama S, Kawai C, et al. Quantitative analysis of contraction band and coagulation necrosis after ischemia and reperfusion in the porcine heart. *Circulation*. 1987;75:1074-82.

[64] Ruiz-Meana M, Garcia-Dorado D, Gonzalez MA, Barrabes JA, Soler-Soler J. Effect of osmotic stress on sarcolemmal integrity of isolated cardiomyocytes following transient metabolic inhibition. *Cardiovasc Res*. 1995;30:64-9.

[65] Garcia-Dorado D, Inserte J, Ruiz-Meana M, Gonzalez MA, Solares J, Julia M, Barrabes JA, Soler-Soler J. Gap junction uncoupler heptanol prevents cell-to-cell progression of hypercontracture and limits necrosis during myocardial reperfusion. *Circulation*. 1997;96:3579-86.

[66] Siegmund B, Klietz T, Schwartz P, Piper HM. Temporary contractile blockade prevents hypercontracture in anoxic-reoxygenated cardiomyocytes. *Am J Physiol*. 1991;260:H426-35.

[67] Lemasters JJ, Bond JM, Chacon E Harper IS, Kaplan SH, Ohata H, Trollinger DR, Herman B, Cascio WE. The pH paradox in ischemia-reperfusion injury to cardiac myocytes. *EXS*. 1996;76:99-114.

[68] Schafer C, Ladilov YV, Siegmund B, Piper HM. Importance of bicarbonate transport for protection of cardiomyocytes against reoxygenation injury. *Am J Physiol*. 2000;278:H1457-63.

[69] Klein HH, Pich S, Bohle RM, Lindert-Heimberg S, Nebendahl K. Na(+)/H(+) exchange inhibitor cariporide attenuates cell injury predominantly during ischemia and not at onset of reperfusion in porcine hearts with low residual blood flow. *Circulation*. 2000;102:1977-82.

[70] Zeymer U, Suryapranata H, Monassier JP, Opolski G, Davies J, Rasmanis G, Linssen G, Tebbe U, Schroder R, Tiemann R, Machnig T, Neuhaus KL; ESCAMI Investigators. The Na(+)/H(+) exchange inhibitor eniporide as an adjunct to early reperfusion therapy for acute myocardial infarction. Results of the evaluation of the safety and cardioprotective effects of eniporide in acute myocardial infarction (ESCAMI) trial. *J Am Coll Cardiol*. 2001;38:1644-50.

[71] Hempel A, Friedrich M, Schluter KD, Forssmann WG, Kuhn M, Piper HM. ANP protects against reoxygenation-induced hypercontracture in adult cardiomyocytes. *Am J Physiol*. 1997;273:H244-9.

[72] Mentzer RM Jr, Lasley RD, Jessel A, Karmazyn M. Intracellular sodium hydrogen exchange inhibition and clinical myocardial protection. *Ann Thorac Surg*. 2003;75:S700-8.

[73] Allen DG, Xiao XH. Role of the cardiac Na+/H+ exchanger during ischemia and reperfusion. *Cardiovasc Res*. 2003;57:934-41.

[74] Ruiz-Meana M, Garcia-Dorado D, Pina P, Inserte J, Agullo L, Soler-Soler J. Cariporide preserves mitochondrial proton gradient and delays ATP depletion in cardiomyocytes during ischemic conditions. *Am J Physiol*. 2003;285:H999-1006.

[75] Ladilov Y, Haffner S, Balser-Schafer C, Maxeiner H, Piper HM. Cardioprotective effects of KB-R7943: a novel inhibitor of the reverse mode of Na+/Ca2+ exchanger. *Am J Physiol*. 1999;276:H1868-76.

[76] Forman MB, Puett DW, Virmani R. Endothelial and myocardial injury during ischemia and reperfusion: pathogenesis and therapeutic implications. *J Am Coll Cardiol*. 1989;13:450-9.

[77] Entman ML, Youker K, Shoji T, Kukielka G, Shappell SB, Taylor AA, Smith CW. Neutrophil induced oxidative injury of cardiac myocytes. A compartmented system requiring CD11b/CD18-ICAM-1 adherence. *J Clin Invest*. 1992;90:1335-45.

[78] Romson JL, Hook BG, Kunkel SL, Abrams GD, Schork MA, Lucchesi BR. Reduction of the extent of ischemic myocardial injury by neutrophil depletion in the dog. *Circulation*. 1983;67:1016-23.

[79] Litt MR, Jeremy RW, Weisman HF, Winkelstein JA, Becker LC. Neutrophil depletion limited to reperfusion reduces myocardial infarct size after 90 minutes of ischemia. Evidence for neutrophil-mediated reperfusion injury. *Circulation*. 1989;80:1816-27.

[80] Libersan D, Quan E, Merhi Y, Uzan A, Laperriere L, Latour JG. Intravenous aspirin at reperfusion does not reduce infarct size in the dog with a residual critical stenosis. *J Cardiovasc Pharmacol*. 1999;34:575-83.

[81] Tofukuji M, Metais C, Collard CD, Morse DS, Stahl GL, Nelson DP, Li J, Simons M, Sellke FW. Effect of sialyl Lewis(x) oligosaccharide on myocardial and cerebral injury in the pig. *Ann Thorac Surg*. 1999;67:112-9.

[82] Briaud SA, Ding ZM, Michael LH, Entman ML, Daniel S, Ballantyne CM. Leukocyte trafficking and myocardial reperfusion injury in ICAM-1/P-selectin-knockout mice. *Am J Physiol*. 2001;280:H60-7.

[83] Barrabes JA, Garcia-Dorado D, Oliveras J, Gonzalez MA, Ruiz-Meana M, Solares J, Burillo AG, Lidon RM, Antolin M, Castell J, Soler-Soler J. Intimal injury in a transiently occluded coronary artery increases myocardial necrosis. Effect of aspirin. *Pflugers Arch*. 1996;432:663-70.

[84] Laws KH, Clanton JA, Starnes VA, Lupinetti FM, Collins JC, Oates JA, Hammon JW Jr. Kinetics and imaging of indium-11-labeled autologous platelets in experimental myocardial infarction. *Circulation*. 1983;67:110-6.

[85] Seligmann C, Kupatt C, Becker BF, Zahler S, Beblo S. Adenosine endogenously released during early reperfusion mitigates postischemic myocardial dysfunction by inhibiting platelet adhesion. *J Cardiovasc Pharmacol*. 1998;32:156-63.

[86] Heindl B, Zahler S, Welsch U, Becker BF. Disparate effects of adhesion and degranulation of platelets on myocardial and coronary function in postischaemic hearts. *Cardiovasc Res*. 1998;38:383-94.

[87] Mirabet M, Garcia-Dorado D, Inserte J, Barrabes JA, Lidon RM, Soriano B, Azevedo M, Padilla F, Agullo L, Ruiz-Meana M, Massaguer A, Pizcueta P, Soler-Soler J. Platelets activated by transient coronary occlusion exacerbate ischemia-reperfusion injury in rat hearts. *Am J Physio*l. 2002;283:H1134-41.

[88] Olivetti G, Quaini F, Sala R, Lagrasta C, Corradi D, Bonacina E, Gambert SR, Cigola E, Anversa P. Acute myocardial infarction in humans is associated with activation of programmed myocyte cell death in the surviving portion of the heart. *J Mol Cell Cardiol*. 1996;28:2005-16.

[89] Anversa P, Cheng W, Liu Y, Leri A, Redaelli G, Kajstura J. Apoptosis and myocardial infarction. *Basic Res Cardiol*. 1998;93:8-12.

[90] Yaoita H, Ogawa K, Maehara K, Maruyama Y. Apoptosis in relevant clinical situations: contribution of apoptosis in myocardial infarction. *Cardiovasc Res*. 2000;45:630-41.

[91] Ekhterae D, Lin Z, Lundberg MS, Crow MT, Brosius FC 3rd, Nunez G. ARC inhibits cytochrome c release from mitochondria and protects against hypoxia-induced apoptosis in heart-derived H9c2 cells. *Circ Res*. 1999;85:70-7.

[92] Ohno M, Takemura G, Ohno A, Misao J, Hayakawa Y, Minatoguchi S, Fujiwara T,Fujiwara H. "Apoptotic" myocytes in infarct area in rabbit hearts may be oncotic myocytes with DNA fragmentation: analysis by immunogold electron microscopy combined with in situ nick end-labeling. *Circulation*. 1998;98:1422-30.

[93] Uchiyama T, Otani H, Okada T, Ninomiya H, Kido M, Imamura H, Nogi S, Kobayashi Y. Nitric oxide induces caspase-dependent apoptosis and necrosis in neonatal rat cardiomyocytes. *J Mol Cell Cardiol*. 2002;34:1049-61.

[94] Condorelli G, Roncarati R, Ross J Jr, Pisani A, Stassi G, Todaro M, Trocha S, Drusco A, Gu Y, Russo MA, Frati G, Jones SP, Lefer DJ, Napoli C, Croce CM. Heart-targeted overexpression of caspase3 in mice increases infarct size and depresses cardiac function. *Proc Natl Acad Sci U S A*. 2001;98:9977-82.

[95] Chen Z, Chua CC, Ho YS, Hamdy RC, Chua BH. Overexpression of Bcl-2 attenuates apoptosis and protects against myocardial I/R injury in transgenic mice. *Am J Physiol*. 2001;280:H2313-20.

[96] Bolli R, Jeroudi MO, Patel BS, DuBose CM, Lai EK, Roberts R, Mc Cay PB. Direct evidence tht oxygen derived free radicals contribute to postischemic myocardial dysfunction in the intact dog. *Proc Natl Acad Sci*. 1989;86:4695-4699.

[97] Dhalla NS, Elmoselhi AB, Hata T, Makino N. Status of myocardial antioxidants in ischemia-reperfusion injury. *Cardiovasc Res*. 2000;47:446-56.

[98] Semenza GL. Cellular and molecular dissection of reperfusion injury: ROS within and without. *Circ Res*. 2000;86:117-8.

[99] Goldhaber JI, Weiss JN. Oxygen free radicals and cardiac reperfusion abnormalities. *Hypertension*. 1992;20:118-27

[100] Arduini A, Mezzetti A, Porreca E, Lapenna D, DeJulia J, Marzio L, Polidoro G, Cuccurullo F. Effect of ischemia and reperfusion on antioxidant enzymes and mitochondrial inner membrane proteins in perfused rat heart. *Biochim Biophys Acta*. 1988;970:113-21.

[101] Ferrari R, Ceconi C, Curello S, Guarnieri C, Caldarera CM, Albertini A, Visioli O. Oxygen-mediated myocardial damage during ischaemia and reperfusion: role of the cellular defences against oxygen toxicity. *J Mol Cell Cardiol*. 1985;17:937-45.

[102] Yue TL, Ma XL, Wang X, Romanic AM, Liu GL, Louden C, Gu JL, Kumar S, Poste G, Ruffolo RR Jr, Feuerstein GZ. Possible involvement of stress-activated protein kinase signaling pathway and Fas receptor expression in prevention of ischemia/reperfusion-induced cardiomyocyte apoptosis by carvedilol. *Circ Res*. 1998;82:166-74.

[103] Galang N, Sasaki H, Maulik N. Apoptotic cell death during ischemia/reperfusion and its attenuation by antioxidant therapy. *Toxicology*. 2000;148:111-8.

[104] Rajesh KG, Sasaguri S, Suzuki R, Maeda H. Antioxidant MCI-186 Inhibits Mitochondrial Permeability Transition Pore and Up regulates Bcl-2 Expression. *Am J Physiol*. 2003.

[105] Shimojo T, Hiroe M, Ishiyama S, Ito H, Nishikawa T, Marumo F. Nitric oxide induces apoptotic death of cardiomyocytes via a cyclic-GMP-dependent pathway. *Exp Cell Res*. 1999;247:38-47.

[106] Taimor G, Hofstaetter B, Piper HM. Apoptosis induction by nitric oxide in adult cardiomyocytes via cGMP signaling and its impairment after simulated ischemia. *Cardiovasc Res*. 2000;45:588-94.

[107] Ing DJ, Zang J, Dzau VJ, Webster KA, Bishopric NH. Modulation of cytokine induced cardiac myocyte apoptosis by nitric oxide, Bak, and Bcl-x. *Circ Res*. 1999;84:21-33.

[108] Liu P, Hock CE, Nagele R, Wong PY. Formation of nitric oxide, superoxide, and peroxynitrite in myocardial ischemia-reperfusion injury in rats. *Am J Physiol*. 1997;272:H2327-36.

[109] Song W, Lu X, Feng Q. Tumor necrosis factor-alpha induces apoptosis via inducible nitric oxide synthase in neonatal mouse cardiomyocytes. *Cardiovasc Res*. 2000;45:595-602.

[110] Neyses L, Vetter H. Action of atrial natriuretic peptide and angiotensin II on the myocardium:studies in isolated rat ventricular cardiomyocytes. *Biochem Biophys Res Commun*. 1989;163:1435-43.

[111] Okamura T, Miura T, Takemura G, Fujiwara H, Iwamoto H, Kawamura S, Kimura M,Ikeda Y, Iwatate M, Matsuzaki M. Effect of caspase inhibitors on myocardial infarct size and myocyte DNA fragmentation in the ischemia-reperfused rat heart. *Cardiovasc Res.* 2000;45:642-50.

[112] Elsasser A, Suzuki K, Lorenz-Meyer S, Bode C, Schaper J.The role of apoptosis in myocardial ischemia: a critical appraisal. *Basic Res Cardiol*. 2001;96:219-26.

[113] Misao J, Hayakawa Y, Ohno M, Kato S, Fujiwara T, Fujiwara H. Expression of bcl-2 protein, an inhibitor of apoptosis, and Bax, an accelerator of apoptosis, in ventricular myocytes of human hearts with myocardial infarction. *Circulation*. 1996;94:1506-12.

[114] Matsushita K, Umezawa A, Iwanaga S, Oda T, Okita H, Kimura K, Shimada M, Tanaka M, Sano M, Ogawa S, Hata J. The EAT/mcl-1 gene, an inhibitor of apoptosis, is up-regulated in the early stage of acute myocardial infarction. *Biochim Biophys Acta*. 1999;1472:471-8.

[115] Depre C, Tomlinson JE, Kudej RK, Gaussin V, Thompson E, Kim SJ, Vatner DE, Topper JN, Vatner SF. Gene program for cardiac cell survival induced by transient ischemia in conscious pigs. *Proc Natl Acad Sci U S A*. 2001;98:9336-41.

[116] Li PF, Dietz R, von Harsdorf R. Reactive oxygen species induce apoptosis of vascular smooth muscle cell. *FEBS Lett*. 1997;404:249-52.

[117] Stangel M, Zettl UK, Mix E, Zielasek J, Toyka KV, Hartung HP, Gold R. H2O2 and nitric oxide-mediated oxidative stress induce apoptosis in rat skeletal muscle myoblasts. *J Neuropathol Exp Neurol*. 1996;55:36-43.

[118] Curello S, Ceconi C, Cargnoni A, Cornacchiari A, Ferrari R, Albertini A. Improved procedure for determining glutathione in plasma as an index of myocardial oxidative stress. *Clin Chem*. 1987;33:1448-9.

[119] Wildhirt SM, Suzuki H, Horstman D, Weismuller S, Dudek RR, Akiyama K, Reichart B. Selective modulation of inducible nitric oxide synthase isozyme in myocardial infarction. *Circulation*. 1997;96:1616-23.

[120] Oltvai ZN, Milliman CL, Korsmeyer SJ. Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. Cell. 1993;74:609-19.

[121] Tsujimoto Y. Role of Bcl-2 family proteins in apoptosis: apoptosomes or mitochondria? *Genes Cells*. 1998;3:697-707.

[122] Deveraux QL, Roy N, Stennicke HR, Van Arsdale T, Zhou Q, Srinivasula SM, Alnemri ES, Salvesen GS, Reed JC. IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases. *EMBO J*. 1998;17:2215-23.

[123] Hofstaetter B, Taimor G, Inserte J, Garcia-Dorado D, Piper HM. Inhibition of apoptotic responses after ischemic stress in isolated hearts and cardiomyocytes. *Basic Res Cardiol*. 2002;97:479-88.

[124] Taimor G, Lorenz H, Hofstaetter B, Schluter KD, Piper HM. Induction of necrosis but not apoptosis after anoxia and reoxygenation in isolated adult cardiomyocytes of rat. *Cardiovasc Res*. 1999;41:147-56.

[125] Zhao ZQ, Nakamura M, Wang NP, Wilcox JN, Shearer S, Ronson RS, Guyton RA, Vinten-Johansen J. Reperfusion induces myocardial apoptotic cell death. *Cardiovasc Res*. 2000;45:651-60.

[126] Amrani M, Gray CC, Smolenski RT, Goodwin AT, London A, Yacoub MH. The effect of L-arginine on myocardial recovery after cardioplegic arrest and ischemia under moderate and deep hypothermia. *Circulation*. 1997;96:274-9.

[127] Weyrich AS, Ma XL, Lefer AM. The role of L-arginine in ameliorating reperfusion injury after myocardial ischemia in the cat. *Circulation*. 1992;86:279-88.

[128] Maulik N, Engelman DT, Watanabe M, Engelman RM, Rousou JA, Flack JE 3rd, Deaton DW, Gorbunov NV, Elsayed NM, Kagan VE, Das DK. Nitric oxide/carbon monoxide. A molecular switch for myocardial preservation during ischemia. *Circulation*. 1996;94:398-406.

[129] Tajima M, Bartunek J, Weinberg EO, Ito N, Lorell BH.Atrial natriuretic peptide has different effects on contractility and intracellular pH in normal and hypertrophied myocytes from pressure overloaded hearts. *Circulation*. 1998;98:2760-4.

[130] Shah AM, Spurgeon HA, Sollott SJ, Talo A, Lakatta EG. 8-bromo-cGMP reduces the myofilament response to Ca2+ in intact cardiac myocytes. *Circ Res*. 1994;74:970-8.

[131] Ito N, Bartunek J, Spitzer KW, Lorell BH. Effects of the nitric oxide donor sodium nitroprusside on intracellular pH and contraction in hypertrophied myocytes. *Circulation*. 1997;95:2303-11.

[132] Cody RJ, Atlas SA, Laragh JH, Kubo SH, Covit AB, Ryman KS, Shaknovich A, Pondolfino K, Clark M, Camargo MJ, et al.Atrial natriuretic factor in normal subjects and heart failure patients. Plasma levels and renal, hormonal, and hemodynamic responses to peptide infusion. *J Clin Invest*. 1986;78:1362-74.

[133] Elsner D, Muders F, Muntze A, Kromer EP, Forssmann WG, Riegger GA. Efficacy of prolonged infusion of urodilatin [ANP-(95-126)] in patients with congestive heart failure. *Am Heart J*. 1995;129:766-73.

[134] Padilla F, Garcia-Dorado D, Agullo L, Inserte J, Paniagua A, Mirabet S, Barrabes JA, Ruiz-Meana M, Soler-Soler J. L-Arginine administration prevents reperfusion-induced cardiomyocyte hypercontracture and reduces infarct size in the pig. Cardiovasc Res. 2000;46:412-20.

[135] Padilla F, Garcia-Dorado D, Agullo L, Barrabes JA, Inserte J, Escalona N, Meyer M, Mirabet M, Pina P, Soler-Soler J.Intravenous administration of the natriuretic peptide urodilatin at low doses during coronary reperfusion limits infarct size in anesthetized pigs.Cardiovasc Res. 2001;51:592-600.

[136] Watano T, Kimura J, Morita T, Nakanishi H. A novel antagonist, No. 7943, of the Na+/Ca2+ exchange current in guinea-pig cardiac ventricular cells. Br J Pharmacol. 1996 119:555-63.

[137] Iwamoto T, Watano T, Shigekawa M. A novel isothiourea derivative selectively inhibits the reverse mode of Na+/Ca2+ exchange in cells expressing NCX1. J Biol Chem. 1996;271:22391-7.

[138] Kimura J, Watano T, Kawahara M, Sakai E, Yatabe J. Direction-independent block of bi-directional Na+/Ca2+ exchange current by KB-R7943 in guinea-pig cardiac myocytes. Br J Pharmacol. 1999;128:969-74.

[139] Mukai M, Terada H, Sugiyama S, Satoh H, Hayashi H. Effects of a selective inhibitor of Na+/Ca2+ exchange, KB-R7943, on reoxygenation-induced injuries in guinea pig papillary muscles.J Cardiovasc Pharmacol. 2000;35:121-8.

[140] Nakamura A, Harada K, Sugimoto H, Nakajima F, Nishimura N. Effects of KB-R7943, a novel Na+/Ca2+ exchange inhibitor, on myocardial ischemia/reperfusion injury. Nippon Yakurigaku Zasshi. 1998;111:105-15.

[141] Klein HH, Pich S, Lindert S, Nebendahl K, Warneke G, Kreuzer H. Treatment of reperfusion injury with intracoronary calcium channel antagonists and reduced coronary free calcium concentration in regionally ischemic, reperfused porcine hearts. J Am Coll Cardiol. 1989;13:1395-401.

[142] Kusuoka H, Camilion de Hurtado MC, Marban E. Role of sodium/calcium exchange in the mechanism of myocardial stunning: protective effect of reperfusion with high sodium solution. J Am Coll Cardiol. 1993;21:240-8.

[143] Ladilov YV, Siegmund B, Piper HM. Protection of reoxygenated cardiomyocytes against hypercontracture by inhibition of Na+/H+ exchange. Am J Physiol. 1995;268:H1531-9.

[144] Magee WP, Deshmukh G, Deninno MP, Sutt JC, Chapman JG, Tracey WR. Differing cardioprotective efficacy of the Na+/Ca2+ exchanger inhibitors SEA0400 and KB-R7943. Am J Physiol Heart Circ Physiol. 2003;284:H903-10.

AGRADECIMIENTOS:

Al Dr. David García-Dorado, director de esta tesis, por haber confiado en mi durante todos estos años y haberme permitido participar en su ambicioso y difícil proyecto: la creación y consolidación de un laboratorio altamente competitivo. Todo lo que he aprendido en investigación cardiovascular se lo debo a él.

A la Dra. Margarita Sentís, ejemplo de profesionalidad tanto en su faceta hospitalaria como docente, por haber aceptado ser tutora de esta tesis doctoral.

La colaboración con el grupo del Profesor HM Piper ha resultado ser realmente fructífera y ha de servir de estímulo para mantenerla en el futuro. Un agradecimiento muy especial para Gerhild Taimor, Bettina Hofstaetter, Yuri Ladilov y Claudia Schafer, partícipes de los estudios relacionados con la apoptosis y el transportador Na^+/Ca^{2+} en miocitos aislados y, por supuesto, para el Profesor HM Piper.

El Dr. Luis Agulló, insustituible compañero de despacho, ha colaborado en los estudios del GMPc, y los doctores Marisol Ruiz-Meana, José Antonio Barrabés y Ferran Padilla lo han hecho en los estudios del transportador Na^+/Ca^{2+} realizados en miocitos aislados y en el modelo porcino. Por su inestimable ayuda les estoy especialmente agradecido.

A todos los miembros del Laboratorio del Cardiología Experimental a los que he tenido el placer de conocer desde que comencé en 1994, culpables de que todos estos años hayan pasado sin darme cuenta.

Por último, quiero expresar mi gratitud al Dr. Jordi Soler-Soler, Jefe del Servicio de Cardiología del Hospital Vall d'Hebron por su entusiasta y efectivo apoyo a la creación y mantenimiento del Laboratorio de Cardiología Experimental en el que se ha desarrollado la presente tesis.