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Hypertrophiemechanismen ventrikulärer Herzmuskelzellen

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Abkürzungen

AC	Adenylatzyklase
ACE	Angiotensin Converting Enzym
AKT / PKB	Proteinkinase B
ANP / ANF	Atriales Natriuretisches Peptid / Faktor
AP1	activator-protein 1
AT-1	Angiotensin II Rezeptor Typ 1
ATF-2	activating transcription factor 2
ATRAP	Angiotensin Typ1 Rezeptor assoziiertes Protein
BIM	Bisindolylmaleimid
BNP	Brain Natriuretisches Peptid
cAMP	zyklisches Adenosin-mono-phosphat
CRE	cAMP response element
c-fos	fos-Onkogen
c-jun	jun-Proto-Onkogen (ju-nana)
DAG	Diacylglycerol
DGK	Diacylglycerol Kinase
ERK / p42/44	Extrazellulär regulierte Kinase
FCS	Fetales Kälberserum
FOXO	forkhead transcription factor
GATA	globin transcription factor 1
GLUT	Glukose Transporter
IP_3	Inositol-tri-phosphat
JNK	c-Jun N-terminale Kinase
L-NNA	L-Nitro-Arginin
MHC	myosin heavy chain
β MHC	myosin heavy chain β
MKP	MAP-Kinase Phosphatase
MR-1	myofibrillogenesis regulator-1

NFAT	nuclear activator of transactivation
NF-kappaB	nuclear factor kappaB
NHE-1	Natrium-Protonen-Austauscher 1
NO	Stickstoffmonoxid
eNOS	endotheliale NO-Synthase
iNOS	induzierbare NO-Synthase
nNOS	neuronale NO-Synthase
NOX	NAD(P)H-Oxidase
p38	p38 MAP-Kinase
p70S6-Kinase (p70S6-K)	p70 S6 ribosomal Proteinkinase
p110α	PI3-kinase class-1 isoform p110α
p110β	PI3-kinase class-1 isoform p110β
PI3-Kinase (PI3-K)	Phosphatidylinositide-3-Kinase
PIP_2	Phosphatidyl-inositol-2-phosphat
PKC	Proteinkinase C
PLC	Phospholipase C
PPARα	peroxisome proliferator-activated receptor
RAMP	receptor-associated modifying proteins
RLF	Ral-specific guanine nucleotide exchange factor
ROS	reaktive Sauerstoffspezies (O_2-Radikal, Wasserstoffperoxid)
siRNA	small inhibitory RNA
SMAD	small mother against decapentaplegia
SOCS	store operated calcium channels
SOD	Superoxid-Dismutase
TGFβ_1	Transforming growth factor β1
TNFα	Tumor necrosis factor α

1 Einleitung

Herz-Kreislauf-Erkrankungen stellen in Europa und Nordamerika die Erkrankungen dar, die mit dem höchsten Mortalitätsrisiko einhergehen. Risikofaktoren für eine klinisch manifeste Herzinsuffizienz sind neben dem Herzinfarkt, der Hypertonie und dem Diabetes auch Myokarditiden und Kardiomyopathien [Gurfinkel et al., Kannel et al. (2000)]. Man kann die Herzinsuffizienz pathophysiologisch als Änderung der Vorlast, der Nachlast, der Kontraktilität und der Herzfrequenz zusammenfassen. Die häufigste Ursache der Herzinsuffizienz ist zweifellos der durch Arteriosklerose hervorgerufene Herzinfarkt mit nachfolgendem Untergang von Herzmuskelzellen und einer daraus resultierenden Funktionseinschränkung mit verminderter Ejektionsfraktion. Alle anderen Ursachen einer Herzinsuffizienz sind deutlich seltener zu finden.

Zu unterscheiden ist die systolische von der diastolischen Herzinsuffizienz. Die systolische Herzinsuffizienz geht mit einer verminderten Pumpleistung und einer exzentrischen Hypertrophie einher. Bei der diastolischen Herzinsuffizienz ist die Ejektionsfraktion nur mäßig erniedrigt, die Steifigkeit der Ventrikel jedoch deutlich erhöht. Ursache einer diastolischen Funktionsstörung ist v. a. die arterielle Hypertonie mit nachfolgender Linksherzinsuffizienz und konzentrischer Hypertrophie. Auf Ebene der einzelnen Herzmuskelzelle lässt sich ebenfalls die konzentrische von der exzentrischen Hypertrophie unterscheiden. Die konzentrische Hypertrophie ist durch die parallele Anlagerung neuer kontraktiler Elemente und damit Dickenzunahme gekennzeichnet, wobei die exzentrische Hypertrophie durch eine Längenzunahme charakterisiert ist.

Als Vorstufe der nicht durch einen Myokardinfarkt ausgelösten Herzinsuffizienz steht in vielen Fällen initial die Hypertonie mit nachfolgender Hypertrophie des Herzens. Natürlich kann eine Herzhypertrophie auch ohne eine Erhöhung des Blutdruckes erfolgen; auf Grund von Stenosen oder Insuffizienzen der beiden Taschenklappen (Valvulae semilunares). Die Hypertonie wird durch die WHO klar definiert. Als hyperton wird ein Blutdruck ab 140/90 mmHg definiert. Blutdruckwerte darüber gehen signifikant mit erhöhten kardiovaskulären

Folgeerkrankungen einher. Hier seien neben kardiovaskulären auch zerebrovaskuläre und nephrologische Erkrankungen genannt. Als Folge der durch einen erhöhten Gefäßwiderstand ausgelösten Hypertonie reagiert der linke oder rechte Ventrikel mit einer Größenzunahme der einzelnen Herzmuskelzelle, um aufgrund des La Place Gesetzes trotz erhöhter Füllmenge die Wandspannung konstant und die Auswurfleistung aufrecht zu erhalten. Diese Phase der Herzhypertrophie stellt in der Regel einen Kompensationsmechanismus dar, der die Lebensqualität des Patienten über lange Zeit aufrechterhalten kann.

Die Herzhypertrophie ist nicht nur charakterisiert durch die Größenzunahme der einzelnen Herzmuskelzelle und eine verstärkte Proteinsynthese sondern auch durch die Re-Expression fetaler Isoformen von MHC, Troponin und die ventrikuläre Re-Expression von ANP und BNP.

Als weiteres Merkmal der Hypertrophie sind Veränderungen in der Zusammensetzung der extrazellulären Matrix der Ventrikel beschrieben, um die erhöhte mechanische Belastung zu kompensieren. Als Hauptbestandteil der extrazellulären Matrixproteine ist Kollagen zu nennen, welches vermehrt gebildet wird und somit zur Fibroseentstehung wesentlich beiträgt. Eine Veränderung der extrazellulären Matrix hat sowohl Auswirkungen auf die systolische als auch die diastolische Funktion der Ventrikel.

1.1. Das adrenerge System

Eine dauerhafte Aktivierung des sympathischen Nervensystems führt in der Regel zu kardiovaskulären Folgeerkrankungen. Ebenso lässt sich aber auch als Folge vieler kardiovaskulärer Erkrankungen eine nachfolgende gesteigerte Aktivität des Sympathikus feststellen. Unter physiologischen Gesichtspunkten ist eine Sympathikusaktivierung dann sinnvoll, wenn z. B. im Zuge der Orthostasereaktion, bei erhöhter körperlicher Belastung und bei psychischem Stress dieser aktiviert wird, um das Herz-Kreislaufsystem dahingehend zu aktivieren, dass Herzfrequenz und Blutdruck erhöht werden. Dauert dieser Zustand längere Zeit an, so können diese physiologischen Regulationsmechanismen pathologischen Charakter annehmen. Folge hiervon sind Hypertonie und Herzhypertrophie mit nachfolgendem Herzinfarkt

und Herzinsuffizienz. Schon lange ist bekannt, dass nicht nur β1-adrenerge Rezeptoren von Herzmuskelzellen exprimiert werden, sondern auch alle anderen bekannten adrenergen Rezeptortypen. Singuläre Aktivierung dieser einzelnen Rezeptortypen führt *in-vitro* an kultivierten Herzmuskelzellen zu ganz unterschiedlichen molekularen und morphologischen Änderungen. Eine adrenerge Stimulation hat in adulten Herzmuskelzellen vielgestaltige Auswirkungen. So bewirkt die alleinige α-adrenerge Stimulation mit Phenylephrin hypertrophes Wachstum und eine Verbesserung der Herzfunktion [Sugden et al. (2001)]. Alleinige β-adrenerge Stimulation mit Isoprenalin z. B. führt in frisch isolierten Herzmuskelzellen (sog. Kurzzeitkulturen) zu keinem hypertrophen Wachstum, jedoch in Langzeitkulturen [Fuller et al., Zhou et al.]. Charakteristisches Kennzeichen einer adrenergen Beteiligung an der Hypertrophie und Insuffizienz ist die Re-Expression fetaler Gene sowohl *in-vivo* am Gesamtherzen als auch *in-vitro* an isolierten Herzmuskelzellen. Zu nennen sei hier ANF, β MHC, α smooth muscle actin und Kreatinkinase B [Ardati et al., Waspe et al., Long et al., Schluter et al. (1992)].

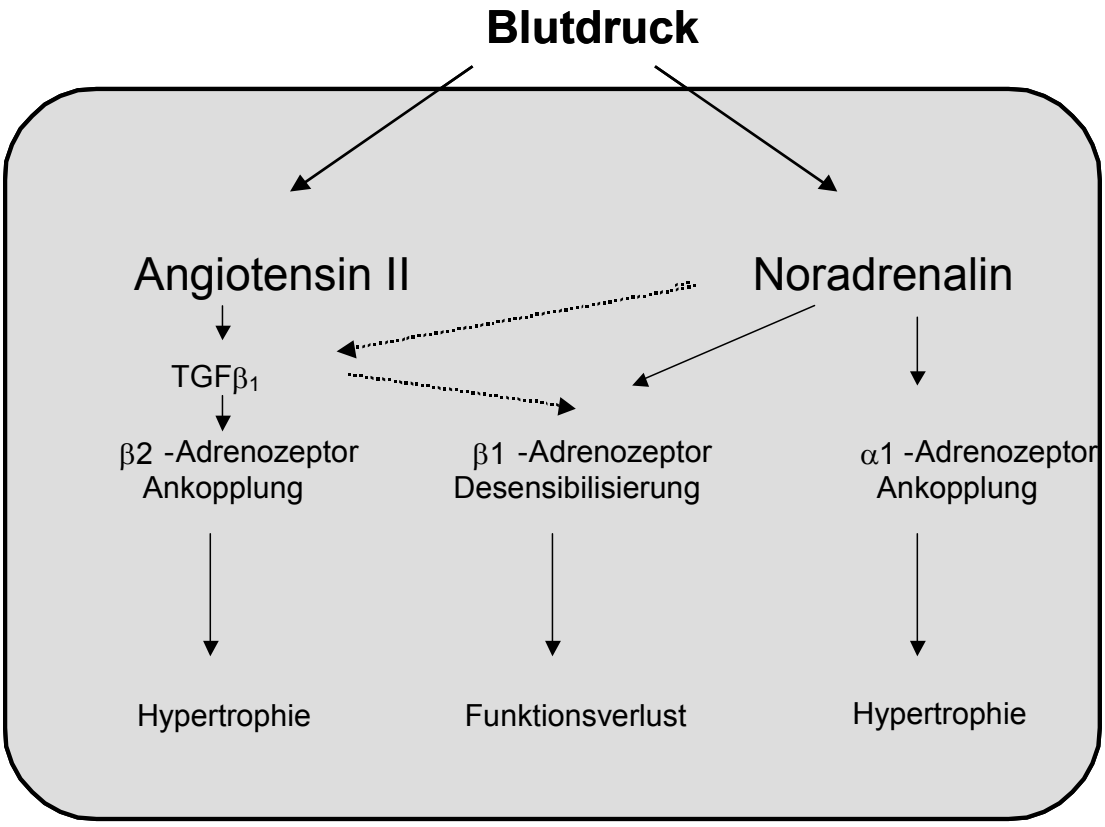

Abb. 1: Vernetzung adrenerger und Angiotensin II-induzierter hypertropher Signalwege in Kardiomyozyten

α-Adrenorezeptoren

α-Adrenorezeptoren können in verschiedene Subtypen unterteilt werden, nämlich in α1 und α2 Rezeptoren, wovon jeweils noch 3 weitere Subtypen existieren [Brodde et al.]. Für die hypertrophe Antwort des Herzens auf Norepinephrin (α und β Rezeptoren) ist vor allem der α1A Rezeptorsubtyp verantwortlich [Ponicke et al.]. α-Adrenorezeptoren sind G-Protein-gekoppelte Rezeptoren, die an ein Gαq Protein binden und hier über Phospholipase C, Inositol-3-phosphat (IP3) und Diacylglycerol (DAG) zu einer Aktivierung der Proteinkinase C (PKC) führen. Versuche an transgenen Tieren konnte die Bedeutung der Proteinkinase C für die Hypertrophieentstehung zeigen. Alleinige Stimulation dieser Kinase selbst führt schon zu hypertrophem Wachstum [Bowman et al.]. Auch *in-vitro* löste der Einsatz von Phorbolester, einem direkten Aktivator der PKC, myokardiale Hypertrophie aus [Schafer et al. (2002)]. Ruf et al. und Rohde et al. konnten zeigen, dass die PKCδ in Bezug auf hypertrophes Wachstum eine wichtige Rolle spielt.

Signalschritte unterhalb der Gαq-Kaskade müssen unterteilt werden in solche, die zu einer gesteigerten Proteinsynthese und solche, die zu einer veränderten Genexpression führen. Beteiligt an einer solchen hypertrophieassoziierten Proteinkinase C-abhängigen Signalkaskade sind die PI3-Kinase mit anschließender Phosphorylierung der AKT/PKB und nachfolgender $p70^{S6}$-Kinase Phosphorylierung [Sadoshima et al., Boluyt et al. (1997), Schluter et al. (1998)]. Gerade der PI3-Kinase/AKT Signalweg ist ein weit verbreiterter Signalweg und wird ebenfalls von anderen hypertrophen Stimuli genutzt. Sehr kontrovers wird in der Literatur über die Beteiligung von Mitgliedern der Mitogen-aktivierten Proteinkinasen an der α-adrenerg-vermittelten Hypertrophie diskutiert. Bekannt ist, dass Phenylephrin – ein α-adrenerger Agonist – die „extracellular regulated kinase“ (ERK) aktiviert, eine Beteiligung am hypertrophen Wachstum ist indes nicht gesichert. In neonatalen Myozyten scheint eine Beteiligung wahrscheinlich [Glennon et al., Thorburn et al.], in adulten eher unwahrscheinlich [Schluter et al. (1999), Ponicke et al.]. Sehr unwahrscheinlich ist eine Beteiligung der beiden anderen Mitogen-aktivierten Proteinkinasen – der p38 MAP-Kinase und der c-Jun N-terminalen Kinase (JNK) – an der α-adrenerg vermittelte Hypertrophieentstehung.

Die Beteiligung weiterer hypertrophierelevanter Signalschritte am α-adrenerg-vermittelten Wachstum ist Gegenstand derzeitiger Forschung und noch nicht abschließend geklärt. So aktiviert α-adrenerge Stimulation den Natrium-Protonen-Austauscher (NHE-1), die Phospholipase D, einige G-Protein assoziierte „Ral-specific guanine nucleotide exchange“ Faktoren (RLF), reaktive Sauerstoffspezies und spannungsabhängige Kalziumkanäle [Mier et al., Schafer et al. (2002), Eble et al., Post et al., Amin et al.]. Kontroverse Ergebnisse liegen hinsichtlich der Frage vor, inwieweit diese Signalschritte kausal an der Hypertrophieentstehung beteiligt sind. Die *in-vitro* gewonnenen Ergebnisse sind weitgehend auch auf die *in-vivo* Situation übertragbar.

β-Adrenorezeptoren

Die *in-vitro* Übertragbarkeit ist hinsichtlich der *in-vivo* gewonnenen Daten bei β-adrenerger Stimulation nicht gegeben, wahrscheinlich wegen der *in-vivo* durch β-adrenerge Stimulation modulierten Hämodynamik, die *in-vitro* in kultivierten Herzmuskelzellen nicht auftritt. Sowohl β1 als auch β2 Rezeptoren werden von Kardiomyozyten exprimiert, wobei β1-adrenrge Rezeptoren sehr viel häufiger vertreten sind. In neonatalen Kardiomyozyten löst die β-adrenerge Stimulation hypertrophes Wachstum vermittelt durch den positiv kontraktilen Effekt aus [Simpson et al.]. Selektive Stimulation der β1-adrenergen Rezeptoren übt einen nur moderaten Effekt auf die Proteinsynthese in adulten Myozyten aus [Schafer et al. (2000)]. Unter physiologischen Bedingungen bewirkt Norepinephrin (Noradrenalin) vorzugsweise über α-Rezeptoren hypertrophes Wachstum [Schafer et al. (2001)]. Die nach selektiver β1-adrenerger Stimulation induzierten Signalwege sind indes wenig bekannt. β1-Adrenerge Stimulation aktiviert zwar den cAMP-abhängigen Signalweg, hypertrophe Signalmechanismen scheinen allerdings cAMP unabhängig zu sein [Schafer et al. (2000)]. Auch scheint eine Beteiligung Mitogen-aktivierter Proteinkinasen – hier speziell der ERK und der p38 MAP-Kinase – nicht wahrscheinlich [Sabri et al. (2000), Schafer et al. (2001), Wang et al. (1998), Crespo et al.]. Als ein beteiligtes Signalmolekül ist der Natrium-Protonen-Austauscher 1 (NHE-1) zu nennen. Selektive Inhibierung des NHE-1 reduziert das β1-adrenerg-vermittelte hypertrophe Wachstum [Bisognano et al.]. Unter pathophysiologischen Bedingungen kommt es bei einem druckbelasteten Herzen zu einer

Herabregulierung und damit Desensitivierung von β1 Rezeptoren [Leineweber et al., Mondry et al., Galinier et al.]. Aus den genannten Gründen scheinen β1-adrenerge Rezeptoren weniger relevant für die druckinduzierte Hypertrophieentstehung zu sein.

Obwohl β2 Rezeptoren in geringerer Zahl von Herzmuskelzellen exprimiert werden als β1 Rezeptoren ist ihre Rolle im Hypertrophiegeschehen sehr interessant. *In-vitro* verursacht die β2-adrenerge Stimulation (im Gegensatz zur *in-vivo* Situation) mit Isoprenalin nur unter bestimmten Voraussetzungen hypertrophes Wachstum [Pinson et al., Dubus et al.], nämlich dann, wenn die Herzmuskelzellen über sechs Tage mit TGFβ$_1$ vorinkubiert werden. Nur unter diesen Kulturbedingungen bewirkt selektive β2-Rezeptorstimulation eine Größenzunahme der Herzmuskelzelle, an der kausal TGFβ$_1$ beteiligt ist [Schluter et al. (1995)]. Von Herzmuskelzellen sezerniertes und inaktives TGFβ$_1$ wird durch Proteasen des Zellkulturmediums aktiviert und kann somit parakrin und autokrin auf die Zelle zurückwirken. Der Mechanismus, der für diese Ankopplung an den hypertrophen Signalweg verantwortlich ist, ist weitgehend unbekannt. Wahrscheinlich ist diese Ankopplung zwischen der cAMP-abhängigen Aktivierung stressinduzierter MAP-Kinasen und nachfolgender PI3-Kinase Aktivierung lokalisiert. Interessant ist, dass im Falle der Ankopplung β-adrenerger Stimulation an hypertrophe Signalwege, gleiche Signalmoleküle rekrutiert werden, wie nach α-adrenerger Stimulation: nämlich die PI3-Kinase, die AKT/PKB und die p70^{S6}-Kinase. Interessant ist auch die Verbindung zwischen adrenergem System und dem Renin-Angiotensin-Aldosteron-System. Gesichert ist, dass Angiotensin II selbst eine verstärkte Expression von TGFβ$_1$ verursacht und zwar dann, wenn eine kompensierte Herzinsuffizienz in eine dekompensierte Form übergeht (siehe unten).

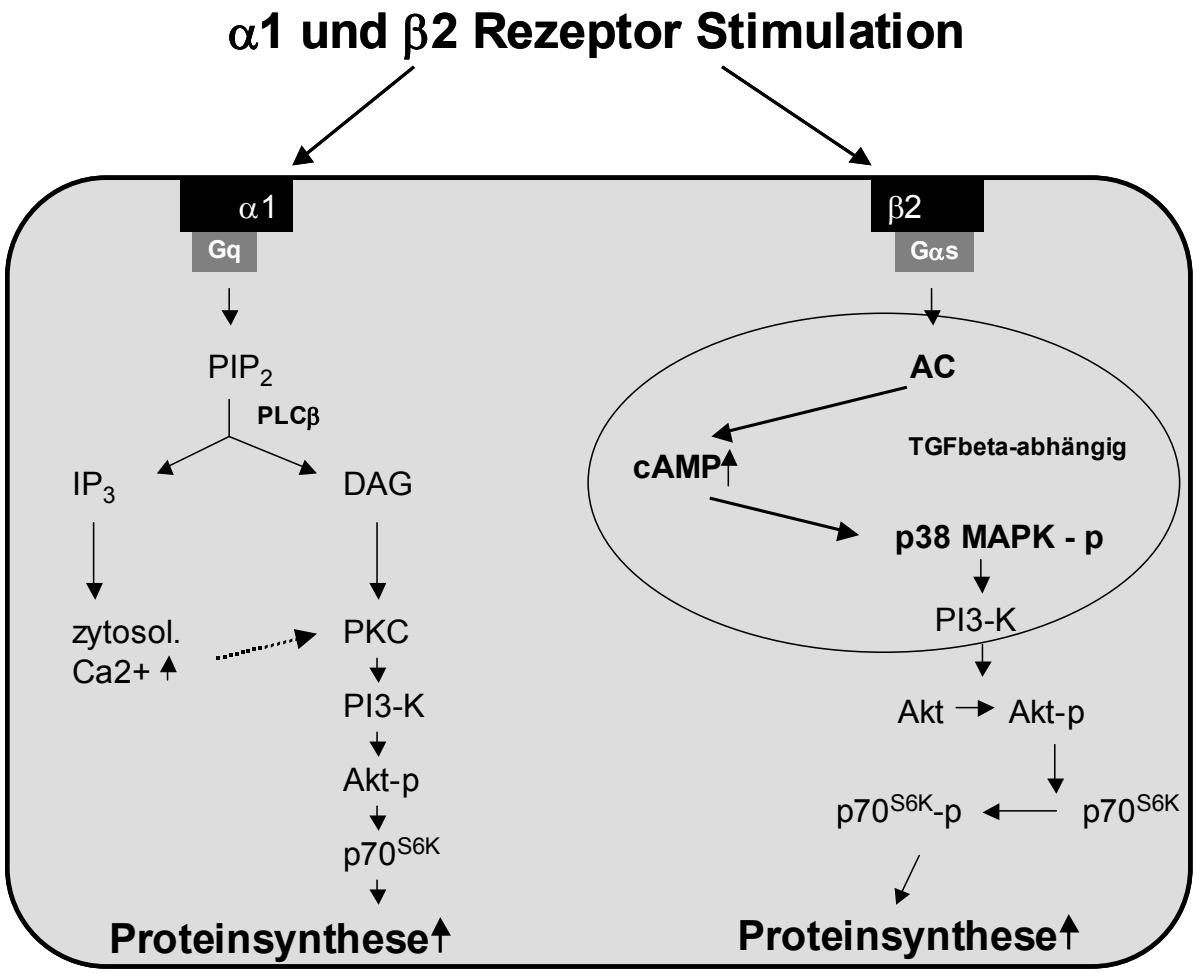

Abb. 2: **α- und β-adrenerge hypertrophe Signalwege in Kardiomyozyten**

1.2. Das Renin-Angiotensin-Aldosteron-System (RAAS)

Zu einem der prominentesten kardiovaskulären und am meisten erforschten Hormonen gehört zweifellos Angiotensin II, die aktive Komponente des Renin-Angiotensin-Aldosteron-Systems. Die direkte Wirkung von Aldosteron, dem zweiten aktiven Hormon des RAAS, auf einzelne Herzmuskelzellen bzw. auf das Gesamtherz ist momentan Gegenstand intensiver Forschung. Forschungsergebnisse mit oder über Angiotensin II hingegen sind seit 1959 in ca. 40200 Manuskripten zu finden. Zwei wichtige kardiovaskuläre Medikamente sind aus diesen Bemühungen hervorgegangen: ACE-Inhibitoren und AT1-Rezeptor Blocker, die beide die Wirkungen von Angiotensin II unterbrechen und verhindern. Die Wirkungen von Angiotensin II sind ebenfalls sehr vielschichtig und innerhalb der gängigen kardialen Zellkulturmodelle unterschiedlich. So reagieren neonatale Herzmuskelzellen mit ausgeprägtem hypertrophen Wachstum [Aceto et al., Baker et al.], wohingegen adulte Myozyten einen nur moderaten Anstieg in Zellgröße und Proteinumsatz zeigen [Fuller et al.]. Den vorherrschenden Effekt von Angiotensin II auf das Herz oder einzelne Herzmuskelzellen ist die Steigerung der Zytokinexpression [Rosenkranz et al.]. Hieraus sind alle weiteren Wirkungen von Angiotensin II zu erklären. So verschlechtert chronische (24h) Stimulation adulter Myozyten mit Angiotensin II deren kontraktile Funktion [Domenighetti et al.] ebenso wie es zu einem verstärkten Remodelling mit einhergehender Fibroseentstehung kommt [Zhu et al.]. Beide Effekte werden zu mindestens zum Teil durch den Transforming growth factor β mediiert, einem Zytokin, welches durch Angiotensin II vermehrt exprimiert und aus der Herzmuskelzelle freigesetzt wird, um dann auf diese selbst zu wirken.

Hypertrophie

Obwohl Angiotensin II in neonatalen Rattenmyozyten hypertrophes Wachstum und eine verstärkte Synthese an Protein und mRNA induziert [Aceto et al., Barker et al.], sind diese Befunde in adulten Myozyten nicht reproduzierbar [Fuller et al.], was eine direkte und relevante Beteiligung an der nicht druckinduzierten Hypertrophie ausschließt. Diese Befunde werden zusätzlich von Hamawaki et al. und Harada et al. untermauert, die zeigen konnten, dass Mäuse trotz der Medikation mit AT1-Rezeptorblockern eine druckinduzierte Hypertrophie entwickeln. Andererseits zeigte

Ehmke et al., dass direkte Applikation von Angiotensin II in der Tat zur Hypertrophie des Herzens führt, jedoch unabhängig von einem erhöhten Blutdruck. Ebenso sind ACE-Hemmer in der Lage, eine druckinduzierte Hypertrophie zu verhindern [Yamamoto et al.]. Die beteiligten pro-hypertrophen Signalwege sind in den letzten Jahren hinreichend – vor allem an neonatalen Kardiomyozyten - untersucht. Über die Aktivierung des Angiotensin 1-Rezeptors (AT-1) mit folgender Aktivierung des G-Proteins Gα12/13 [Nishida et al.] kommt es zu einer verstärkten Generierung reaktiver Sauerstoffspezies (ROS), gebildet von einer Isoform der NAD(P)H-Oxidase, der NOX2 [Grieve et al.]. Shih et al. und Laufs et al. konnten zeigen, dass Statine kleine GTPasen (Rac und Rho) inhibieren, die eine Rolle als zytosolische Untereinheit der NAD(P)H-Oxidase spielen. Die anti-hypertrophe Wirkung von Statinen lässt sich durch eine verminderte Radikalbildung über Inhibierung von Rac erklären. Darüber hinaus sind Statine auch in der Lage, weiter distal die ERK zu inhibieren und zeitgleich einen weiteren anti-hypertrophen Signalweg zu aktivieren [Yao et al.]. Als „downstream“ Molekül der ROS konnte die p38 MAP-Kinase identifiziert werden [Nishida et al.]. Inhibierung des AT-1-Signalweges führt somit zu einer reduzierten p38 MAP-Kinase Phosphorylierung. Dies beinhaltet auch die Aktivierung des Angiotensin Typ 1 Rezeptor assoziierten Proteins (ATRAP) [Tanaka et al.] oder die Aktivierung der MAP-Kinase Phosphatase (MKP) [Palm-Leis et al.]. Zusätzlich gibt es Hinweise darauf, dass ein weiteres Mitglied der Familie der Mitogen-aktivierten Proteinkinasen durch Angiotensin II aktiviert wird, nämlich die JNK. Das dritte Mitglied dieser Familie, die ERK, wird ebenfalls aktiviert [Booz et al., Shih et al.]. Nishida et al. konnte zeigen, dass Angiotensin II eindeutig die p38 MAP-Kinase und die ERK in einem ROS-abhängigen Signalweg aktiviert, aber nicht die JNK. Zusätzlich verhindert die Überexpression der Diacylglycerol kinase (DGK) ζ die Gαq-abhängige Aktivierung der ERK [Arimoto et al.]. Die DGK ζ katalysiert die Bildung von Diacylglycerol, einem second messenger, der notwendig ist für die Aktivierung der Proteinkinase C (PKC). Angiotensin II ist in der Lage, verschiedene PKC Isoformen zu aktivieren [Qin et al.], von denen die PKCε für die Regulierung der Hypertrophie verantwortlich ist [Iwata et al.]. Diese Ergebnisse legen den Schluss nahe, dass der Angiotensin Typ 1 Rezeptor entweder an das Gαq Protein koppelt, um über einen PKC-abhängigen Weg die ERK zu aktivieren, oder an das Gα12/13 Protein koppelt, um hier in einem ROS-abhängigen Weg die p38 MAP-Kinase zu aktivieren. Weiter distal aktiviert Angiotensin II ebenfalls radikalabhängig

verschiedene Transkriptionsfaktoren, wie z. B. das „activator protein-1“ (AP1), den „nuclear factor kappa B“ (NF-kappaB) [Wu et al., Purcell et al., Kawano et al., Freund et al.] oder den „nuclear activator of transactivation“ (NFAT) [Hunton et al.]. Yu et al. konnte eine Beteiligung von Calcineurin an der Angiotensin II-vermittelten kardialen Hypertrophie nachweisen. Angiotensin II erhöht die Caplain Aktivität, welche zu einer verstärkten Proteolyse der auto-inhibitorischen Domäne von Calcineurin führt. Daraufhin kann Calcineurin in den Kern translozieren, um eine veränderte pro-hypertrophe Genregulierung zu initiieren [Burkard et al.]. Angiotensin II kann außerdem sogenannte „store operated calcium channels” (SOCS) aktivieren, deren Aktivierung durch das Ausströmen von Kalzium aus dem Sarkoplasmatischen Retikulum gesteuert wird. Sowohl NFAT als auch Caplain können die Calcineurin Beteiligung erklären. Inwieweit der „myofibrillogenesis regulator-1“ (MR-1) [Liu et al.] an dem Angiotensin II-induzierten hypertrophem Wachstum beteiligt ist, ist noch nicht hinreichend geklärt.

Angiotensin II kann aber auch direkt anti-hypertroph wirken. So desensibilisiert Angiotensin II in einem PKC- und SOCS-abhängigen Weg seinen eigenen Typ 1 Rezeptor [Chen et al. (2002), Calegari et al.]. Da Angiotensin II sowohl über Gα12/13 als auch über Gαq wirkt, liegt die Vermutung nahe, dass über Gα12/13 und die NAD(P)H-Oxidase pro-hypertrophe Signalwege, aber über Gαq anti-hypertrophe Wege aktiviert werden. Zusätzlich aktiviert Angiotensin II auch die „receptor-associated modifying proteins“ (RAMP) 1 und 3. RAMP 1 und 3 sensitivieren Adrenomedullin Rezeptoren, welche die cAMP-Spiegel in Myozyten erhöhen. Erhöhte cAMP-Spiegel wirken als negative Regulatoren pro-hypertropher Signalwege [Chakravarty et al.].

Neben dem Typ 1 Rezeptor wird von Myozyten auch der Typ 2 Rezeptor exprimiert und von Angiotensin II aktiviert. Opie et al. zeigte einen durch Rezeptor Typ 2 Aktivierung ausgelösten inhibierenden Effekt auf Typ 1-abhängige Prozesse. Dieser Effekt kann z. T. durch eine Angiotensin II-induzierte Calcineurin/NFAT abhängige Hochregulierung der endothelialen NO Synthase erklärt werden [Ritter et al.]. Andererseits hat D’Amore et al. ein Ankopplung des Typ 2 Rezeptors an pro-hypertorphe Signalwege gezeigt. Diese Studie unterstützt klinische Beobachtungen die zeigen, dass AT1-Rezeptorblocker gegenüber ACE-Inhibitoren keinen positiveren

therapeutischen Nutzen haben. Dies müsste der Fall sein, wenn Angiotensin II Typ 2 Rezeptoren tatsächlich einen anti-hypertorphen Effekt hätten.

Zytokinexpression

Zytokine, wie der Transforming growth factor β (TGFβ), der Tumor necrosis factor α (TNFα) oder Interleukine (vor allem IL-1β, IL-2 und IL-6) spielen eine wichtige Rolle in der Pathogenese und Entwicklung der Herzhypertrophie mit daraus resultierender Herzinsuffizienz [Mehra et al.]. Neben Angina Pectoris, entzündlichen Myokarditiden und der kongestiven Herzinsuffizienz sind neurohumorale Faktoren verantwortlich für eine verstärkte Zytokinexpression. Ein aktiviertes Renin-Angiotensin-System nimmt eine herausragende Rolle bei der Bildung von Zytokinen ein [Kalra et al., Rosenkranz et al.]. Ursprünglich wurden klassische Immunzellen für die Bildung pro-inflammatorischer Zytokine verantwortlich gemacht. Doch in den letzten Jahren konnte gezeigt werden, dass Zytokine im Herzen selbst und sogar von einzelnen Myozyten exprimiert werden. Diese sind dann involviert in Apoptosemechanismen [Euler-Taimor et al.], Hypertrophie [Rosenkranz et al., Schultz et al., Lim et al.], Remodelling [Lijnen et al.], kontraktile Dysfunktion [Mehra et al.] und die Beeinflussung adrenerger Signalwege [Schluter et al. (1995)]. Valide Daten bezüglich Kardiomyozyten existieren vor allem für $TGF\beta_1$, in nur geringerem Maße auch für TNFα. $TGF\beta_1$ wirkt hier autokrin oder parakrin auf Myozyten selbst. Inwieweit Zytokine direkt die Entwicklung einer Herzinsuffizienz mediieren ist bislang noch nicht hinreichend geklärt [Mehra et al.], dennoch ist es unumstritten, dass hohe Zytokinspiegel im Plasma eng mit dem Auftreten einer Herzinsuffizienz korrelieren [Testa et al., Deswal et al.] und sogar prognostische Relevanz besitzen, wie in der Vesnarianone Studie gezeigt [Deswal et al.]. Wie oben bereits beschrieben, besitzen Substanzen, die Angiotensin II inhibieren (ACE-Inhibitoren und AT1-Rezeptor Blocker), hohes therapeutisches Potenzial, was zum einen auf hämodynamische Verbesserungen und zum anderen auf verminderte Zytokinexpression zurückgeführt werden kann.

Isolierte Myozyten reagieren innerhalb von 10 Stunden nach Angiotensin II Stimulation mit einer erhöhten $TGF\beta_1$ Expression sowohl auf Protein- als auch auf mRNA-Ebene [Gray et al., Wenzel et al.]. Dieser Effekt ist an die Aktivierung des Typ

1 Rezeptors, die Aktivierung der Gα12/13 und der NAD(P)H-Oxidase Aktivierung gekoppelt [Nishida et al.]. Das von den Zellen gebildete TGFβ_1 wird in das Kulturmedium abgegeben [Taimor et al. (1999)] und von Proteasen aktiviert, die in dem Kulturmedium vorhanden sind. Wie oben beschrieben wirkt TGFβ_1 auf vielfältige Weise auf die Myozyten selbst zurück. Als wichtiges Signalmolekül sind in der Angiotensin II-induzierten Steigerung der TGFβ_1 Expression freie reaktive Sauerstoffspezies zu sehen [Wu et al.]. Sowohl *in-vivo* als auch *in-vitro* werden diese Radikale von der NAD(P)H-Oxidase, einem membrangebundenen Flavoenzym gebildet [Hintgten et al., Clempus et al., Wenzel et al.]. Diverse NOX transgene Mäuse mit unterschiedlich veränderten Expressionen von Untereinheiten der NAD(P)H-Oxidase [Dikalova et al.], die Applikation von Inhibitoren der NAD(P)H-Oxidase (Inhibitoren oder Antioxidantien) oder das Herabregulieren verschiedener Untereinheiten durch Antisense Oligonukleotide [Wenzel et al.] demonstrieren den Zusammenhang zwischen Angiotensin II, Radikalen und der NAD(P)H-Oxidase sowohl im Tier [Matsuno et al.] als auch in der Zellkultur. Auch hier sind Statine wiederum in der Lage, die Radikalbildung zu verhindern, indem sie die Translokation von Rac (einer zytosolische Untereinheit der NAD(P)H-Oxidase) zur Zellmembran verhindern. Nur aufgrund dieser Translokation ist eine Radikalbildung möglich. Die Medikation von Tieren mit Statinen reduziert deren oxidativen Stress [Stoll et al.], Nakagami et al.]. Auch in kultivierten Myozyten reduziert die Applikation von Statinen oder anderen Farnesyltransferase Inhibitoren (BMS161953) sowohl die Radikalbildung als auch die TGFβ_1 Expression [Wenzel et al., unpublizierte Daten].

Wie bereits erwähnt, zeigen unterschiedlichste Studien die p38 MAP-Kinase als ein mögliches „down-stream“ Molekül der NAD(P)H-Oxidase [Shen et al., Sano et al.]. Angiotensin II kann, wie ebenfalls bereits erwähnt, alle Mitglieder der Familie der Mitogen-aktivierten Proteinkinasen aktivieren. Dabei scheint ERK vor allem an dem hypertrophen Wachstum [Shih et al.], die JNK vor allem an Apoptosemechanismen [Mascareno et al.] und die p38 MAP-Kinase vor allem an der Zytokinexpression [Shen et al., Wenzel et al.] beteiligt zu sein. Da die p38 MAP-Kinase in vielen unterschiedlichen Signalwegen beteiligt ist, stellt sich natürlich die Frage, ob hier spezielle Isoformen involviert sind. So ist z. B. bekannt, dass an pro-apoptotischen und anti-hypertophen Signalwegen die p38α, wohingegen die p38β eher an anti-apoptotischen und pro-hypertrophen Wegen beteiligt ist [Sabri et al.

(2006)]. Welche Isoform in der Angiotensin II-induzierten Steigerung der TGFβ_1 Expression involviert ist, ist indes nicht bekannt.

Die Proteinkinase C (PKC) spielt eine wichtige Rolle für die Angiotensin II-induzierten Signalwege. Sowohl die Angiotensin II-induzierte TGFβ_1 als auch TNFα Expression sind PKC-abhängige Vorgänge, sowohl im Gesamtherzen als auch in isolierten Kardiomyozyten [Kalra et al., Wenzel et al.]. Angiotensin II aktiviert vor allem die Ca^{2+}-unabhängigen PKC-Isoformen δ und ε [Ruf et al.]. Mollnau et al. untersuchte die Bedeutung der PKC für die Regulierung der NAD(P)H-Oxidase mit nachfolgender Radikalbildung durch die Medikation von Tieren mit dem PKC-Inhibitor Chelerythrine. Diese Arbeitsgruppe konnte zeigen, dass NOX1 eine wichtige Rolle in der durch Angiotensin II ausgelösten endothelialen Dysfunktion spielt. Dieser Effekt ist PKC-abhängig. Im Gegensatz dazu zeigte Palomeque et al. einen PKC-abhängigen Verlust der kontraktilen Funktion in Kardiomyozyten nach Angiotensin II Stimulation. Diese Arbeitsgruppe konnte eine ROS-Beteiligung allerdings ausschließen. Die ROS-abhängige Zytokinexpression adulter Myozyten nach Angiotensin II Stimulation ist ebenfalls PKC-abhängig, wobei es zu einer ROS-abhängigen PKC-Aktivierung kommt [Wenzel et al.].

Die PI3-Kinase ist ein multifunktionales Enzym, welches ebenfalls in Angiotensin II-induzierten Signalwegen involviert ist. Der Zusammenhang zwischen ROS-Generierung und PI3-Kinase Aktivierung wird in der Literatur kontrovers diskutiert. Einige Autoren finden eine PI3-Kinase-abhänige gesteigerte Bildung von ROS, andere nicht [Arcaro et al., Karlsson et al.]. Mittlerweile kristallisiert es sich heraus, dass die Herkunft der gebildeten Radikale verantwortlich ist für eine Beteiligung der PI3-Kinase. Die unterschiedlichen Isoformen der NAD(P)H-Oxidase können in zwei Gruppen unterteilt werden: einige setzten ROS intrazellulär frei (NOX1, NOX4), andere geben die gebildeten Radikale nach extrazellulär ab (NOX2). Im Falle der intrazellulären Abgabe von Radikalen scheint der Prozess PI3-Kinase abhängig zu sein, im Falle der extrazellulären Abgabe PI3-Kinase unabhängig [Karlsson et al.]. Da Myozyten sowohl NOX1 und NOX4, aber auch NOX2 exprimieren, und somit ihre Radikale zumindestens z. T. intrazellulär abgeben, scheint eine PI3-Kinase Beteiligung wahrscheinlich. Darüber hinaus konnte Anderson er al. eine Beteiligung der PI3-Kinase Isoform p110α an pro-hypertrophen

Signalwegen zeigen. Crackower et al. machte die PI3-kinase Isoform p110γ für eine verminderte cAMP Konzentration verantwortlich, die in einer schlechteren kontraktilen Funktion mündet.

Im Falle der Angiotensin II-induzierten TGFβ_1 Expression spielen Transkriptionsfaktoren ebenfalls eine wichtige Rolle. Wang et al. (2005) fand eine erhöhte Aktivität des Transkriptionsfaktors GATA-4 in neonatalen Kardiomyozyten nach Angiotensin II Stimulation. Auch FOXO3a, ein Mitglied der Familie der „forkhead transcription factors“ und AKT sind in Angiotensin II-induzierten Signalwegen beschrieben [Skurk et al.]. Die Aktivierung des „peroxisome proliferator-activated receptor“ (PPARα) mit Fenofibrate verhindert die durch Angiotensin II-Infusion ausgelöste Inflammation und Fibrose in Ratten [Diep et al.]. Darüber hinaus induziert Angiotensin II die Bindung von ATF-2/c-jun an die CRE Stelle „(cAMP response element)“, welche verantwortlich ist für die TNFα Induktion in kardialen Fibroblasten [Sato et al.]. Sowohl in neonatalen als auch in adulten Kardiomyozyten erhöht Angiotensin II die Bindungsaktivität des Transkriptionsfaktors AP-1 (activator-protein 1). Diese Aktivierung ist redoxabhängig und essentiell für die TGFβ_1 Expression [Wu et al., Wenzel et al.]. Zusätzlich ist c-fos, eine mögliche Untereinheit von AP-1, heraufreguliert. Die Inhibierung der AP-1 Aktivität durch Decoy Oligonukleotide und Antisense Oligonukleotide gegen c-fos verhindert die Angiotensin II-induzierte Zytokinexpression.

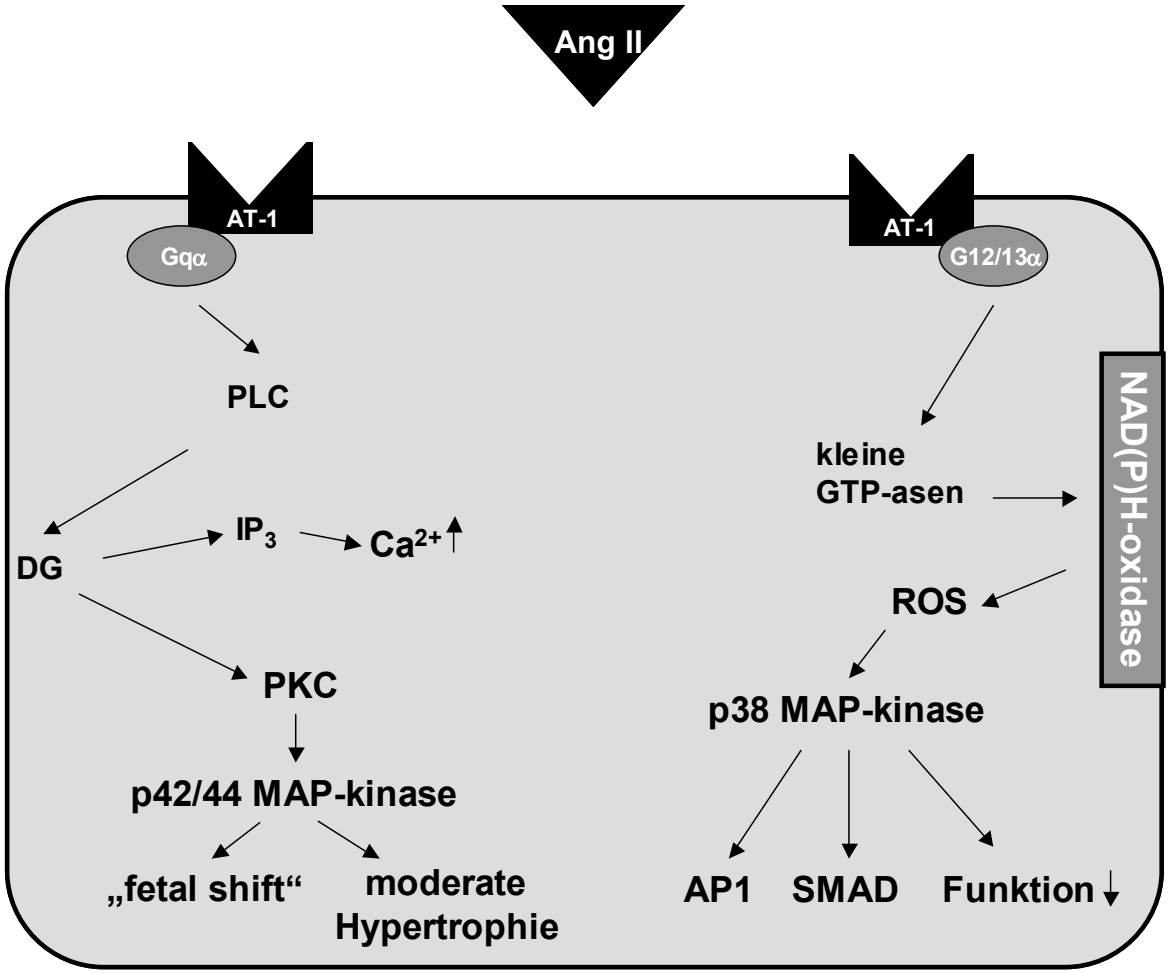

Abb. 3: Angiotensin II-induzierte Signalwege in Kardiomyozyten

1.3. Das metabolische Syndrom / Hyperglykämie

In den letzten Jahren ist in den sogenannten Industrieländern in verstärktem Maße ein Krankheitskomplex entstanden, der aus der großen Verfügbarkeit hochwertiger Nahrungsmittel bei gleichzeitigem Mangel an Bewegung resultiert und in einem erhöhten Risiko an Hypertronie und Diabetes zu erkranken mündet: dem metabolischen Syndrom. Hauptauslöser dieser „Stoffwechselstörung“ ist Adipositas, wobei eingelagertes abdominales Fett selbst hormonell aktiv ist und somit zur Aufrechterhaltung der veränderten Stoffwechsellage beiträgt. Die Folgen sind Hypertonie, Hyperinsulinämie, Hyperglykämie, Hypercholesterinämie und Hyerlipidämie mit resultierenden Schäden an Herz und Gefäßen. Erhöhte Blutzuckerspiegel haben nicht nur Auswirkungen auf das Gefäßendothel, sondern auch direkt auf das Herz und hier sogar auf die einzelne Herzmuskelzelle. In der Framingham Studie konnte ein deutlich erhöhtes Risiko diabetischer Patienten, eine Herzinsuffizienz zu entwickeln festgestellt werden gegenüber Patienten mit normalen Blutglukosewerten [Kannel et al. (1991)]. Diese durch klinische Studien gewonnenen Erkenntnisse werden durch *in-vivo* und *in-vitro* Versuche vor allem an Mäusen und Ratten bestätigt. Herzen transgener, diabetischer Mäuse zeigen sowohl systolische als auch diastolische Insuffizienz [Severson et al.]. Herzmuskelzellen, isoliert aus diabetischen Ratten, deren Diabetes durch eine einmalige Streptozotocin Injektion ausgelöst wurde, sind in ihrer kontraktilen Funktion signifikant eingeschränkt bei veränderten Ca^{2+}-Transienten. Ähnliche Effekte verursacht auch die direkte Stimulation adulter Kardiomoyzyten isoliert aus normotensiven Wistar Ratten mit hohen Konzentrationen an extrazellulärer Glukose. Auch hier ist sowohl eine veränderte Signaltransduktion als auch eine verschlechterte kontraktile Funktion zu beobachten. Da sowohl GLUT1 als auch GLUT4 in Kardiomyozyten exprimiert werden [Davey et al.], ist Insulin nicht unbedingt vonnöten, um eine direkte Wirkung von Glukose auf die einzelne Zelle zu untersuchen. Eine Reihe wichtiger Signaltransduktionsschritte sind sowohl in Herzmuskelzellen als auch in Zellen anderer Geweben charakterisiert. So konnte Wilmer et al. eine direkte Aktivierung der ERK und der p38 MAP-Kinase durch Hyperglykämie in humanen Mesangialen Zellen nachweisen. Die JNK, das dritte Mitglied der Familie der Mitogen-aktivierten Proteinkinasen, ist direkt nicht beteiligt an Hyperglykämie-induzierten Änderungen der Signaltransduktion. Die Beteiligung der p38 MAP-Kinase legt die Vermutung

nahe, dass es sich hier um einen redoxsensitiven Prozess handelt. Susztak et al. zeigte in Nierenpodozyten eine gesteigerte Apoptose, welche durch reaktive Sauerstoffspezies (ROS) und eine Aktivierung der p38 MAP-Kinase vermittelt wird. Des Weiteren konnte Igarashi et al. in mikrovaskulären Endothelzellen zeigen, dass die Hyperglykämie-induzierte p38 MAP-Kinase Aktivierung ein PKC-abhängiger Schritt ist. Eine moderate Hyperglykämie ist PKC-abhängig, eine starke Hyperglykämie jedoch PKC-unabhängig. Eine gegenseitige Beeinflussung der Angiotensin II-induzierten Signalwege und der durch Hyperglykämie ausgelösten Veränderungen postuliert Privratsky et al. So konnten die Autoren zeigen, dass eine Blockade des Angiotensin II-induzierten Signalweges mit Angiotensin II Typ-1Rezeptor Blockern den durch Hyperglykämie ausgelösten Verlust der kontraktilen Funktion des Herzens verhindert. Dieser Weg ist ebenfalls redoxsensitiv. Relativ wenig ist über die Beteiligung von Transkriptionsfaktoren in Kardiomyozyten bekannt. Aus anderen Zelltypen ist die Beteiligung des „nuclear factor-kappa B“ [Chen et al. (2006)], des „peroxisome proliferator-activated receptor α“ [Park et al.] und des „nuclear factor of activated T cells” [Nilsson et al.] bekannt. In Kardiomyozyten ist lediglich eine verstärkte „nukleäre O-GlcNAcylation“ nach Diabetes mit daraus resultierender Hyperglykämie gezeigt [Clark et al.]. All diese Untersuchungen legen den Schluss nahe, dass neben der durch Veränderungen des vaskulären Systems hervorgerufenen Kardiomyopathie ein direktes, durch Hyperglykämie verursachtes Remodelling mit einhergehendem Funktionsverlust des Herzen besteht.

1.4. NO und Kardiomyozyten

Stickstoffmonoxid (NO) wurde ursprünglich in Endothelzellen entdeckt, wo es einen gefäßrelaxierenden Effekt ausübt, indem es zu einer Erschlaffung von Glattmuskelzellen der Gefäßwand führt. eNOS defiziente Mäuse entwickeln sowohl einen Bluthochdruck als auch eine moderate kardiale Hypertrophie. Untersuchungen der letzten Jahre zeigen, dass NO nicht nur wichtig in Gefäßen ist, sondern als Signalmolekül in einer Vielzahl von Zellsystemen eine Rolle spielt. Die NO-Synthase (NOS), als NO produzierendes Enzym wird in nahezu allen Organen des Körpers exprimiert und kann in drei Isoformen unterteilt werden: die endotheliale NOS (eNOS), die induzierbare NOS (iNOS) und die neuronale NOS (nNOS). Adulte Kardiomyozyten exprimieren vor allem die eNOS und die nNOS, wobei die eNOS mit Calveolin-3 assoziiert ist [Feron et al.], während die nNOS am Sarcoplasmatischen Retikulum lokalisiert ist und die Aktivität Ca^{2+}-sensitiver Proteine moduliert [Sears et al.]. Die Aktivität der eNOS nimmt direkt Einfluss auf die β-adrenerge Signaltransduktion. Im Zuge der hypertrophen Kardiomyopathie wird die Expression von eNOS herabreguliert, die von nNOS jedoch heraufreguliert [Bayraktutan et al., Piech et al., Massion et al.]. Unabhängig vom blutdrucksteigernden Effekt bewirkt die experimentelle Inhibierung der endogenen NO Bildung ein hypertrophes Wachstum in isolierten Kardiomyozyten [Sanada et al.]. Die hierzu führenden Mechanismen sind weitgehend ungeklärt. NO kann mit gleichzeitig gebildetem ROS zu Peroxynitrit reagieren, wobei dieses Reaktionsprodukt für die Zelle ausgesprochen schädlich ist, da es z. B. zu DNA Schäden führen kann. Es ist bekannt, dass es in Zellen eine „Redox-Balance“ gibt, die für physiologisch ablaufende Signalprozesse wichtig ist. Eine Störung dieser Balance resultiert in veränderten Signaltransduktionsschritten. So kann eine reduzierte eNOS Aktivität die Menge an ROS erhöhen, welche dann weitere Signalmoleküle aktivieren, wie z. B. die p38 MAP-Kinase, die ihrerseits in pro-hypertrophe Signalwege und Zytokinexpression eingebunden ist.

1.5. Isolierte Kardiomyozyten

Da es *in-vivo* schwierig ist, neurohumorale von mechanischen Effekten abzugrenzen, wurden in der vorliegenden Studie die Vorteile des Zellkulturmodells genutzt. Im Gegensatz zu *in-vivo* Methoden ist man hier in der Lage, mit definierten Medien und genau bekannten Wirkstoffkonzentrationen, neurohumorale Faktoren unabhängig von der Hämodynamik zu untersuchen. Durch selektive Anheftung isolierter Herzmuskelzellen an FCS-vorbehandelte Kulturschalen ist es möglich, das Vorhandensein weiterer kardialer Zellen nahezu vollständig auszuschließen [Jacobson et al.]. Dies ist präparationsbedingt in Kulturen neonataler Herzmuskelzellen nicht möglich.

Da die myokardiale Hypertrophie klinisch vorwiegend im Erwachsenenalter auftritt, ist es sinnvoll, im Zellkulturmodell terminal differenzierte, adulte Herzmuskelzellen zu verwenden. Zudem differiert die Wachstumsregulation neonataler Herzmuskelzellen vielfältig zur Wachstumsregulation adulter Herzmuskelzellen. Aus diesen Gründen wurde in der vorliegenden Arbeit auf Kardiomyozyten adulter Ratten zurückgegriffen. Verglichen mit neonatalen Kardiomyozyten, deren Wachstumsregulation bereits gut erforscht ist, sind adulte Kardiomyozyten bisher nur wenig als experimentelles Modell genutzt worden.

1.6. Zielsetzung

Diese Arbeit soll einen Beitrag zum Verständnis der Mechanismen der Hypertrophieinduktion und gesteigerten Zytokinexpression in ventrikulären Herzmuskelzellen leisten. Dazu sollen im Konkreten folgende Fragen analysiert werden:

1. Ist die PI3-Kinase beteiligt an der Angiotensin II-induzierten gesteigerten Zytokinexpression? Wenn ja, ist es möglich spezifische Isoformen zu ermitteln, die den Angiotensin II-induzierten von dem α-adrenergen Weg abgrenzen?

2. Welche Rolle spielen die Mitglieder der Mitogen-aktivierten Proteinkinasen an der adrenerg-vermittelten Hypertrophie? Was ist der Unterschied zwischen Kurz- und Langzeitkulturen im Hinblick auf die Ankopplung β-adrenerger Signalwege an Hyertrophiemechanismen?

3. Welche Auswirkungen hat unmittelbare Hyperglykämie auf die Signaltransduktion und kontraktile Funktion adulter Herzmuskelzellen?

4. Welche Rolle spielt NO bei der Hypertrophieentstehung und der Zytokinexpression *in-vitro* in kultivierten Kardiomyozyten und *in-vivo* in NO-defizienten Mäusen?

2. *Ergebnisse und Diskussion*

2.1. Angiotensin II, TGFβ_1 und involvierte Signalmoleküle

Angiotensin II hat an adulten Herzmuskelzellen im Gegensatz zu neonatalen Herzmuskelzellen keine nennenswerte hypertrophe Wirkung [Ruf et al.]. Die Hauptwirkung von Angiotensin II am Herzen beruht sowohl *in-vivo* als auch *in-vitro* auf einer gesteigerten Expression von Zytokinen. Als ein wichtiges Zytokin ist hier der Transformig growth factor β_1 (TGFβ_1) zu nennen, der im Herzen vor allem am Übergang von kompensierter zu dekompensierter Herzinsuffizienz vermehrt exprimiert wird [Boluyt et al. (1995)]. Als ein wichtiges Schlüsselenzym für die Angiotensin II-induzierte Antwort in Kardiomyozyten ist die NAD(P)H-Oxidase zu nennen, welche ähnlich wie in Glattmuskelzellen zu einer intrazellulären Freisetzung von reaktiven Sauerstoffspezies (ROS) führt. Diese Sauerstoffradikale dienen sowohl in Glattmuskelzellen als auch in adulten Kardiomyozyten als Signaltransduktions-moleküle. Weiter führen diese ROS zu einer Proteinkinase C-abhängigen Phosphorylierung der p38 MAP-Kinase. Angiotensin II bewirkt in Herzmuskelzellen eine Aktivierung der Proteinkinase C Isoformen δ und ε. Als weiterer Signalschritt in der Angiotensin II-vermittelten Stimulation der TGFβ_1-Expression konnte die Aktivierung des Transkriptionsfaktors AP1 ermittelt werden. Eine Beteiligung von c-fos an der Zusammensetzung dieses Transkriptionsfaktors (Homo- oder Heterodimer) konnte charakterisiert werden [Wenzel et al.].

Die initialen Signalschritte zwischen dem Angiotensin II Rezeptor Typ1 und der NAD(P)H-Oxidase sind in Kardiomyozyten nicht bekannt. In anderen Zelltypen, wie z. B. Gattmuskelzellen oder embryonalen Stammzellen wurde eine Beteiligung der PI3-Kinase an redoxsensitiven Signalschritten gezeigt [Sauer et al.]. Rabkin et al. konnte in Hühnerkardiomyozyten zeigen, dass Angiotensin II direkt die PI3-Kinase aktiviert. Um zu überprüfen, ob in dem hier verwendeten Zellkulturmodell die Angiotensin II-induzierte Generierung von ROS PI3-Kinase-abhängig ist, wurden zwei verschiedene Inhibitoren der PI3-Kinase eingesetzt: Wortmannin (100 nM) und Ly 294002 (100 μM). Inhibierung der PI3-Kinase reduziert die ROS Bildung, die p38 MAP-Kinase Aktivierung und auch die TGFβ_1 Expression und scheint somit als

initiales Signalmolekül zwischen dem Angiotensin II Rezeptor und der NAD(P)H-Oxidase zu stehen. Auch weitere wichtige kardiale Signalwege benutzen die PI3-Kinase als Signalmolekül, führen jedoch nicht zur Zytokinexpression sondern zur Hypertrophie. Um diesbezüglich eine Abgrenzung zwischen dem α-adrenerg-vermittelten Signalweg, an dessen Signalkaskade ebenfalls die PI3-Kinase beteiligt ist, zu untersuchen, wurde eine Isoformen-Spezifität der PI3-Kinase in beiden Signalwegen dargestellt. Versuche auf mRNA Ebene und auf Protein Ebene zeigen, dass alle vier Sub-Isoformen der Class-1 Isoform in adulten Herzmuskelzellen exprimiert werden. Um eine spezifische Herabregulierung der unterschiedlichen Sub-Isoformen durchzuführen, wurden Antisense Oligonukleotide hergestellt, mit deren Hilfe die PI3-Kinase Isoformen p110α und p110β signifikant vermindert exprimiert wurden. Mit Hilfe dieser Sense- und Antisense-Oligonukleotide konnte für den Angiotensin II-induzierten Signalweg die PI3-Kinase Isoform β (p110β) und für den α-adrenerg-vermittelten Signalweg die PI3-Kinase Isoform α (p110α) verantwortlich gemacht werden. Diese Ergebnisse stimmen mit Aussagen von Karlsson et al. überein, der postuliert, dass die p110α Isoform in hypertrophen Signalwegen involviert ist, wobei die Isoformen p110δ und p110γ an der Immunabwehr und an inflammatorischen Prozessen beteiligt sind.

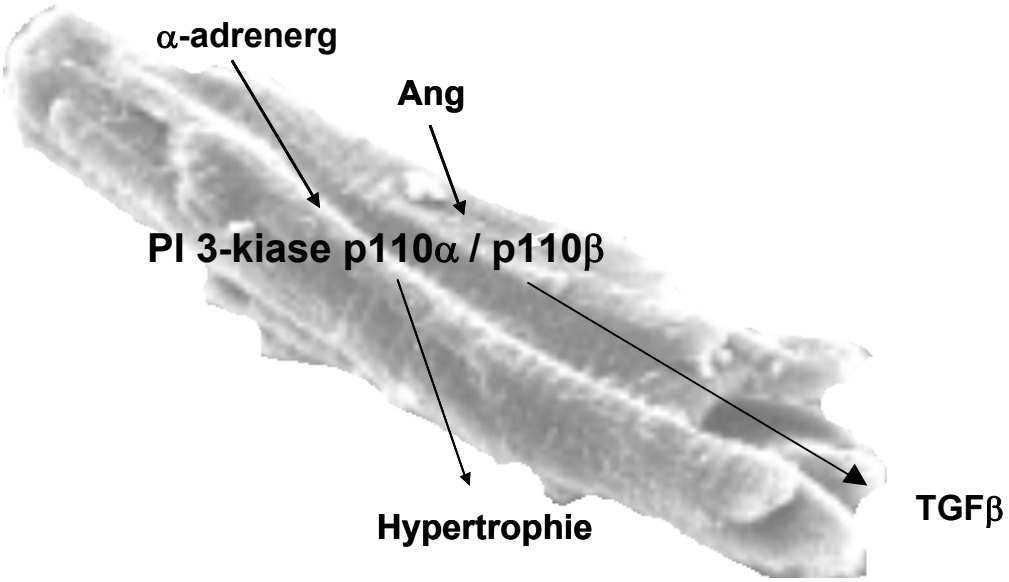

Abb. 4: Beteiligung verschiedener PI3-Kinase Isoformen an unterschiedlichen Signaltransduktionswegen in adulten Herzmuskelzellen.

2.2. p38 MAP-Kinase und Hypertrophie

Die p38 MAP-Kinase stellt ein wichtiges Signalmolekül in verschiedenen zellphysiologischen Prozessen adulter Herzmuskelzellen dar. Die p38 MAP-Kinase ist ein Mitglied der Familie der Mitogen-aktivierten Proteinkinasen. Neben ihr gehören noch die ERK (p42/44 MAP-Kinase) und die JNK zur dieser Familie. Eine Beteiligung der ERK an der adrenerg-vermittelten Hypertrophie sowie eine Beteiligung von JNK im Apoptosegeschehen der Herzmuskelzelle konnte gezeigt werden [Taimor et al. (2001)]. Da α-adrenerge Stimulation zu einer p42/44 MAP-Kinase-abhängigen Re-Expression hypertrophieassoziierter Gene führt, wurde in der Arbeit die Beteiligung der p42/44 MAP-Kinase und der p38 MAP-Kinase an der β-adrenerg-vermittelten Hypertrophie untersucht. Im Kulturmedium vorhandene Proteasen aktivieren das von den Herzmuskelzellen selbst sezernierte und inaktive TGFβ_1. Dieses aktivierte TGFβ_1 wiederum ist verantwortlich für die hypertrophe Ankopplung β-adrenerger Stimulation. Die Kultivierung adulter Kardiomyozyten über einen Zeitraum von sechs Tagen bewirkt eine selektive Aktivierung der p38 MAP-Kinase. Die beiden anderen Mitglieder dieser Proteinkinase Familie, ERK und JNK, werden nicht aktiviert. Ebenso sind weder die PKC noch die Proteinkinase B (PKB) in ihrer Aktivität beeinflusst. β-Adrenerge Stimulation erhöht in Kurzzeitkulturen und in Langzeitkulturen die p38 MAP-Kinase Aktivität – in Langzeitkulturen über das schon erhöhte Niveau hinaus. Die p38 MAP-Kinase ist demnach kausal an der β-adrenerg-vermittelten Hypertrophie beteiligt, da deren Hemmung mit SB202190 auch die β-adrenerg-vermittelte Steigerung der Proteinbiosynthese mindert. Einen direkten Einfluss auf die verbesserte β-adrenerge Ankopplung bei Anwesenheit von aktivem TGFβ_1 konnte jedoch nicht gezeigt werden, da die erhöhte p38 MAP-Kinase Phosphorylierung auch nach dem Abfangen des TGFβ_1 in der Zellkultur erhalten bleibt. Darüber hinaus konnte die Aktivierung der p38 MAP-Kinase in anderen Hypertrophie-induzierenden Signalwegen gezeigt werden. Diese MAP-Kinase ist ebenfalls in der durch Hyperosmolarität, Hyperglykämie (siehe weiter unten) und mechanischen Stress hervorgerufenen Hypertrophie beteiligt; aber nicht am α-adrenerg-vermittelten hypertrophen Wachstum. Elektrische Stimulation von Kardiomyozyten erhöht ebenfalls sowohl die p38 MAP-Kinase Aktivierung als auch die Proteinsynthese.

2.3. Hyperglykämie und Kardiomyozyten

Hyperglykämie, häufig eine Folge des nicht oder falsch eingestellten Diabetes mellitus, resultiert vorwiegend in der Gykosylierung verschiedener Proteine mit zu meist irreversiblen Schäden am Gefäßendothel und Nerven. Zudem werden direkt Signaltransduktionswege aktiviert. Da in anderen Zellkulturen Glukose einen direkten Einfluss auf Signalschritte ausübt [Igarashi et al., Wilmer et al.], sollte die negative Wirkung von Hyperglykämie auf Kardiomyozyten untersucht wurde. Die Zugabe von 15 mM Glukose in das Kulturmedium adulter isolierter Herzmuskelzellen zeigt direkt keinen Effekt auf hypertrophes Wachstum, jedoch auf deren kontraktile Funktion gemessen mittels Zeilenkamera z. B. als fraktionelle Zellverkürzung (Δl/l). Auf Ebene der involvierten Signalmoleküle aktiviert Glukose konzentrationsabhängig die p38 MAP-Kinase innerhalb von 45 Minuten, wobei die Phosphorylierung nur bei einer Konzentration von 30 mM signifikant erhöht ist. Ebenfalls konzentrationsabhängig steigert Glukose die Bildung von ROS innerhalb von 30 Minuten, gemessen mittels H_2DCF Fluoreszenz. Auch hier zeigt sich ein Maximum bei einer eingesetzten Konzentration an Glukose von 30 mM. Dass Diabetes die Zytokinexpression steigert, konnten schon Ohshiro und Mitarbeiter zeigen. Direkte Hyperglykämie steigert in isolierten Herzmuskelzellen auch die TGFβ_1 Expression. Auch hier zeigt sich ein konzentrationsabhängiger Bezug. Bei einer Konzentration von 30 mM Glukose reagieren die Zellen mit einem deutlichen und signifikanten Anstieg nach 24-stündiger Inkubation. Da sowohl der Angiotensin II-induzierte Signalweg als auch der durch Hyperglykämie-induzierte Signalweg die gleichen Signalmoleküle benutzen, liegt die Vermutung nahe, dass hohe Konzentrationen an Glukose einen direkten Einfluss auf den Angiotensin II Rezeptor ausüben. Schon Privratsky et al. konnte zeigen, dass die Blockierung des Angiotensin II-Rezeptors mit einem AT1-Rezeptor Blocker die durch Hyperglykämie ausgelöste Funktionsverschlechterung isolierter adulter Myozyten verhindert. Aus diesem Grund wurde die Beteiligung der untersuchten Signalwege an der ebenfalls ausgelösten systolischen Funktionsverschlechterung untersucht, da auch Angiotensin II selbst nach 24-stündiger Inkubation eine kontraktile Dysfunktion verursacht [Domenighetti et al.]. Hierzu wurden verschiedene Hemmstoffe der evaluierten Signalmoleküle eingesetzt. Reaktive Sauerstoff Spezies wurden mit Antioxidantien, wie dem Vitamin C,

abgefangen, und die p38 MAP-Kinase wurde mit SB202190 inhibiert. In beiden Fällen war die durch Hyperglykämie ausgelöste Kontraktionsverschlechterung nicht aufhebbar, was hier in diesem Fall gegen einen kausalen Zusammenhang der untersuchten Signalmoleküle und der ausgelösten kontraktilen Dysfunktion spricht. Ebenso spricht gegen einen Zusammenhang, dass die Funktionsverschlechterung schon bei einer Glukosekonzentration von 15 mM ausgelöst wird, wohingegen die untersuchten Signalmoleküle erst bei sehr viel höherer Konzentration (30 mM) aktiviert werden. Vielmehr scheint die Ursache, der durch hohe Glukosespiegel ausgelösten Funktionsverschlechterung in einer veränderten Kalziumhomöostase und/oder einer Proteinkinase C Aktivierung [Adeghate et al., Guo et al., Howarth et al.] zu liegen

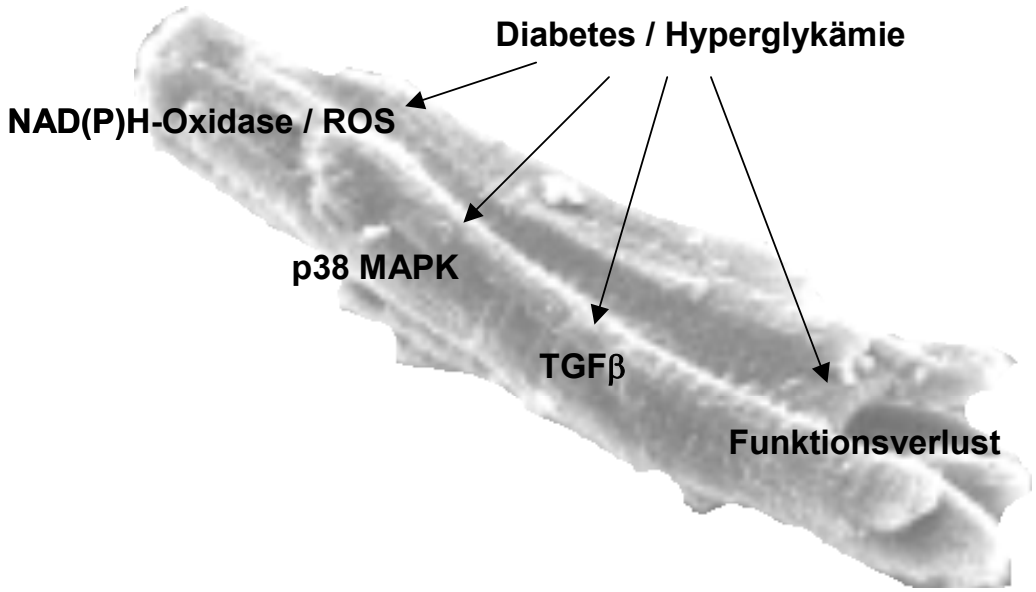

Abb. 5: Direkter Einfluss hoher Glukosekonzentrationen auf unterschiedliche Signalmoleküle in isolierten Kardiomyozyten

2.4. NO und Hypertrophie, Zytokine und „Redox-Balance“

Stickstoffmonoxid, ursprünglich entdeckt als ein in Endothelzellen gebildeter Faktor, welcher eine stark dilatatorische Wirkung sowohl auf venöse als auch auf arterielle Gefäße besitzt, wird von einem Enzym gebildet, welches in drei Isoformen existiert: der endothelialen NO-Synthase (eNOS), der induzierbaren NO-Synthase (iNOS) und der neuronalen NO-Synthase (nNOS). In ventrikulären Herzmuskelzellen werden alle drei Isoformen [Obasanjo-Blackshire et al., Martin et al.] exprimiert, wobei jedoch zum überwiegenden Anteil die eNOS vorhanden ist. Bubikat et al. konnte zeigen, dass eNOS defiziente Mäusen eine kardiale Hypertrophie entwickeln. Isolierte adulte Myozyten zeigen ebenfalls eine Steigerung der Proteinsynthese und damit Hypertrophieausbildung nach Inhibierung der NO Bildung für 24 Stunden. Diese Ergebnisse können auch durch direkte Volumenvermessung verifiziert werden. Inhibierung der p38 MAP-Kinase mit SB 202190 (SB) ebenso wie die Inhibierung der Proteinkinase C mit Bisindolylmaleimid (BIM) verhindert die durch mangelnde NO Verfügbarkeit ausgelöste Hypertrophie. Eine verminderte NO Produktion erhöht die Bildung von ROS. Auch dieser Effekt scheint wichtig für das hypertrophe Wachstum zu sein, da sowohl die Zugabe von Antioxidantien als auch die Applikation eines NAD(P)H-Oxidase Inhibitors (Apocinin) die durch L-Nitro-Arginin (L-NNA) ausgelösten Effekte verhindert. Eine Inhibierung von NO scheint somit die „Redox-Balance“ in Kardiomyozyten zu beeinträchtigen, und direkt in ROS-abhängige Signalwege einzugreifen. Die Verfütterung von Hydralazin an Mäuse mit einer durch die gleichzeitige Verfütterung von L-NNA ausgelösten Herzhypertrophie bestätigen diese Ergebnisse [Sanada et al.]. Darüber hinaus konnte Hsu et al. zeigen, dass direkte Applikation eines NO-Donors die Expression von TGFβ_1 und die Produktion von Kollagen in Kelloid-Fibroblasten steigert. In isolierten adulten Kardiomyozyten ist ein gegenteiliger Effekt zu sehen. Hier steigert die Inhibierung der NO Produktion sowohl die TGFβ_1 Expression als auch die TNFα Freisetzung. Diese Ergebnisse wurden *in-vivo* verifiziert. Neben der entstandenen Hypertrophie ist in isolierten Myozyten von eNOS defizienten (eNOS$^{-/-}$) Mäusen die TGFβ_1 Expression signifikant erhöht, ebenso wie die TNFα Freisetzung. Ebenfalls ist eine erhöhte Phosphorylierung der p38 MAP-Kinase nachzuweisen. Nicht transgene Wurfgeschwister wurden als Kontrollen verwendet. Gleichsinnig hierzu zeigt eine

Inhibierung der NO Produktion in aus eNOS$^{+/+}$ Mäusen isolierten Kardiomyozyten eine erhöhte p38 MAP-Kinase Phosphorylierung und Zytokinexpression. Der wachstumsstimulierende Effekt einer NO Inhibierung scheint also weniger auf einem cGMP-abhängigen Vorgang zu beruhen, als vielmehr auf einer gestörten Redox-Balance mit erhöhten Leveln an ROS und damit einer Anbindung an Redox-abhängige Signalschritte. Fiedler et al. konnte zeigen, dass ein erhöhtes NO Angebot antihypertrophe cGMP-abhängige Effekte auslöst.

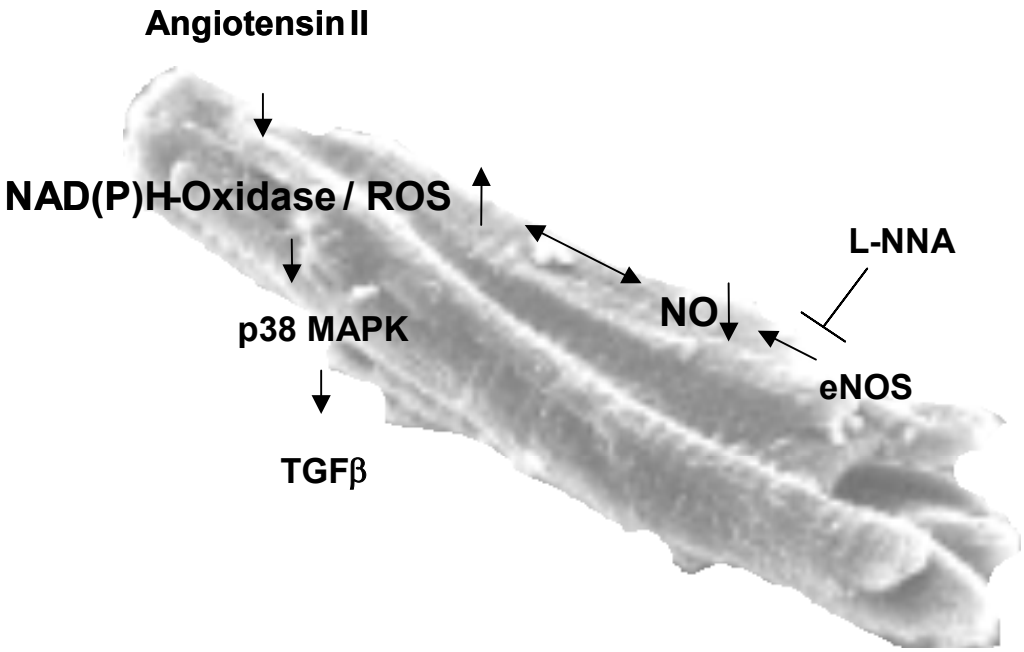

Abb. 6: Redox-Balance in Kardiomyozyten

3 Zusammenfassung

Die unterschiedlichsten Formen der Herzhypertrophie und -insuffizienz haben eines gemeinsam: eine Änderung bestehender oder eine Aktivierung neuer Signalkaskaden auf Ebene der einzelnen Herzmuskelzelle, die zu Funktionsverlust und Remodelling auf Gesamtherzebene führen. Neben dem vegetativen Nervensystem nimmt das Renin-Angiotensin-Aldosteron-System eine herausragende Rolle für diese Prozesse ein, wie auch die zur Zeit möglichen Behandlungen von Herz-Kreislauf-Erkrankungen mit ACE-Hemmern und AT1-Rezeptorblockern zeigen. Die Studie trägt wesentlich dazu bei, die Wirkung von Angiotensin II auf die einzelne Herzmuskelzelle darzustellen und die vielfältigen Interaktionen mit anderen Signalwegen und –molekülen aufzuzeigen. Im Gegensatz zu der *in-vivo* Situation ist in isolierten Herzmuskelzellen eine nur moderate direkte hypertrophe Stimulierbarkeit durch Angiotensin II auszulösen. Vielmehr löst die Applikation von Angiotensin II vor allem eine gesteigerte Zytokinexpression aus. Inwieweit die gesteigerte Expression von TGFβ_1 ursächlich für das einsetzende Remodelling, den Funktionsverlust und die daraus resultierende Herzinsuffizienz wichtig ist, ist noch nicht hinreichend geklärt und Gegenstand eigener aktueller Forschungen. Neben den schon in früheren Arbeiten ermittelten Signalmolekülen, welche an der Angiotensin II-induzierten gesteigerten TGFβ_1 Expression beteiligt sind, zeigt diese Arbeit die initialen Schritte bis zur NAD(P)H-Oxidase Aktivierung nach Rezeptorstimulation auf und identifiziert die PI3-Kinase und die kleine GTPase Rac (Wenzel et al., unpublizierte Daten) als wichtige Bestandteile. Um eine Abgrenzung zu dem α-adrenergen Signalweg zu ziehen, welcher zu einer gesteigerte Proteinsynthese führt und ebenfalls die PI3-Kinase beinhaltet, wurde in dieser Arbeit eine Isoformenspezifität für die PI3-Kinase in den beiden Signalwegen erarbeitet. Für den Angiotensin II-induzierten Signalweg konnte eine Beteiligung der p110β ermittelt werden. Rac, eine zytosolische Untereinheit der NAD(P)H-Oxidase, ist ebenfalls in diesen Weg involviert. Statine hemmen unter anderem die Translokation von Rac zu der Zellmembran und unterbinden somit die ROS Produktion, was unter anderem den pleiotropen Effekt von Statinen erklärt. Delbosc et al. konnte diese *in-vitro* Ergebnisse ebenfalls *in-vivo* bestätigen. Eine Bildung von ROS und NO in Kardiomyozyten legt die Vermutung nahe, dass ähnlich der Situation in Glattenmuskelzellen auch hier eine Gleichgewicht

zwischen unterschiedlichen Radikalen, Radikal produzierenden Enzymen und zelleigenen Antioxidantien, wie z. B. der SOD besteht. Die Arbeit zeigt, dass eine Reduzierung der endogenen NO Produktion unabhängig von cGMP-abhängigen Signalwege in ROS-abhängige Signalweg eingreift und ebenfalls als Endpunkt zu einer gesteigerten Hypertrophie und Zytokinexpression führt. Als ein zentrales, essentielles und ubiquitär vorkommendes Signalmolekül in vielen pathologischen Signalwegen der Herzmuskelzelle ist die p38 MAP-Kinase zu nennen. Die selektive α-adrenerge Stimulation mit Phenylephrin erhöht nicht deren Aktivität. Im Gegensatz dazu erhöhen Hyperglykämie, mechanischer Stress, osmotischer Stress, die Aktivierung des Renin-Angiotensin-Aldosteron-Systems und des β-adrenergen Systems innerhalb kurzer Zeit (30-60 Minuten) die Phosphorylierung und damit Aktivierung dieser Kinase, die typischerweise redoxsensitiv ist [Sugden et al. (2006)]. Aus diesen Gründen besteht die Überlegung, die p38 MAP-Kinase therapeutisch am Menschen *in-vivo* zu hemmen [Kerkela et al.], um damit in viele unterschiedliche pro-hypertrophe Signalwege regulatorisch einzugreifen.

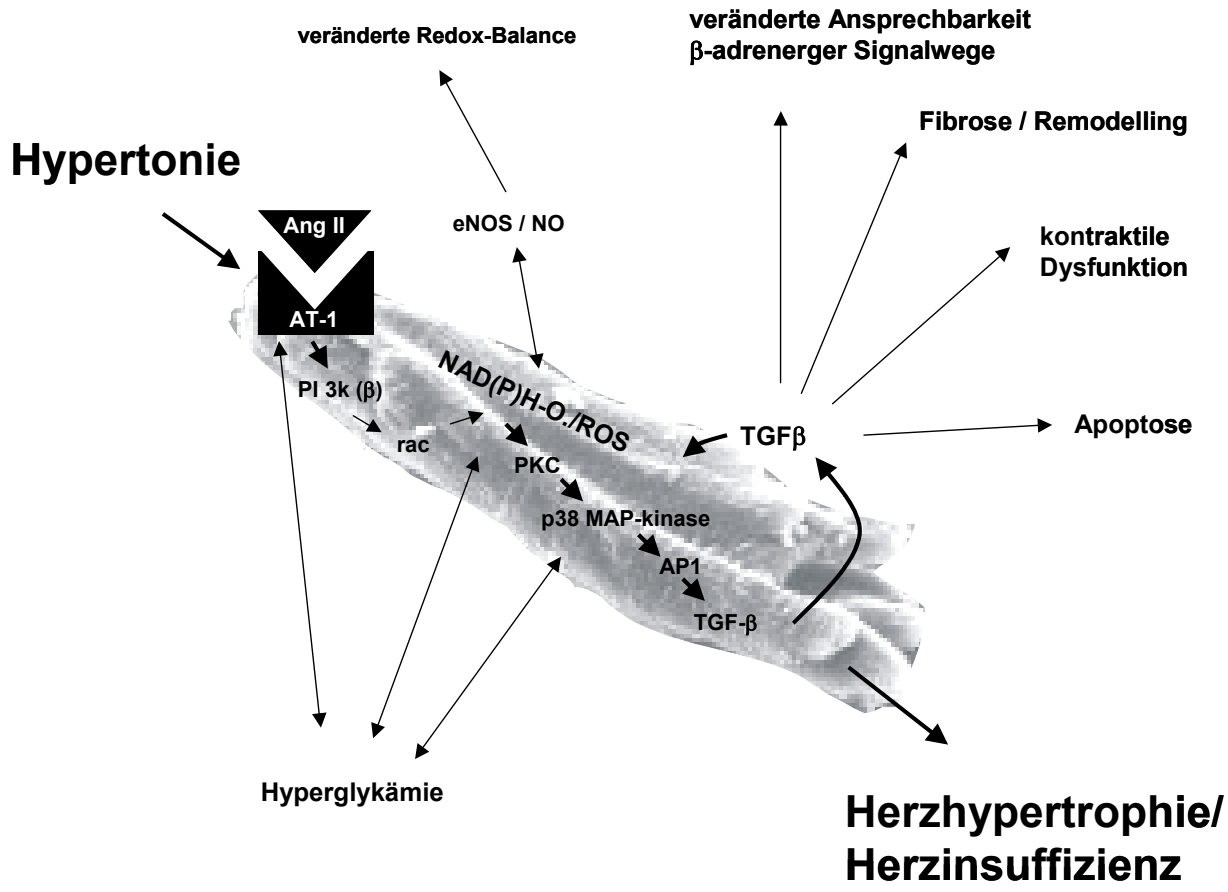

Abb. 7: Zusammenfassende Darstellung der bei der Entwicklung der Herzinsuffizienz beteiligten Signalschritte und deren Interaktionen.

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Clin Exp Pharmacol Physiol; 30:911-918.

6 Anhang

Folgende eigene Originalarbeiten sind in dieser Habilitationsschrift zusammengefasst und diskutiert worden:

1. ***Wenzel S****, Müller C, Piper HM, Schlüter KD*
   p38 MAP-Kinase in cultured adult rat ventricular cardiomyocytes: expression and involvement in the hypertrophic signaling
   Eur J Heart Fail. 2005; 7:453-60.

2. ***Wenzel S****, Soltanpour G, Schlüter KD*
   No correlation between the p38 MAP-kinase pathway and the contractile dysfunction in diabetic cardiomyocytes
   Pflugers Arch. 2005; 451:328-37.

3. ***Wenzel S****,* Abdallah Y, Helmig S, Schafer C, Piper HM, Schlüter KD
   Contribution of PI 3-kinase isoforms to angiotensin II- and alpha-adrenoceptor-mediated signalling pathways in cardiomyocytes
   Cardiovasc Res. 2006; 71:352-62.

4. ***Wenzel S****, Rohde C, Wingerning S, Roth J, Kojda G, Schlüter KD*
   The effect of inhibition of endogenously produced nitric oxide on p38 MAP kinase phosphorylation in adult ventricular cardiomyocytes
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p38 MAP-kinase in cultured adult rat ventricular cardiomyocytes: expression and involvement in hypertrophic signalling

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Abstract

Both α-adrenoceptor- and β-adrenoceptor-stimulation lead to hypertrophic growth of the myocardium. But only β-adrenoceptor-stimulation requires the pre-cultivation of cells with active TGF-β. In order to define signalling molecules that are specifically involved in β-adrenoceptor-dependent hypertrophy, changes in expression and hypertrophic responsiveness during pre-cultivation with TGF-β were investigated. Isolated adult ventricular cardiomyocytes from rats were either cultured in 20% (v/v) foetal calf serum (FCS) to activate autocrine released TGF-β or used without pre-treatment. Protein synthesis was analysed by ^{14}C-phenylalanine incorporation. Expression of signalling molecules was determined by immunoblotting. During cultivation of cardiomyocytes with active TGF-β only the expression of p38 MAP-kinase increased. Subsequent stimulation of β-adrenoceptors induced protein synthesis in a p38 MAP-kinase-dependent way. However, stimulation of β-adrenoceptors activated p38 MAP-kinase irrespective of pre-treatment with TGF-β. In the absence of this cytokine, hyperosmolarity or reconstitution of mechanical activity increased protein synthesis via p38 MAP-kinase activation in freshly isolated cells. In conclusion, activation of p38 MAP-kinase is a newly identified necessary signalling step required for β-adrenoceptor induced hypertrophic growth. Like activation of adenyl cyclase, activation of p38 MAP-kinase is up-stream of the TGF-β-induced coupling to the regulation of protein synthesis. Reconstitution of mechanical activity mimics the co-activation required and induced by TGF-β.

Keywords: Hyperosmolarity; Mechanical forces; Cardiomyocytes; Hypertrophy

1. Introduction

Plasma levels of catecholamines are found to be increased under conditions leading to myocardial hypertrophy and heart failure in vivo [1]. In vitro, α-adrenoceptor stimulation of freshly isolated adult ventricular cardiomyocytes causes hypertrophy in a protein kinase C (PKC), PI 3-kinase and $p70^{S6}$ kinase-dependent manner [2–5]. These cells do not show any hypertrophic response to selective β-adrenoceptor stimulation, in contrast to the in vivo situation, where β-adrenergic stimulation can also cause myocardial hypertrophy [6]. In vitro pre-cultivation of cardiomyocytes in the presence of foetal calf serum restores their in vivo-like behaviour [7]. This culture system therefore permits analysis of the cellular mechanisms by which β_2-adrenoceptor stimulation induces myocardial hypertrophy. Previous studies using this culture system showed that TGF-β, which is released by the cells and activated by proteases present in foetal calf serum, is necessary for the hypertrophic response to β-adrenergic stimulation [7,8]. In contrast to the β-adrenoceptor-dependent pathway, α-adrenoceptor-dependent hypertrophy is not different between cells pre-cultivated with TGF-β or not. The priming effect of TGF-β for cardiac growth is likely to play a role in vivo, since cardiac expression of TGF-β has been found to be increased in patients with idiopathic hypertrophic cardiomyopathy [9] or in spontaneously hypertensive rats during the transition to heart failure [10]. It may therefore be suggested that under these pathological conditions β-adrenergic stimulation contributes to the dysregulation of myocardial growth.

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Some of the signalling molecules required for β-adrenoceptor-dependent stimulation of protein synthesis are in common with those of the α-adrenoceptor-induced pathway: for example, PI 3-kinase and $p70^{S6}$ kinase [2]. PKC-activation is a further step that is localised upstream the above mentioned enzymes but not activated by β-adrenoceptor stimulation. It further suggests, that members of the family of mitogen activated protein kinases, like p38 MAP-kinase and p42/44 MAP-kinase (ERK), are involved in hypertrophic regulation of the heart [11,12]. For example, p38 MAP-kinase activation was found to be critically involved in the induction of hypertrophy in some cases [13] but not in all cases [14]. In the same cell system under investigation, angiotensin II was characterized as a strong activator of p38 MAP-kinase, but increases protein synthesis via p42/p44 MAP-kinase rather than p38 MAP-kinase [15]. This focussed our interest on members of the MAP-kinase family.

In this study, we hypothesized that some of the above mentioned signalling molecules change their expression level during exposure to active TGF-β. If so, such changes in expression might contribute to the priming effect of TGF-β. Therefore, we further investigated whether such factors are linked to β-adrenoceptor-dependent regulation of protein synthesis and whether their regulation depends on TGF-β. In this manuscript we show that p38 MAP-kinase expression increases during the pre-cultivating time in the presence of TGF-β. Subsequently we investigated whether p38 MAP-kinase is causally involved in β-adrenoceptor-dependent protein synthesis requiring such pre-incubation. Finally, we investigated whether the expression level of p38 MAP-kinase is a limiting factor for hypertrophy coupling of p38 MAP-kinase in protein synthesis.

2. Methods

All animal studies were performed in accordance with guidelines described in the NIH Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH, publication no. 85-23, revised 1996).

2.1. Cell isolation, short-term, long-term cultures

Ventricular cardiomyocytes isolated from 200–250 g male Wistar rats were suspended in basal culture medium and plated on 60 mm culture dishes. The culture dishes had been pre-incubated overnight with 4% (v/v) foetal calf serum (FCS) in medium 199. The basal culture medium (CCT) was modified medium 199 including Earl's salts, 2 mM L-carnitine, 5 mM taurine, 100 IU/ml penicillin and 100 μg/ml streptomycin. To prevent growth of nonmyocytes, the media were also supplemented with 10 μM cytosine-β-D-arabinofuranoside (pH 7,4).

Four hours after plating, cultures were washed twice with CCT medium. As a result of the medium change, broken cells were removed resulting in cultures of 93±2% quiescent rod-shaped cells. These cells were either directly stimulated (short-term cultures) or the cardiomyocytes were supplied with CCT medium containing 20% (v/v) FCS and incubated for up to 6 days without change of media (TGF-β pre-treatment protocol). In both cases the experiments were carried out in basic culture medium under serum free conditions (control) or with addition of respective substances.

2.2. Incorporation of ^{14}C-phenylalanine

The rate of protein synthesis was determined by exposing cultures to L-^{14}C-phenylalanine (0.1 μCi/ml) for 24 h. Incorporation of radioactivity into acid insoluble cell mass was determined as described before [7] and normalized to the precursor pool (acid soluble cell mass).

2.3. SDS-gel electrophoresis

Protein extracts from adult ventricular cardiomyocytes for SDS-gel electrophoresis were prepared as described before [16]. Protein extracts (100 μg) were loaded on a 12% (w/v) SDS-page (acryl amide/bisacryl amide 30:1). After electrophoresis, proteins were transferred onto reinforced nitrocellulose by semidry blotting. The sheets were saturated with 2% (w/v) bovine serum albumin and incubated for 2 h with rabbit polyclonal anti rat p42 MAP-kinase, rabbit polyclonal anti protein kinase B, and rabbit polyconal anti rat protein kinase C α and δ. After washing, the sheets were re-incubated with an alkaline phosphatase-labeled goat anti-rabbit IgG. Finally, bands were visualized by alkaline phosphatase activity using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium. p38 MAP-kinase activation was calculated from the amount of phosphorylated p38 MAP-kinase and the total amount of p38 MAP-kinase. Therefore two blots were performed and two different first antibodies were used. First a rabbit polyclonal anti-rat p38 MAP-kinase and second a polyclonal anti-rat phosphorylated p38 MAP-kinase antibody (0.2 μg/ml, RBI). SB202190 (1 μM) was used as a p38 MAP-kinase inhibitor.

2.4. Hyperosmolarity

In order to cause hyperosmolarity, cells were exposed to culture medium containing 150 mM sucrose for 24 h. Following p38 MAP-kinase, activation and hypertrophy were measured as described before.

2.5. Electrical activity

Regular mechanical activity was achieved by electrical stimulation. Cardiomyocytes were stimulated by 0.5 Hz for 2, 4 and 24 h by two wire loops which were arranged parallel lying on the top of the cells and cultivated for up to 24 h with ^{14}C-phenylalanine. The cells were then either used for measuring protein synthesis or p38 MAP-kinase activation. In order to differentiate between electrical

stimulation and mechanical activity, cells were co-incubated with butanedione monoxime (BDM, 20 mM) which prevents cell contraction by inhibiting the actin and myosin interaction induced by electrical stimulation. Controls were incubated for 24 h without electrical stimulation.

3. Statistics

Data are given as means±S.E.M. Statistical comparisons were performed by one-way analysis of variance and use of the Student–Newman–Keuls test for post hoc analysis. In cases in which two groups were compared, conventional *t*-tests were performed. Differences with $p<0.05$ were regarded as statistically significant. All data analyses were performed using SAS software, version 6.11 (SAS Institute, Cary, NC, USA).

4. Results

4.1. Changes in cellular expression of hypertrophy associated signalling molecules during cultivation of adult ventricular cardiomyocytes

In order to determine signalling molecules that are specifically involved in the hypertrophic response of cells pre-treated with TGF-β, cardiomyocyte cultures were used in which the cells were incubated in the presence of 20% foetal calf serum for up to 6 days. As shown previously, the cells release substantial levels of TGF-β into the medium, which is activated by proteases present in the foetal calf serum [17]. p38 MAP-kinase, p42/44 MAP-kinase, protein kinase B (PKB) and protein kinase C (PKC) α and δ were analysed by immunoblotting. Data were normalized to actin expression. Among the signalling molecules under investigation only p38 MAP-kinase expression was increased during pre-treatment. It was elevated after 3 days of cultivation and remained increased thereafter (Fig. 1A,B).

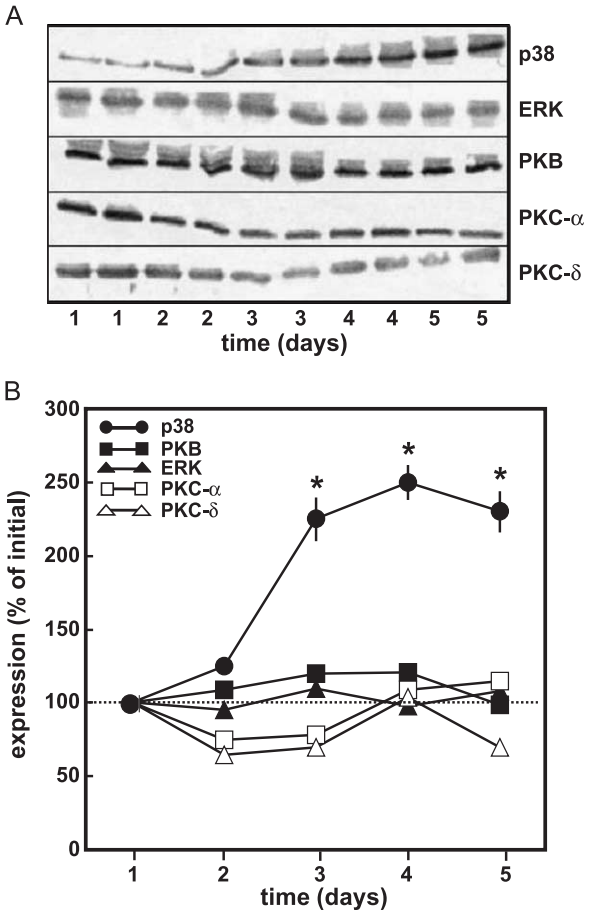

Fig. 1. (A) Representative immunoblots from protein samples of cardiomyocytes. Cardiomyocytes were cultured up to 5 days in the presence of active TGF-β. (B) Influence of culture conditions on the expression of different proteins. Cardiomyocytes were cultured up to 5 days in the presence of active TGF-β. Protein expressions were measured every day. Data are means from $n=3$ cultures, $*p<0.05$ vs. expression at day 1. In case of p38 MAP-kinase S.E.M. is given. In each other case it is omitted for greater clarity.

4.2. p38 MAP-kinase activation by isoprenaline in cardiomyocytes pre-treated with TGF-β and its implication for the regulation of protein synthesis

The experiments performed so far have shown a correlation between upregulation of p38 MAP-kinase (this study) and the induction of hypertrophic responsiveness to β-adrenoceptor stimulation [18,19]. This suggests a causal relationship between both. In order to show a causal link between p38 MAP-kinase activation and the β-adrenoceptor-dependent hypertrophy, cells were used 6 days after cultivation and the hypertrophic responsiveness to β-adrenoceptor stimulation was investigated thereafter by addition of 100 nM isoprenaline. This was done in the presence or absence of the p38 MAP-kinase inhibitor SB202190. This inhibitor, which is known to inhibit angiotensin II induced activation of p38 MAP-kinase in adult ventricular cardiomyocytes [20], was used in a concentration-dependent way (Fig. 2). Based on this concentration–response curve, SB202190 was used at a concentration of 1 μM throughout the subsequent experiments. Cells were also stimulated by phenylephrine (10 μM), an α-adrenoceptor agonist in order to exclude any unspecific effects of SB202190 on stimulation of protein synthesis. The results, which confirm earlier observations, showed that isoprenaline and phenylephrine increased protein synthesis, as determined by ^{14}C-phenylalanine incorporation and total protein content (Fig. 3). However, SB202190 was sufficient to inhibit the hypertrophic response to isoprenaline but did not impair the response to phenylephrine (Fig. 3).

In the next set of experiments we confirmed that isoprenaline activates p38 MAP-kinase. p38 MAP-kinase activation

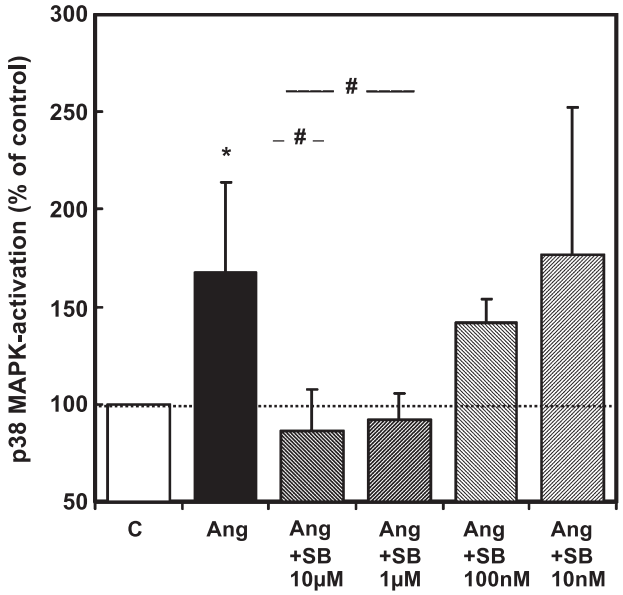

Fig. 2. Concentration response curve for the inhibitory effect of SB202190 on angiotensin II-dependent p38 MAP-kinase activation in adult ventricular cardiomyocytes. Cells were treated with 100 nM angiotensin II and SB202190 at concentrations as indicated. Data are means and S.E.M. from n=4 cultures; *p<0.05 vs. control; $^{\#}p$<0.05 vs. angiotensin II.

was analysed by demonstration of p38 MAP-kinase phosphorylation. Isoprenaline raised the phosphorylation of p38 MAP-kinase within 60 min (Fig. 4). However, activation of p38 MAP-kinase was not restricted to cardiomyocytes pre-incubated with TGF-β (Fig. 4). In contrast to isoprenaline, phenylephrine did not activate p38 MAP-kinase (data not shown).

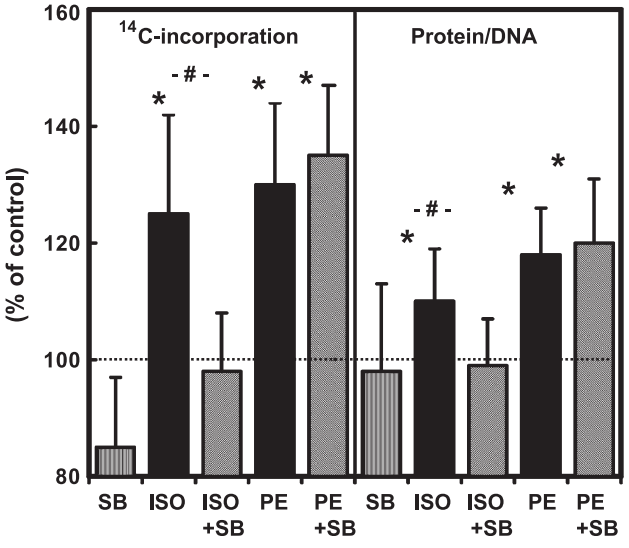

Fig. 3. Effect of adrenoceptor stimulation on protein synthesis and protein mass after 6 days of pre-cultivation with active TGF-β. Cultures were stimulated in the absence or presence of SB202190 (SB, 1 μM), an inhibitor of p38 MAP-kinase, with isoprenaline (ISO, 100 nM) or with phenylephrine (PE, 10 μM). Data are means±S.E.M. from n=4 cultures, *p<0.05 vs. control, $^{\#}p$<0.05 vs. isoprenaline stimulation alone.

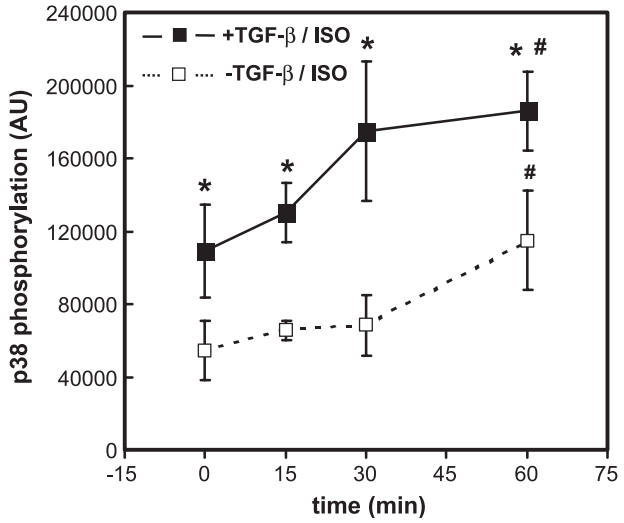

Fig. 4. p38 MAP-kinase phosphorylation of cardiomyocytes pre-cultured in presence of TGF-β or not. Subsequently, cells were stimulated with isoprenaline (ISO, 100 nM). Data are expressed as the amount measured as arbitrary units of phosphorylated p38 MAP-kinase and expressed as means±S.E.M. from n=4 cultures, *p<0.05 vs. control, $^{\#}p$<0.05 vs. time point zero of the respective measurements.

4.3. Influence of TGF-β on the induction of p38 MAP-kinase expression in cultured cardiomyocytes

The aforementioned experiments support our hypothesis, if one assumes that expression of p38 MAP kinase is too low in cardiomyocytes not pre-treated with TGF-β to trigger protein synthesis and that TGF-β is responsible for the induction of p38 MAP-kinase expression in this system. To confirm this, cells were incubated with a neutralizing antibody against active TGF-β for 6 days. However, the

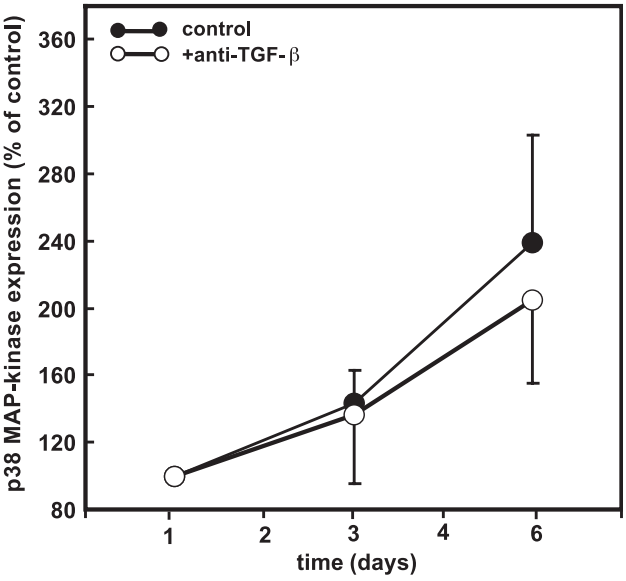

Fig. 5. Supplementation of anti-TGF-β to the culture medium does not change the amount of p38 MAP-kinase expression during cultivation. Data are means±S.E.M. from n=4 cultures.

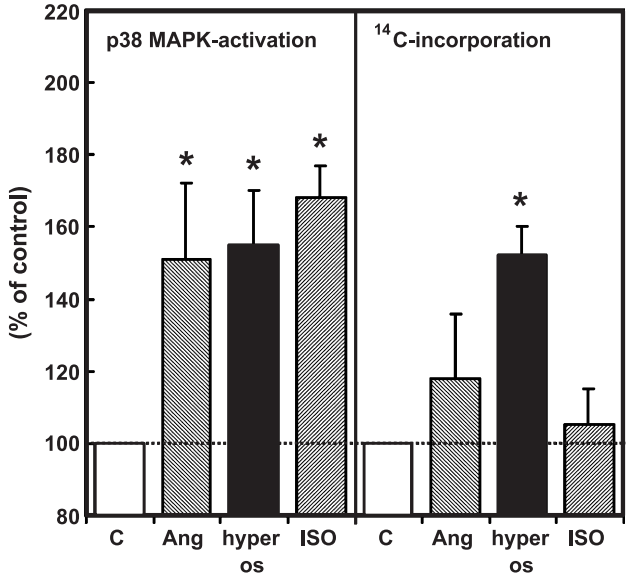

Fig. 6. p38 MAP-kinase activation and protein synthesis in freshly isolated cells. Cells were stimulated with angiotensin II (Ang, 100 nM), hyperosmolarity (hyperos, 150 mM sucrose) or isoprenaline (ISO, 100 nM) for 60 min. p38 MAP-kinase activation was determined by the ratio of phosphorylated to non-phosphoryted p38 MAP-kinase. Data are means±S.E.M. from n=4 cultures, *p<0.05 vs. control.

presence of this neutralizing antibody did not prevent the upregulation of p38 MAP-kinase (Fig. 5).

In order to show that the lower expression level of p38 MAP-kinase in freshly isolated cardiomyocytes is not a limiting factor, we performed experiments with three different stimuli for p38 MAP-kinase activation, namely hyperosmolarity, isoprenaline, and angiotensin II. All

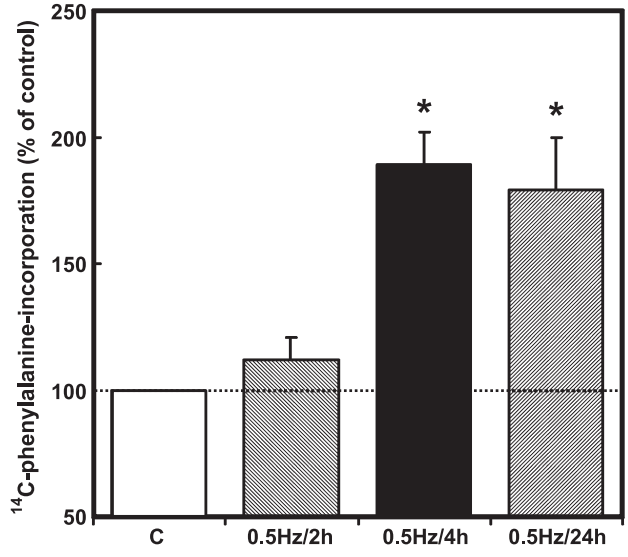

Fig. 8. The influence of reconstitution of mechanical activity by electrical stimulation with 0.5 Hz on protein synthesis. Cells were stimulated for 2, 4 or 24 h and in all cases protein synthesis was determined during a 24-h cultivation period. Data are means±S.E.M. from n=4 cultures, *p<0.05 vs. control.

three agonists activated p38 MAP-kinase (Fig. 6), however, with the exception of hyperosmolarity, induced by 150 mM sucrose added to the medium, none of the other agonists raised ^{14}C-phenylalanine incorporation (Fig. 6). In order to investigate whether hypertrophic growth caused by hyperosmolarity depends on p38 MAP-kinase, cells were co-incubated again with SB202190. SB202190 was able to reduce protein synthesis and protein mass

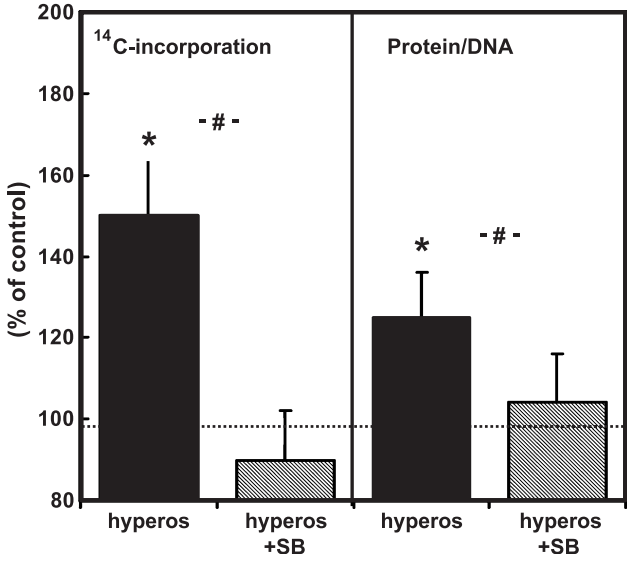

Fig. 7. Influence of SB202190 on hyperosmolarity (hyperos, 150 mM sucrose) dependent increase in protein synthesis and protein mass. Co-incubation of cells with SB202190 (SB, 1 μM) inhibits both parameters of hypertrophic growth significantly. Data are means±S.E.M. from n=4 cultures, *p<0.05 vs. control, $^{\#}p$<0.05 vs. hyperosmolarity alone.

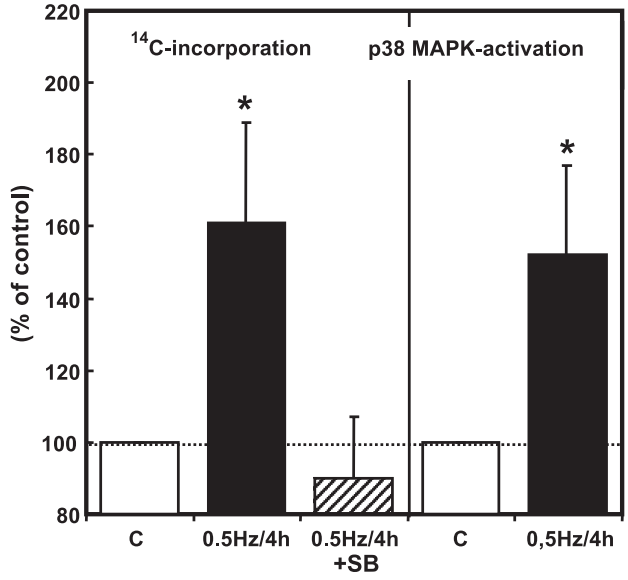

Fig. 9. Influence of electrical stimulation with 0.5 Hz for 4 h on protein synthesis and p38 MAP-kinase activation. Cells were stimulated in presence or absence of SB202190 (SB, 1 μM). Data are means±S.E.M. from n=5 cultures, *p<0.05 vs. control.

after addition of 150 mM sucrose to the cell medium (Fig. 7).

4.4. Reconstitution of mechanical activity increases protein synthesis via p38 MAP-kinase

Although the experiments on cardiomyocytes exposed to hyperosmolarity indicated that p38 MAP-kinase expression level in freshly isolated cardiomyocytes is sufficient to induce hypertrophic growth via this pathway, it remained unclear whether this pathway was related to the regulation of protein synthesis under more physiological or pathophysiological conditions. We hypothesized that mechanical forces generated by hyperosmolarity trigger p38 MAP-kinase-activation and the hypertrophic response. If this is true, mechanical stress, like cell contraction, may also induce hypertrophic growth via this pathway. Therefore, in order to demonstrate a more physiological mechanical intervention than hyperosmolarity, we performed experiments to reconstitute mechanical activity. Reconstitution of the mechanical activity of cardiomyocytes was achieved by electrical stimulation of the cardiomyocytes at 0.5 Hz for 2, 4 and 24 h (Fig. 8). Reconstitution of mechanical activity for 4 h activated p38 MAP-kinase (Fig. 9). In addition, it raised protein synthesis in a p38 MAP-kinase-dependent way (Fig. 9). To provide evidence that mechanical contraction rather than electrical stimulation is responsible for the observed p38-dependent increase in protein synthesis, the interactions of actin and myosin were inhibited by pre-incubation of cells with BDM (20 mM). In the presence of BDM, electrical pacing no longer caused an increase in protein synthesis (Fig. 10). BDM alone decreased protein synthesis by 21±6% ($p<0.05$) in non-stimulated cardiomyocytes.

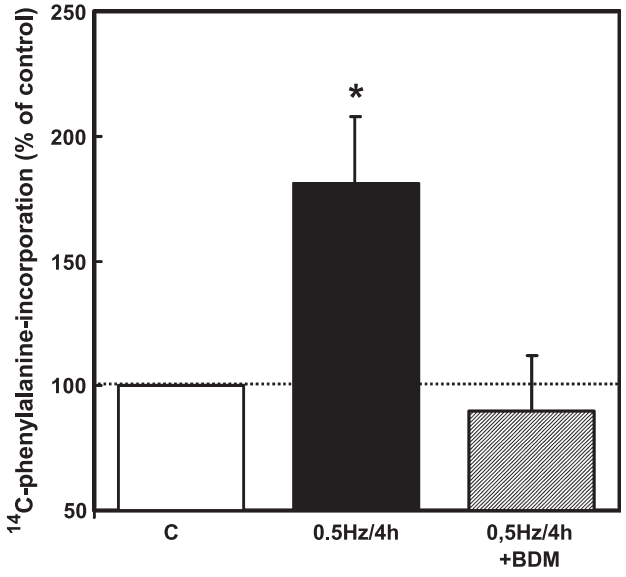

Fig. 10. Influence of BDM (20 mM) on protein synthesis of cardiomyocytes under electrical stimulation. Data are means±S.E.M. from $n=5$ cultures, *$p<0.05$ vs. control.

5. Discussion

In the present study we hypothesized that cultivation of cardiomyocytes in the presence of active TGF-β changes the expression of key signalling molecules involved in the regulation of protein synthesis. This hypothesis was based on our previous findings, that cardiomyocytes pre-cultured with TGF-β develop hypertrophic responsiveness to β-adrenoceptor stimulation that cannot be found in newly isolated cells [8]. The main finding of our study is that under these conditions p38 MAP-kinase expression raises significantly. Upregulation of p38 MAP-kinase has not been investigated in cardiomyocytes under specific conditions before. However, a recent report has shown that mechanical unloading of the heart in patients with a left ventricular assist device increased p38 MAP-kinase expression [21]. This finding supports our report on isolated cells, as these cells do not beat spontaneously. Thus, they have, by virtue of this lack of mechanical activity, a certain type of mechanical unloading. In contrast, the expression value of key signalling molecules involved in the hypertrophic response to other hypertrophic stimuli, i.e. that of α-adrenoceptor stimulation, did not change. In agreement with these observations we did not find any differences in the amount of α-adrenoceptor stimulation or the signalling cascades by which α-adrenoceptor stimulation increases protein synthesis in adult cardiomyocytes. The upregulation of p38 MAP-kinase prompted us to investigate more specifically the role of p38 MAP-kinase activation in β-adrenoceptor-dependent stimulation of protein synthesis.

Data from the present study clearly demonstrates that p38 MAP-kinase activation is permissive for the hypertrophic responsiveness of cardiomyocytes to β-adrenoceptor stimulation. Thus, our study confirms data on mouse cardiomyocytes that also reported that p38 MAP-kinase is permissive but not necessary for the induction of hypertrophy in case of β-adrenoceptor stimulation [18]. It is also clear that p38 MAP-kinase activation is not a prerequisite for induction of hypertrophic responsiveness, as α-adrenoceptor-dependent stimulation is independent from p38 MAP-kinase activation. In fact, signalling molecules of the hypertrophic pathway which are either specifically involved in the PKC-dependent pathway induced by α-adrenoceptor stimulation or which are common for α- or β-adrenoceptor-dependent induction of protein synthesis are not changed during cultivation. This includes different PKC isoforms, the p42/44 MAP-kinase, and PKB. In line with these observations, we could not find any differences in the signalling pathway linked to α-adrenoceptor stimulation in cultured versus freshly isolated cells in several previous studies. The finding that phenylephrine neither activated p38 MAP-kinase nor increased protein synthesis in a p38 MAP-kinase-dependent way is in agreement with these other reports [22,23].

The finding that β-adrenoceptor-dependent stimulation of hypertrophy is dependant on p38 MAP-kinase activation,

is new. Although the upregulation of this kinase during cultivation in parallel with the development of hypertrophic responsiveness to β-adrenoceptor stimulation strongly suggested that the induction of hypertrophic responsiveness to β-adrenoceptor stimulation by TGF-β is mediated by an increased expression induced by the cytokine, our further experiments in this study demonstrated that this is not the case. This is indicated by the following results: First, the upregulation of p38 MAP-kinase is independent from TGF-β, as a neutralizing antibody directed against TGF-β did not prevent upregulation. The same type of antibody has been shown previously to inhibit induction of hypertrophic responsiveness [8]. Second, β-adrenoceptor stimulation activates p38 MAP-kinase in both, freshly isolated cells and pre-treated cells. Third, hypertrophic stimuli generated by electrical stimulation are sufficient to induce hypertrophic stimulation via p38 MAP-kinase activation. All these experiments indicate that the expression level of p38 MAP-kinase in newly isolated cells is not too low for induction of hypertrophic responsiveness. Our study also confirmed earlier studies on neonatal cardiomyocytes that had already shown that electrical pacing induced p38 MAP-kinase phosphorylation [24]. We conclude that β-adrenoceptor-dependent hypertrophy requires co-activation of other factors in addition to p38 MAP-kinase. Similar, morphological changes of cardiomyocytes caused by IL-1 were found to require co-activation of p42/p42 MAP-kinase and p38 MAP-kinase, indicating that p38 MAP-kinase exerts some of its effects in concert with other factors [25]. Therefore, although the present study has identified p38 MAP-kinase activation as a new signalling step specific for β-adrenoceptor-dependent hypertrophy, this step is located up-stream of the TGF-β induced changes in hypertrophic signalling molecules.

The question of whether p38 MAP-kinase activation is part of the hypertrophic response of the heart is still a matter for debate. Recent studies on transgenic mice with cardiac specific expression of dominant negative forms of p38 MAP-kinases have shown that p38 MAP-kinase activation is not necessary to trigger hypertrophy in response to pressure overload [26]. On the other hand, mechanical stretch-induced increase in protein synthesis was suppressed by p38 MAP-kinase inhibition on cardiomyocytes from neonatal and adult rats [27–29]. Our in vitro data on reconstitution of mechanical activity strongly suggest that p38 MAP-kinase is part of a direct mechanical transduction of hypertrophic responses. It seems not to be involved in many cases of receptor-dependent hypertrophic signals, i.e. α-adrenoceptor stimulation (this study and Refs. [18] and [23]), endothelin [30], angiotensin II (this study and Ref. [29]), cocaine [31] or leukemic inhibitory factor [25].

In addition to the adrenoceptor-dependent activation of p38 MAP kinase, this stress activated kinase was also activated by mechanical stress of the cardiomyocytes. This was performed in our experiments either by hyperosmolarity or, alternatively, by reconstitution of mechanical activity by electrical pacing. As the latter approach does not allow us to differentiate between electrical activation and mechanical activation we repeated these experiments in the presence of butanedione monoxime. Using the same approach Kubisch and colleagues, demonstrated that induction of immediate-early response genes linked to cardiac hypertrophy, i.e. egr-1, are induced by reconstitution of pacing induced activity. As shown in these experiments, butanedione monoxime did not alter membrane potential [32]. However, the use of butanedione monoxime may interfere with calcium currents and phosphatases as well, which raises the question of whether BDM differentiates between electrical stimulation and mechanical stimulation [33]. However, our conclusion that mechanical stress activates p38 MAP kinase phosphorylation is based on the combined use of hyperosmolarity and electrical reconstitution.

In summary, this study identifies p38 MAP-kinase activation as a crucial step in the hypertrophic response of rat ventricular cardiomyocytes to β-adrenoceptor stimulation. Since this type of hypertrophy mimics characteristic features of the hypertensive heart at the transition to heart failure, this suggests that the above mentioned step is critical in the transition from compensated hypertrophy to decompensated hypertrophy. It is in line with this suggestion that hypertensive end-organ damage and premature mortality in a rat model of cardiac hypertrophy was found to depend on p38 MAP-kinase activation [34].

Acknowledgements

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CARDIOVASCULAR SYSTEM

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No correlation between the p38 MAPK pathway and the contractile dysfunction in diabetic cardiomyocytes

Hyperglycaemia-induced signalling and contractile function

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Abstract Besides the classical cardiovascular diseases, high levels of blood glucose directly interfere with cardiomyocytes. The mechanisms responsible for this have not yet been explored in detail. This study aims to determine if hyperglycaemia has any impact on prominent signalling molecules and on the contractile function of cardiomyocytes. Freshly isolated cardiomyocytes from adult rats were treated with various concentrations of glucose. Formed free radicals were measured by DCF-fluorescence. TGFβ expression and p38 MAP-kinase (MAPK) activation were measured by Western blotting. The contractile efficiency was determined by measurement of the maximal amount of cell shortening. Glucose (30 mM) caused an increase in formation of radicals, phosphorylation of p38 MAPK, and TGFβ expression. Under conditions of low viscosity (1 cp), contractile responses to hyperglycaemia (15 mM) were not altered in contrast to control. However, enhancement of viscosity (400 cp) effected a limitation of contractile function. The responsiveness to β-adrenoceptor stimulation did not change. Neither inhibition of p38 MAPK with SB 202190 (1 μM) nor inhibition of reactive oxygen species with vitamin C did alter these measured functional parameters. Diabetes mellitus directly influences the activation degree of prominent signalling molecules and the contractile function of adult ventricular cardiomyocytes, which results in facilitating in the development of diabetic cardiomyopathy.

Keywords Reactive oxygen species (ROS) · angiotensin II · TGFβ · Hyperglycaemia · Fractional shortening

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Introduction

In diabetes mellitus, evidence is accumulation that besides the classical pathophysiological changes, such as cardiovascular complications, myocardial dysfunction independent of coronary artery disease occurs [5]. A direct negative influence on the cardiovascular system was detected in patients with type I or II diabetes mellitus [25]. This assumption was confirmed by studies on transgenic mice, diabetic rats, and isolated cardiomyocytes [18, 19]. Hearts from transgenic mice with type I or II diabetes mellitus with reduced glucose utilization showed systolic (reduced left ventricular pressures and decreased cardiac output) as well as diastolic dysfunction (impaired relaxation) [19]. Howarth et al. [9] and Ren et al. [18] induced diabetes in rats by streptozotocin injection. Isolated cardiomyocytes from such hearts showed a significant prolonged myocyte shortening as well as prolonged intracellular Ca^{2+} transients. Chronic exposure of cardiomyocytes from normotensive and non-diabetic rats to high levels of extracellular glucose (25 mM) effected the same myocardial alterations like those found in myocytes isolated from diabetic animals. In diabetic patients, peak values of blood glucose of 400–500 mg/dl were measured. A concentration of 400 mg/dl corresponds to 22.22 mM glucose. In cultured cells, the effects induced by high levels of glucose are not caused by the hyperosmotic impact of high amounts of glucose, because high concentrations of non-metabolizable sugars such as l-glucose or mannitol did not affect contractile dysfunction [18].

High blood glucose has a direct impact on the activation degree of intracellular signalling molecules. Wilmer et al. [28] showed that high levels of extracellular glucose directly activate ERK and the p38 MAPK in human mesangial cells. The hyperglycaemia-induced p38 MAPK activation could not be inhibited by the application of antioxidants or diphenylium iodide. In contrast, in microvascular smooth muscle cells, a hy-

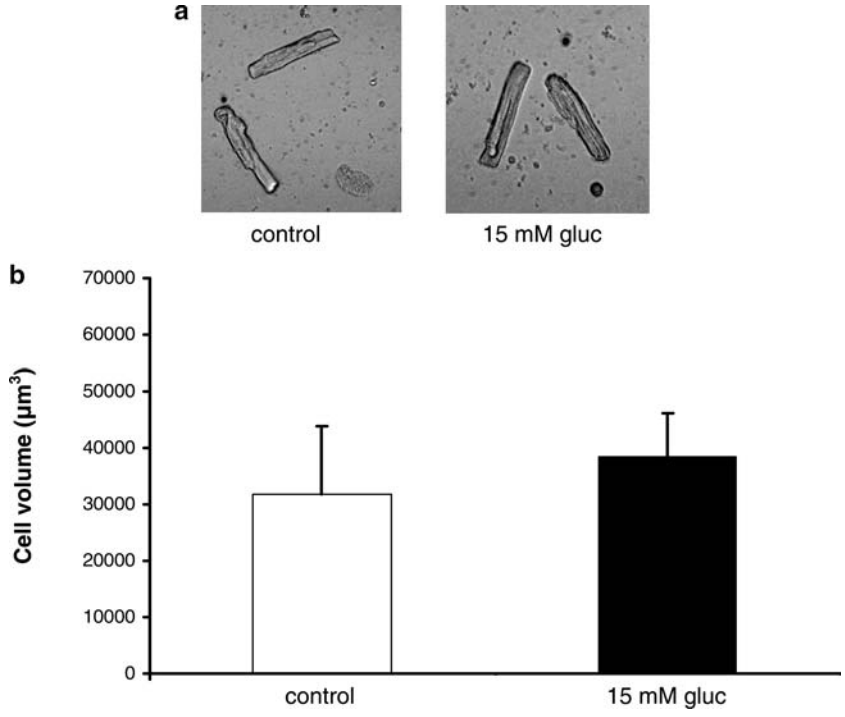

Fig. 1 Hyperglycaemia and cell morphology. **a** Representative pictures of control and hyperglycaemic cardiomycoytes. There was no influence of 15 mM glucose for 24 h on cell volume and morphology. This figure shows representative pictures of cardiomyocytes under control conditions or after glucose treatment.**b** Statistical analysis. The results are displayed as a *bar chart*. Again, no differences in cell volume after glucose induction for 24 h were observed. Data are means ± SEM from $n = 45$ different cells of four different preparations

perglycaemia-induced, redox-sensitive p38 MAPK activation was demonstrated [11]. Hyperglycaemia also activated p38 MAPK in a redox-sensitive manner in endothelial cells. An association of an increased generation of ROS and contractile dysfunction of cardiomocytes was shown [30]. Moreover, an association of the angiotensin II pathway and diabetes in cardiomyocytes was analysed. Privratsky et al. [15] demonstrated that the blockade of the angiotensin II type I-receptor prevented hyperglycaemia-induced contractile dysfunction. This pathway includes an NAD(P)H-oxidase activation and generation of reactive oxygen species (ROS). Nevertheless, the direct influence of hyperglycaemia on adult ventricular cardiomyocytes is not so well understood. We therefore used an insulin-free, in vitro model of isolated adult myocytes and exposed them to pathophysiological amounts of extracellular glucose to exclude vascular- and insulin-induced effects. The aim of the present study was to determine the direct hyperglycaemic effects on

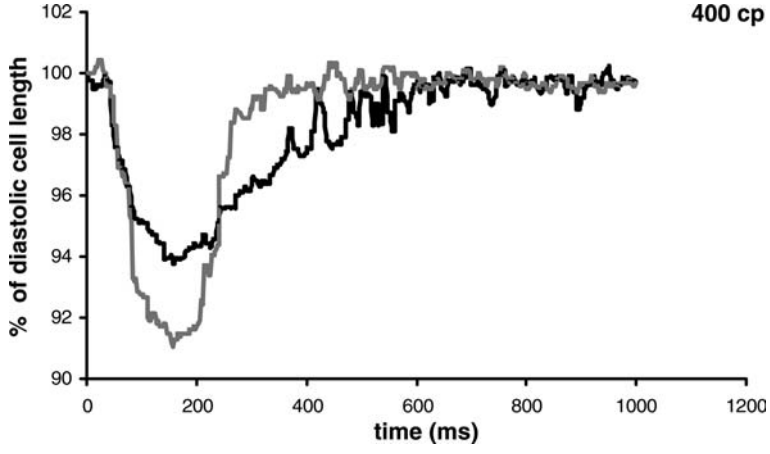

Fig. 2 Representative single-cell measurement. This is a section of 1 min of the measurement of contractile function. At a frequency of 1 Hz and a viscosity of 400 cp, pre-incubation of cardiomyocytes (15 mM glucose, 24 h) reduced cell shortening. The *black line* characterizes the contractile dysfunction of hyperglycaemic cardiomyocytes. The *grey line* is the control line

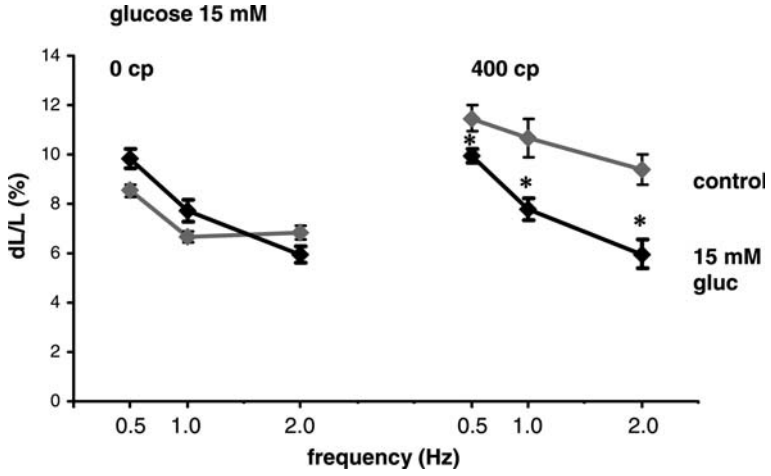

Fig. 3 Statistical analysis of cell shortening after hyperglycaemia. As a measure of contractile function, this figure shows the influence of high levels of extracellular glucose on cell shortening. Chronic exposure of cardiomyocytes to 15 mM of glucose for 24 h under load-free conditions was not able to reduce contractile function. The presence of a viscous load (400 cp) reduced contractile function of hyperglycaemic cardiomyocytes at all examined frequencies. Data are means ± SEM from $n = 35$ different cells of six different preparations; * $P < 0.05$ vs control

contractile functions and important intracellular signalling pathways in myocytes. The signalling molecules investigated in this study are restricted to those intracellular molecules found in the angiotensin II-induced pathway in adult ventricular cardiomocytes because there is some evidence that these molecules are also involved in the hyperglycaemic pathway [21, 22, 23].

Methods

All animal studies were performed in accordance with guidelines described in the NIH Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH, publication no. 85–23, revised 1996).

Cell isolation, short term

Ventricular cardiomyocytes were isolated from 200–250 g male Wistar rats, suspended in basal culture medium, and plated on 60 mm culture dishes. The culture dishes had been pre-incubated overnight with 4% (v/v) foetal calf serum (FCS) in medium 199. The basal culture medium (CCT) was modified medium 199 including Earl's salts, 2 mM L-carnitine, 5 mM taurine, 100 IU/ml penicillin and 100 µg/ml streptomycin. To

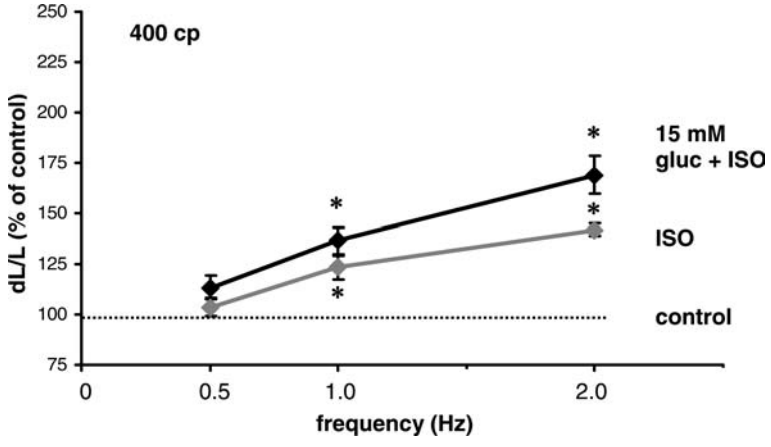

Fig. 4 Responsiveness to β-adrenoceptor stimulation. In order to test the influence of chronic hyperglycaemia on the β-adrenoceptor-mediated improvement of contractile function, cells were chronically treated with 15 mM glucose (24 h) and additionally stimulated with the β-adrenoceptor agonist isoprenaline (ISO 100 nM, 5 min prior to the beginning of the measurement). Chronic exposure to 15 mM glucose did not alter the positive effect of β-adrenoceptor stimulation on contractile function. Data are means ± SEM from $n = 30$ different cells of five different preparations; * $P < 0.05$ vs control

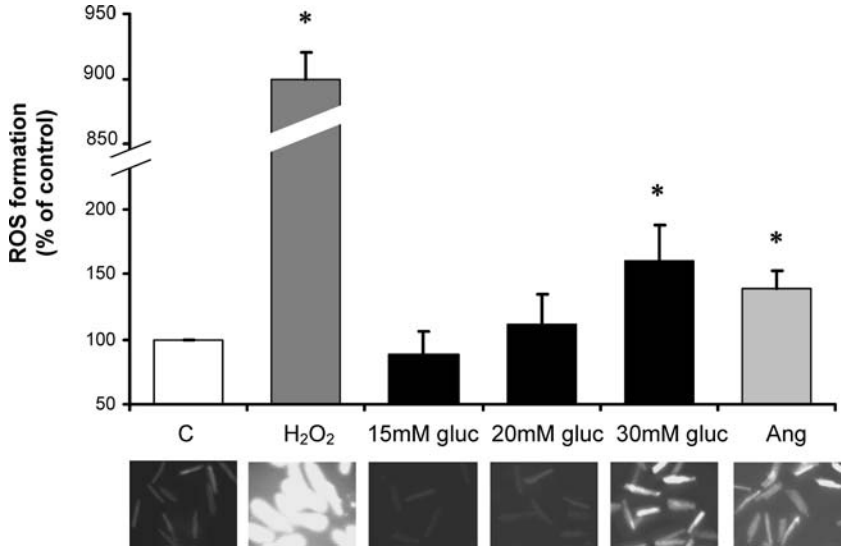

Fig. 5 Hyperglycaemia and ROS formation. Effect of hyperglycaemia on ROS formation. Production of radicals was measured 15 min under untreated test conditions. Additionally, hyperglycaemia-induced (glucose concentration: 15, 20, and 30 mM) formation of radicals was determined for 30 min. Only hyperglycaemia caused with 30 mM glucose was able to increase formation of radicals significantly. For comparison, hydrogen peroxide (H_2O_2; 1 μM)-induced ROS formation is shown. Beneath the *bar chart*, representative fluorescence pictures of ROS formation after 30 min of treatment with respective substances and concentrations are shown. Data are means ± SEM from $n = 50$ different cells of eight different preparations; * $P < 0.05$ vs control. Angiotensin II (Angi, 100 nM) is also given as a positive control

prevent growth of nonmyocytes, media were also supplemented with 10 μM cytosine-β-D-arabinofuranoside (pH 7.4).

Four hours after plating, cultures were washed twice with CCT medium. As a result of the medium change, broken cells were removed, yielding cultures of 93 ± 2% quiescent, rod-shaped cells. These cells were stimulated directly.

Determination of cell volume and morphology

Myocyte growth was determined on phase-contrast micrographs recorded on tape using a CCD-video camera. Cell volumes were calculated by the following formula: volume = $(\text{radius})^2 * \pi *$ length, assuming a cylindrical cell shape.

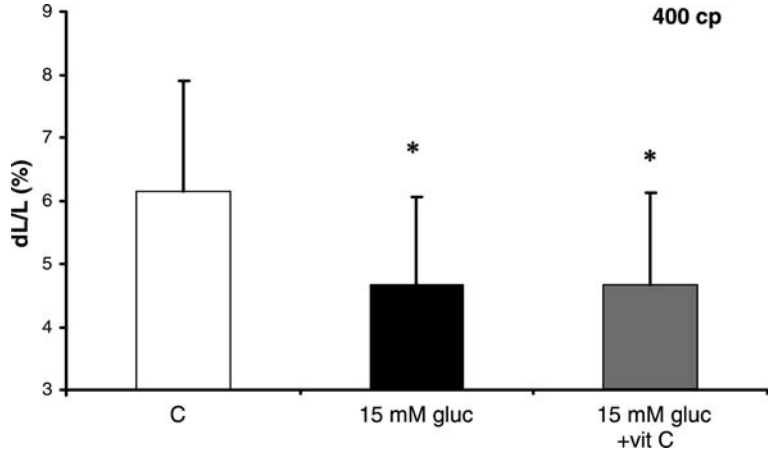

Fig. 6 No correlation between ROS and contractile dysfunction. Effect of scavenging free radicals with antioxidants on contractile dysfunction. In order to examine the correlation between contractile dysfunction and ROS formation, cells were incubated with a glucose concentration of 15 mM alone or in the presence of the antioxidant vitamin C (*vit C*, 10 μM) for 24 h. Scavenging ROS did not prevent hyperglycaemia-induced contractile dysfunction. Data are means ± SEM from $n = 30$ different cells of five different preparations;* $P < 0.05$ vs control

SDS-gel electrophoresis

Protein extracts from adult ventricular cardiomyocytes for SDS-gel electrophoresis were prepared as described before [20]. Protein extracts (100 μg) were loaded on a 12% (w/v) SDS-polyacrylamide gel (acrylamide : bis-acrylamide 30 : 1). After electrophoresis, proteins were transferred onto reinforced nitrocellulose membranes by semidry blotting. The membranes were saturated with 2% (w/v) bovine serum albumin and incubated for 2 h with rabbit polyclonal anti-rat anti-TGFβ antibodies. After washing, the membranes were re-incubated with an alkaline phosphatase-labelled goat anti-rabbit IgG. Finally, bands were visualized by alkaline phosphatase activity by using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium. p38 MAPK activation was investigated by comparing the amount of phosphorylated p38 MAPK to the total amount of p38 MAPK. Therefore, two blots were performed and two different first antibodies were used: first, a rabbit polyclonal anti-rat p38 MAPK and, second, a polyclonal anti-rat phosphorylated p38 MAPK antibody (0.2 μg/ml, RBI). As a p38 MAPK inhibitor, SB202190 (1 μM) was used based on the concentration-response curve described earlier [27].

Measurement of ROS

ROS generation in cells was assessed using the probe 2,7-dichlorofluorescein diacetate (H_2DCF-DA) (Molecular Probes). This membrane-permeable diacetate form of the dye was added to the culture medium at a final concentration of 10 μM. Within the cell, esterases cleave the acetate groups on DCFH-diacetate, thus trapping the reduced probe (DCFH) intracellularly. ROS in the cells oxidize DCFH, yielding the fluorescent product DCF. Our previous studies of the behaviour of DCFH in cardiomyocytes revealed that the probe is readily oxidized by H_2O_2 or hydroxyl radical, but is relatively

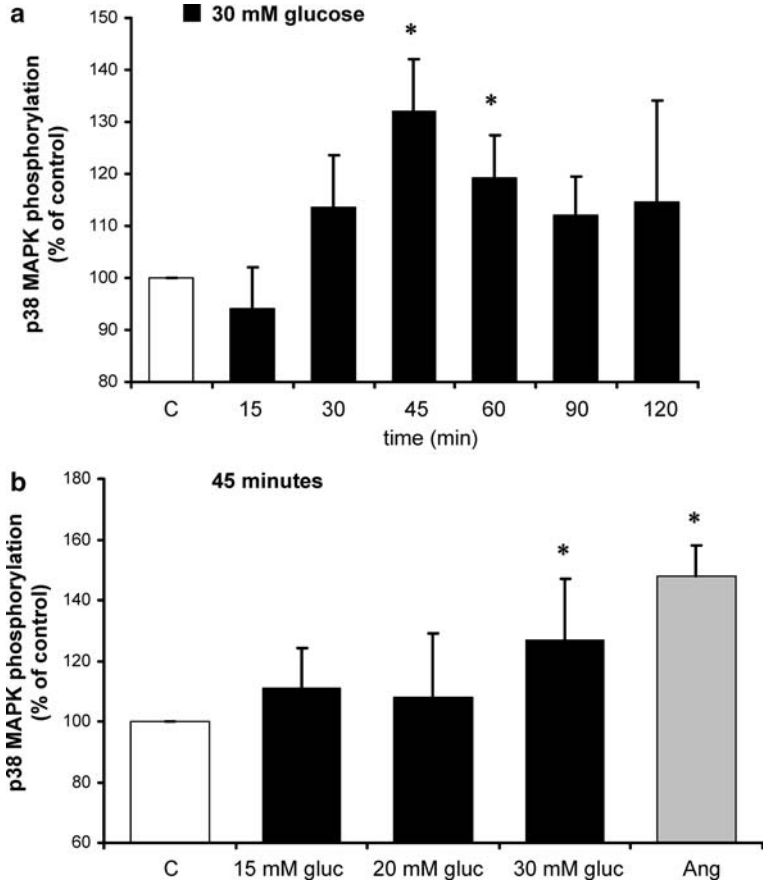

Fig. 7 Hyperglycaemia and p38 MAPK. **a** Time-dependent p38 MAPK phosphorylation. Effects of hyperglycaemia on p38 MAPK phosphorylation. Cells were incubated with a glucose concentration of 30 mM for 15, 30, 45, 60, 90, and 120 min. There is a transient increase in phosphorylation of MAPK shown with a maximum after 45 min of glucose incubation. Data are means ± SEM from $n = 6$ cultures; * $P < 0.05$ vs control. **b** Concentration-dependent p38 MAPK phosphorylation. Effect of hyperglycaemia on p38 MAPK phosphorylation. Cells were incubated with different concentrations of glucose (15, 20, and 30 mM) for 45 min. p38 MAPK activation was determined by the ratio of phosphorylated to non-phosphorylated p38 MAPK. Only 30 mM glucose was able to increase p38 MAPK phosphorylation significantly. Fifteen and 20 mM of glucose had no effects. Data are means ± SEM from $n = 5$ cultures; * $P < 0.05$ vs control. Angiotensin II (Angi, 100 nM) was additionally given as control

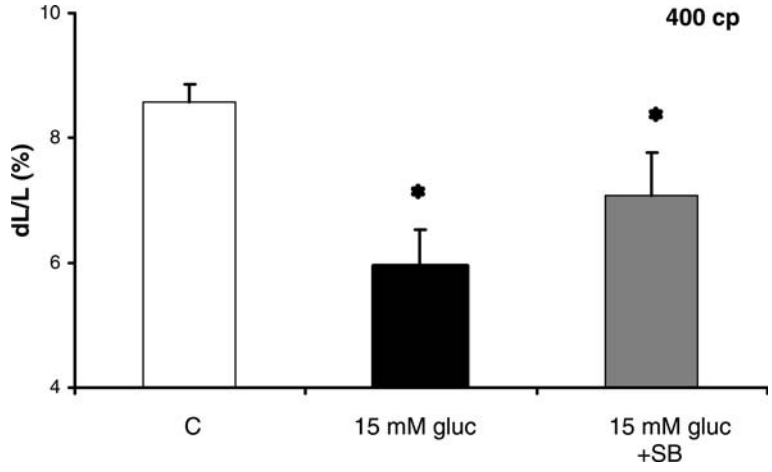

Fig. 8 No correlation between p38 MAPK phosphorylation and contractile dysfunction. Effect of p38 MAPK inhibition on contractile dysfunction. In order to examine the correlation between contractile dysfunction and p38 MAPK phosphorylation, cells were incubated with a glucose concentration of 15 mM alone or in the presence of the p38 MAPK inhibitor SB202190 (*SB*, 1 μM) for 24 h. p38 MAPK inhibition did not prevent hyperglycaemia-induced contractile dysfunction. Data are means ± SEM from $n = 30$ different cells of five different preparations;* $P < 0.05$ vs control

insensitive to superoxide [2, 24]. Fluorescence was measured using an excitation wavelength of 485 nm. Fluorescence intensity was assessed in several (five to nine) myocytes per measurement, and background fluorescence was identified as an area without cells. Intensity values are reported as the percentage of the increase in fluorescence during a first period with no stimulation compared with a second period where glucose or angiotensin II was added. This percentage then was normalized to control measurements.

Determination of cell contraction

Cells were allowed to contract at room temperature and analysed using a cell-edge detection system. Cells were stimulated via two AgCl electrodes with biphasic electrical stimuli composed of two equal but opposite rectangular 50-V stimuli of 0.5 ms duration. Each cell was stimulated at 0.5, 1, and 2 Hz for 1 min. Every 15 s, the next five contractions were averaged. The mean of these four measurements at a given frequency was used to define the contractility of a given cell. Cell lengths were measured at a rate of 500 Hz via a line camera. As parameters of contractile function, fractional shortening, velocity of relaxation, and velocity of contraction were measured.

Statistics

Data are given as means ± SEM from '*n*' different culture preparations. Statistical comparisons were performed by one-way analysis of variance and use of the Student-Newman–Keuls test for post hoc analysis. In cases in which two groups were compared, conventional *t*-tests were performed. Differences with $P < 0.05$ were regarded as statistically significant. All data analyses were performed using SPSS version 11.5 for Windows.

Results

Morphology of hyperglycaemic cardiomyocytes in contrast to control cardiomyocytes

In order to exclude a change in morphology of hyperglycaemic cardiomyocytes, phase-contrast micrographs of the cells were recorded on tape, using a CCD-video camera. Cell volume and morphology of cardiomyocytes was examined. Hyperglycaemic cardiomyocytes did not show any differences concerning cell volume and morphology in comparison to control cells (Fig. 1a, b).

Hyperglycaemia and contractile function

Contractile function was measured as percent cell shortening normalized to diastolic cell length (dl/l). Cells were paced at a frequency of 0.5, 1.0, or 2.0 Hz and experiments were performed under load-free conditions (viscosity of 1 cp) or under viscous loading (400 cp). Cells were stimulated with 15 or 30 mM glucose for 24 h. Chronic hyperglycaemia without heightened viscosity had no effect on contractile function at a glucose concentration of 15 or 30 mM. Enhancement of viscosity degraded cell shortening at a frequency of 0.5, 1.0, or 2.0 Hz. Glucose increased the loss of contractile function to a maximal amount at 15 mM. There was no augmentation possible with higher concentrations of added glucose (30 mM, data not shown). In Fig. 2, a representative individual measurement is displayed. Figure 3 shows the statistical analysis of the influence of hyperglycaemia on cell shortening.

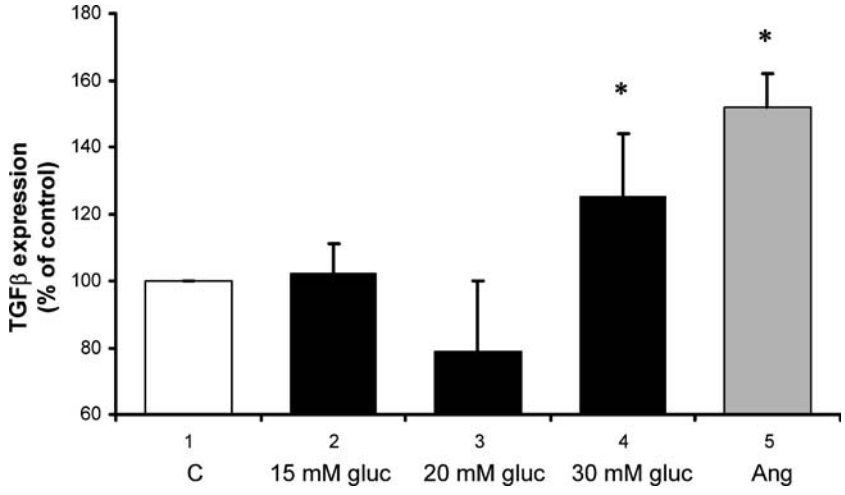

Fig. 9 Hyperglycaemia and TGFβ expression. Effect of hyperglycaemia on TGFβ expression. Cells were incubated with different concentrations of glucose (15, 20, and 30 mM) for 24 h. TGFβ expression was determined by Western blotting. The values were normalized to actin values. Only 30 mM glucose was able to induce TGFβ expression. Data are means ± SEM from $n = 4$ cultures, * $P < 0.05$ vs control. Angiotensin II (Angi, 100 nM) was additionally given as control

Effects of hyperglycaemia on the responsiveness to β-adrenoceptor stimulation

β-Adrenoceptors are the most abundant adrenoceptors of the heart. They are important for the regulation of sympathetic responses such as inotropy, chronotropy, dromotropy, and lusitropy. In order to test the influence of hyperglycaemia on the ability to increase contractile function by β-adrenoceptor stimulation, cells were exposed to hyperglycaemia for 24 h, and subsequently, isoprenaline (100 nM) was added. Isoprenaline, a selective β-adrenoceptor agonist, usually increases contractile function. Chronic hyperglycaemia (15 mM, 24 h) did not affect this positive contractile effect at a viscosity of 400 cp. In Fig. 4 the statistical analysis of cell shortening is displayed.

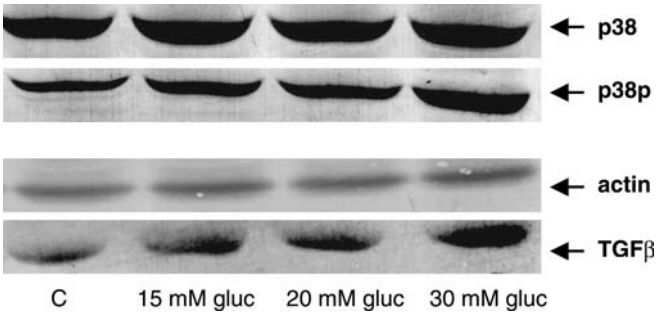

Fig. 10 Hyperglycaemia and signalling pathways. Representative immunoblots from protein samples of cardiomyocytes. Cardiomyocytes were cultured up to 45 min in the case of p38 MAPK phosphorylation or up to 24 h in the case of TGFβ expression in the presence of different concentrations of glucose. Both p38 MAPK phosphorylation and TGFβ expression were increased by 30 mM glucose. Lower glucose concentrations (15, 20 mM) had no effect on both parameters. In the case of p38 MAPK, the phospho-specific p38 MAPK (p38p) values were normalized to the total expression of p38 MAPK (p38). TGFβ values were normalized to actin values

Effects of hyperglycaemia on ROS formation

A concentration–response curve with 15, 20, and 30 mM glucose was created to measure ROS formation. A maximum in generation of radicals was examined at a concentration of 30 mM (Fig. 5). Hyperglycaemia (30 mM) caused a significant increase in formation of radicals measured by H_2DCF fluorescence in a time period of 30 min. Glucose was added to the culture medium after a period of 15 min. The angiotensin II-induced effect on the formation of radicals was observed as control.

Role of ROS on contractile function

As described before, hyperglycaemia is able to increase formation of ROS significantly. In order to investigate the relationship between ROS and contractile dysfunction, free radicals were scavenged with the antioxidant vitamin C (vit C, 10 μM), and hyperglycaemia-induced contractile function was determined (Fig. 6). As contractile dysfunction was strongly affected at the frequency of 2 Hz, we electrically paced cells at this frequency and existing viscosity of 400 cp. Cells were incubated with 15 mM glucose alone or in the presence of vitamin C for 24 h. There was no improvement of contractile function as measured by cell shortening compared to those cells not simultaneously incubated with vitamin C (Fig. 6). Vitamin C alone had no effect on contractile function (data not shown).

Effects of hyperglycaemia on p38 MAPK phosphorylation

Extracelullar glucose at 30 mM increased activation of p38 MAPK significantly in contrast to a physiological glucose concentration of 5 mM. After stimulation with glucose, cells were harvested in a time-dependent manner (15, 30, 45, 60, 90, and 120 min), and the degree of phosphorylation was examined (Fig. 7a). Phosphorylation of p38 MAPK significantly increased by about 30% after 45 min of glucose incubation and was normalized to the total amount of p38 MAPK protein. Glucose at 15 and 20 mM did not increase phosphorylation of p38 MAPK (Figs. 7b and 10). The angiotensin II-induced phosphorylation of p38 MAPK is also shown as a positive control.

Role of p38 MAPK on contractile function

As described before, hyperglycaemia is able to increase phosphorylation of p38 MAPK significantly. In order to assess the relationship between p38 MAPK and contractile dysfunction, this kinase was inhibited with SB202190 (SB 1 μM) and additionally contractile function was determined. As contractile dysfunction was strongly affected at the frequency of 2 Hz, we electrically paced cells at this frequency and existing viscosity of 400 cp. Cells were incubated with 15 mM glucose in the prescence of SB202190 for 24 h. There was no improvement of contractile function measured as cell shortening compared to those cells not simultaneous stimulated with SB (Fig. 8). SB incubation alone had no effect on contractile function (data not shown).

Effects of hyperglycaemia on TGFβ expression

High glucose levels (30 mM) were able to augment TGFβ expression significantly. TGFβ was measured at the protein level by western blotting. Values were normalized to actin values. Protein levels were measured after a hyperglycaemia incubation of 24 h (Figs. 9 and 10). Lower glucose concentrations (15 and 20 mM) did not raise TGFβ expression. The angiotensin II-induced increase in TGFβ expression was measured as a positive control.

Discussion

The Framingham study clearly shows that people with diabetes mellitus develop heart failure more often than non-diabetic patients. These patients suffer from cardiovascular diseases, neuropathies, diastolic dysfunction, and cardiomyopathies [12].

Hyperglycaemia and contractile function

One of the basic definitions of cardiomyopathy is contractile dysfunction [3, 7, 10]. Many in vivo as well as in vitro studies have dealt with the question of whether high levels of blood glucose have direct effects on tissues, aside from the classical atherosclerotic changes and independent of effects that were caused by insulin [6]. Diabetes in vivo as well as hyperglycaemia in vitro have an influence on contractile function of the heart or isolated cardiomyocytes. Dyntar et al. [4] reported that hyperglycaemia had a dramatic destructive effect on the myofibrillar apparatus in cardiomyocytes. Isolated cardiomyocytes from streptozocin-treated diabetic rats showed a strong contractile dysfunction [17]. Wold et al. [29] analysed the direct effect of streptozotocin injection on contractile parameters and signalling molecules. They found that the injection of this compound alone alters contractile function via a p38 MAPK- and redox-sensitive signalling pathway. To exclude such influences and in vivo regulatory mechanisms, the present study used the model of isolated myocytes from non-diabetic rats and simulated diabetes by addition of glucose to the culture medium. Several experiments excluded the hyperosmotic effect of high glucose concentrations. We found that even low concentrations of extracellular glucose alter contractile function of single cardiomyocytes isolated from non-diabetic rats. No further increase in contractile dysfunction was elicited by higher glucose concentrations (30 mM). In our cellular model of hyperglycaemia, a glucose concentration of 15 mM for 24 h did not impair contractile function when the culture medium had low viscosity. However, if viscosity was increased to 400 cp—and therefore the force that myocytes had to apply—hyperglycaemic cardiomyocytes (15 and 30 mM glucose) reduced their amount of fractional shortening significantly at all examined frequencies of beating. Kent et al. [13] discussed the effects of high viscosity on the assignment of cardiomyocytes. With this model, the in vivo situation could be imitated more correctly. Hyperglycaemia also reduced the time of contraction significantly at a frequency of 2 Hz and a viscosity of 400 cp. The time of relaxation was not influenced, which indicates a primary contractile dysfunction (data not shown).

In order to additionally assess the functionality of hyperglycaemic cardiomyocytes besides the mentioned contractile parameters, the β-adrenoceptor-mediated responsiveness was measured. Hyperglycaemia for 24 h did not alter the positive inotropic effect of β-adrenoceptor stimulation. In contrast, in mouse myocytes diabetes impaired β-adrenoceptor-mediated responsiveness in contractile function [14].

Hyperglycaemia and signalling pathways

Studies performed in vivo or in vitro highlighted some signalling steps, which become activated by diabetes or high blood glucose levels independent of the osmotic influence of glucose. As Privratsky et al. [15] demonstrated that the angiotensin II-induced signalling pathway could be influenced by hyperglycaemia in

ventricular cardiomyocytes, it becomes a matter of particular interest to identify important intracellular molecules directly influenced by hyperglycaemia. Former studies of our laboratory [26] identified ROS, the p38 MAPK, and TGFβ as important molecules in the angiotensin II-induced signalling pathway in adult myocytes. The present study dealt with the question whether they were also affected by high levels of extracellular glucose as it is found in diabetes. We demonstrated for the first time that all molecules analysed are activated in the same time-dependent manner as in the angiotensin II-induced pathway. Glucose at 30 mM is able to increase the formation of ROS, the phosphorylation of p38 MAPK, and the expression of TGFβ. Lower glucose concentrations were not able to influence the activation degree of the molecules examined in a significant way, despite their effect on contractile responsiveness. As Privrastky et al. [15] reported that blockade of the AT1-receptor prevented hyperglycaemia-induced contractile dysfunction, the question arises whether these intracellular molecules have any relationship to the concomitant contractile dysfunction. Neither addition of a radical scavenger (vitamin C) nor inhibition of p38 MAPK with SB202190 prevented hyperglycaemia-induced loss of contractile function. These results suggested that the intracellular signalling molecules examined in this study are not involved to the contractile dysfunction. Additionally, our results confirmed the findings that, on the one hand, hyperglycaemia interferes with angiotensin II signalling (the same intracellular signalling molecules in the same time frame), and, on the other hand, that hyperglycaemia has a direct negative effect on heart function at the single-cell level [15, 16]. The fact that it is necessary to add high glucose concentrations to the culture medium in order to activate p38 MAPK-dependent pathways but not to induce contractile dysfunction supports the hypothesis that the molecules examined and the contractile dysfunction do not have any connection. Contractile dysfunction was even caused at the lower glucose concentration of 15 mM.

Concluding remarks

In summary, this study helps to clarify the direct effects of high levels of blood glucose, as it is found in diabetic patients, on single cardiomyocytes. This study clearly demonstrates the loss in contractile function after 24 h of hyperglycaemia. Moreover, it shows that glucose is able to affect prominent intracellular signalling molecules that are also activated in the angiotensin II-induced pathway. Some studies have suggested a correlation between high levels of glucose and angiotensin II, and our results support these observations. However molecules such as ROS, p38 MAPK, and TGFβ are not responsible for the contractile dysfunction. Alterations in the Ca^{2+} homeostasis and/or protein kinase C activation [8] might be responsible [1].

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Contribution of PI 3-kinase isoforms to angiotensin II- and α-adrenoceptor-mediated signalling pathways in cardiomyocytes☆

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Abstract

Objective: Angiotensin II stimulation increases the formation of reactive oxygen species (ROS), the phosphorylation of p38 mitogen-activated protein kinase (MAPK), and the expression of transforming growth factor beta (TGFβ) in adult cardiomyocytes. The aim of this study was to determine the involvement of PI 3-kinase and to specify the participation of different isoforms in the angiotensin II-induced formation of ROS in comparison to the hypertrophic pathway triggered by α-adrenoceptor stimulation.
Methods: Freshly isolated myocytes were used to examine formation of ROS via H_2DCF fluorescence. p38 MAPK phosphorylation, $p70^{S6}$-kinase phosphorylation, PI 3-kinase, and TGFβ expression were measured by Western blotting. Sense and antisense oligonucleotides were used to down-regulate diverse PI 3-kinase isoforms. Hypertrophy was measured by ^{14}C-phenylalanine incorporation and cell volume.
Results: Inhibition of PI 3-kinase by Ly294002 or wortmannin, two inhibitors, decreased formation of ROS, phosphorylation of p38 MAPK, and TGFβ expression. Down-regulation of the p110β isoform by antisense oligonucleotides inhibited the angiotensin II-induced signalling pathway but not the α-adrenoceptor-mediated hypertrophic growth of cardiomyocytes. In contrast, down-regulation of the p110α isoform decreased the α-adrenoceptor-mediated hypertrophic growth of cardiomyocytes but did not affect the angiotensin II-mediated signalling pathway.
Conclusion: Thus, our study identifies an involvement of PI 3-kinase in the angiotensin II-induced formation of ROS and provides a biochemical basis for ligand-specific responses for angiotensin II and α-adrenoceptor stimulation as relates to hypertrophy.

Keywords: Reactive oxygen species; p38 MAPK; TGFβ; Antisense oligonucleotides; Hypertrophy

1. Introduction

Cardiac hypertrophy is not only a functionally useful adaptation to an enhanced workload [1], but also one of the most critical clinical complications of cardiovascular disorders [2]. Nevertheless, the cellular mechanisms leading to myocardial hypertrophy are incompletely understood. To date, a number of in vivo animal models have been established to examine the role of an activated renin–angiotensin system in cardiac hypertrophy and heart failure. For example, treatment of stroke-prone spontaneously hypertensive rats with an angiotensin converting enzyme inhibitor (ACE inhibitor) or angiotensin II type1 (AT1 blocker) receptor blocker decreased elevated TGFβ expression [3] and prolonged survival rates, which indicates a dependency between the angiotensin II and TGFβ pathways. These in vivo data have been verified in vitro by the use of isolated adult ventricular cardiomyocytes. Our own data demonstrated that angiotensin II is able to increase TGFβ expression in single cardiomyocytes. Formation of radicals by activation of NAD(P)H oxidase and phosphorylation of p38 mitogen-activated protein kinase (MAPK) seem to be key steps in this

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pathway [4]. Nevertheless, much less is known about the initial steps in this signalling pathway that leads to an activation of NAD(P)H oxidase.

Other cell systems and signalling pathways utilise PI 3-kinase as a signalling molecule for reactive oxygen species (ROS) formation [5]. Furthermore, it is known that angiotensin II directly increases PI 3-kinase activity in embryonic chicken cardiomyocytes [6]. Therefore, we were specifically interested in the question of whether the angiotensin II-dependent formation of ROS is also mediated via induction of the PI 3-kinase pathway in adult ventricular cardiomyocytes. Beyond this, PI 3-kinase participates in many different signalling pathways leading to a multiplicity of end points [7,8]. Important for the aim of this study was the identification of PI 3-kinase as a signalling molecule in another heart-specific signalling pathway that involves coupling between G-proteins and receptors. In adult ventricular cardiomyocytes, stimulation of α-adrenoceptors with phenylephrine leads to PI 3-kinase activation, $p70^{S6}$-kinase phosphorylation, and marked hypertrophic growth of cells [9]. Nevertheless, angiotensin II increases protein synthesis in a less efficient way in adult cardiomyocytes in comparison to phenylephrine stimulation [10]. We hypothesised that an isoform-specificity of PI 3-kinase may be responsible for the ligand-specific differences.

The class I PI 3-kinase isoform is the best known isoform [7]. This class can be subdivided into two groups (Ia and Ib) based on structure. All members possess regulatory (p85, p101) and catalytic (p110) subunits [7]. The p85 regulatory subunit of the class Ia isoform is expressed in cardiomyocytes [11]. Less is known about the expression of the p101 regulatory subunit of the class Ib isoform. All members of the class I isoform have a p110 catalytic subunit [7]. This subunit is classified into α, β, δ, and γ isoforms of p110. For this reason, we dealt with the question of whether there is any difference in requirements for diverse p110 catalytic subunits of PI 3-kinase that could be responsible for the different downstream signalling pathways between angiotensin II and α-adrenoceptor stimulation.

In summary, our study investigated whether the PI 3-kinase pathway is involved in the angiotensin II-mediated formation of radicals and examined the involvement of an idiosyncratic PI 3-kinase isoform in adult ventricular cardiomyocytes.

2. Methods

All animal studies were performed in accordance with guidelines described in the NIH Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH, publication no. 85-23, revised 1996).

2.1. Cell isolation, short-term cultures

Ventricular cardiomyocytes were isolated from 200 to 250 g male Wistar rats, suspended in basal culture medium, and plated on 60 mm culture dishes as described in detail in Ref. [12]. The culture dishes had been pre-incubated overnight with medium 199 containing 4% (*v*/*v*) foetal calf serum (FCS). The basal culture medium (CCT) was modified medium 199 including Earl's salts, 2 mM L-carnitine, 5 mM taurine, 100 IU/ml penicillin and 100 μg/ml streptomycin. To prevent growth of nonmyocytes, media were also supplemented with 10 μM cytosine-β-D-arabinofuranoside (pH 7.4).

Cultures were washed twice with CCT medium after 4 h of plating. As a result of the medium change, broken cells were removed. This resulted in cultures of 93±2% quiescent rod-shaped cells. These cells were stimulated directly.

2.2. SDS-gel electrophoresis

After the particular stimulation, cells were lysed in lysis buffer [composition: 50 mmol/l Tris/HCl, pH 6.7, 2% (*w*/*v*) SDS, 2% (*v*/*v*) mercaptoethanol, and 1 mmol/l sodium orthovanadate]. Afterwards, nucleic acids were digested with benzonase (Merck, Darmstadt, Germany) [13]. Protein extracts (100 μg) were loaded on a 12.5% (*w*/*v*) SDS-gel (acryl amide:bisacryl amide 30:1). After electrophoresis, proteins were transferred onto reinforced nitrocellulose by semi-dry blotting. The sheets were saturated with 2% (*w*/*v*) bovine serum albumin and incubated for 2 h with the diverse polyclonal primary antibodies. After washing, the sheets were re-incubated with an alkaline phosphatase-labelled secondary antibody. Finally, bands were visualised by alkaline phosphatase activity using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium. p38 MAPK activation as well as $p70^{S6}$-kinase phosphorylation were determined by a ratio of the amount of phosphorylated protein to the total amount of that protein. Therefore, two blots were performed and two different primary antibodies were used.

2.3. Measurement of reactive oxygen species (ROS)

ROS generation in cells was assessed using the probe 2,7-dichlorofluorescein (DCF) (Molecular Probes). The membrane-permeable diacetate form of the dye (reduced DCF, DCF-DA) was added to the culture medium with a final concentration of 10 μM. Within the cell, esterases cleave the acetate groups on DCFH diacetate, thus trapping the reduced probe (DCFH) intracellularly. ROS in the cells oxidize DCFH, yielding the fluorescent product DCF. Fluorescence intensity was measured in up to ten different cells per preparation, and background was identified as an area without cells. Fluorescence was analysed using a fluorescence microscope combined with a video imaging system (T.I.L.L. Photonics). Cardiomyocytes, cultured on round glass coverslips, were incubated (30 min) in Hepes-buffered medium containing H_2DCF-DA.

2.4. RT-PCR

Total RNA from cardiomyocytes was extracted with RNA-Clean (AGS, Heidelberg, Germany) as described by the manufactures. Reverse transcription reactions were performed for 1 h at 37 °C in a final volume of 10 μl RNA, 100 ng oligo(dT) (Boehringer Mannheim, Germany), 1 mM dNTPs (Gibxo-BRL), 8 U RNAse Block (Promega, Mannheim, Germany), and 60 U M-MLV reverse transcriptase (Gibco-BRL). Aliquots (1.5 μl) of the synthesised cDNA were used for polymerase chain reaction in a final volume of 10 μl, containing 10 μl of 10xPCR-Buffer, 5.8 μl of a. b., 3 μl of primer pairs (100 μM), 0.4 μl dNTPs (10 mM), 0.15 μl $MgCL_2$ (50 mM), 0.5 μl 1% W1, and 0.2 μl Taq-polymerase (Gibco-BLR, 5 U/μl). Amplification was performed under the following cycle conditions: 1 min 93 °C, 1 min 60 °C, and 3 min 72 °C. Oligonucleotide primers had the following sequences: β-actin forward: 5′-GAAGTGTGACGTTGA-CATCCG-3′, reverse: 5′-TGCTGATCCACATCTGCTGG-3′; p110α forward: 5′-GGTGACTGTGTGGGACT TATTG-3′, reverse: 5′-ATGTAGTGTGTGGCTGTTGAAC-3′; p110β forward: 5′-TGTGCCCTCTCCAGATTCC-3′, reverse: 5′-GACAGTATGCCTCTAGGATGAC-3′; p110δ forward: 5′-TTCCACGGCAATGAGATG-3′, reverse: 5′-CTTCTCC-ACGACAGCATAG-3′; p110γ forward: 5′-TCTGGTTCTT-GCGAAGTGAG-3′, reverse: 5′-GCTGCGTGAAGTCCTG-TAG-3′. After amplification, reaction products were separated on a 2% agarose gel, stained with ethidium bromide, and photographed under UV illumination. As a loading control, β-actin was additionally amplified. As a negative control the reaction mixture was run without cDNA. All primers were purchased from Invitrogen.

2.5. Incorporation of ^{14}C-phenylalanine

To measure the rate of protein synthesis, the incorporation of ^{14}C-phenylalanine was determined by exposing cultures to L-^{14}C-phenylalanine (0.1 μCi/ml) for 24 h. Incorporation of radioactivity into acid-insoluble cell mass was determined as described before [14].

2.6. Determination of cell volume

Myocyte growth was determined on phase-contrast micrographs recorded on tape using a CCD-video camera. Cell volume was determined by the following formula: cell volume = $(\text{radius})^2 * \pi *$ length. To calculate radius of cardiomyocytes the widest point of the cells was measured and half of that value was used.

2.7. Antisense experiments

In order to down-regulate the expression of the PI 3-kinase isoforms in adult ventricular cardiomyocytes, cells were incubated for 24 h with 10 μg/ml phosphorothioated antisense or sense oligonucleotides in 20-mer lengths that corresponded to the region of the translation initiation site of the proteins. These are: PI 3-kinase p110α sense: TEF ZEA TGG TCT TGG AEE OFT; antisense: AZE OOT CCA AGA CCA TOF ZOA; PI 3-kinase p110β sense: TZZ EZC AAG TCG ATG OFE OT; antisense: AEO ZEC ATC GAC TTG FOF FA; PI 3-kinase p110γ sense: TFO FZC CGG GAT GCC OZO FT; antisense: AZE FEG GCA TCC CGG FZE ZA; PI 3-kinase p110δ sense: AFF EOA ACA TGG CGG OOF OA; antisense: TEZ EEC CGC CAT GTT EOZ ZT. To assess transfection of cardiomoycytes by oligonucleotides, FITC-labelled oligonucleotides were directly added to the medium and fluorescence was measured by 484 nm (Fig. 1). All oligonucleotides were purchased from Invitrogen.

2.8. Statistics

Data are given as means ± s.e.m. from *n* different culture preparations or single cells. Statistical comparisons were

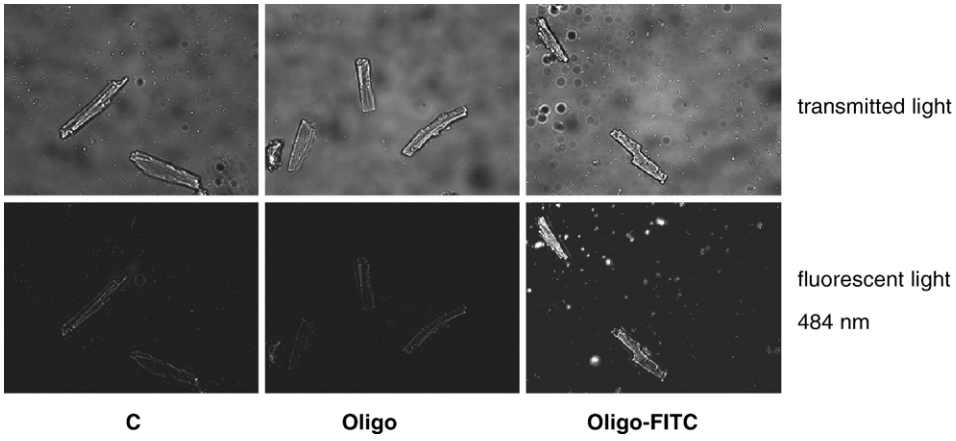
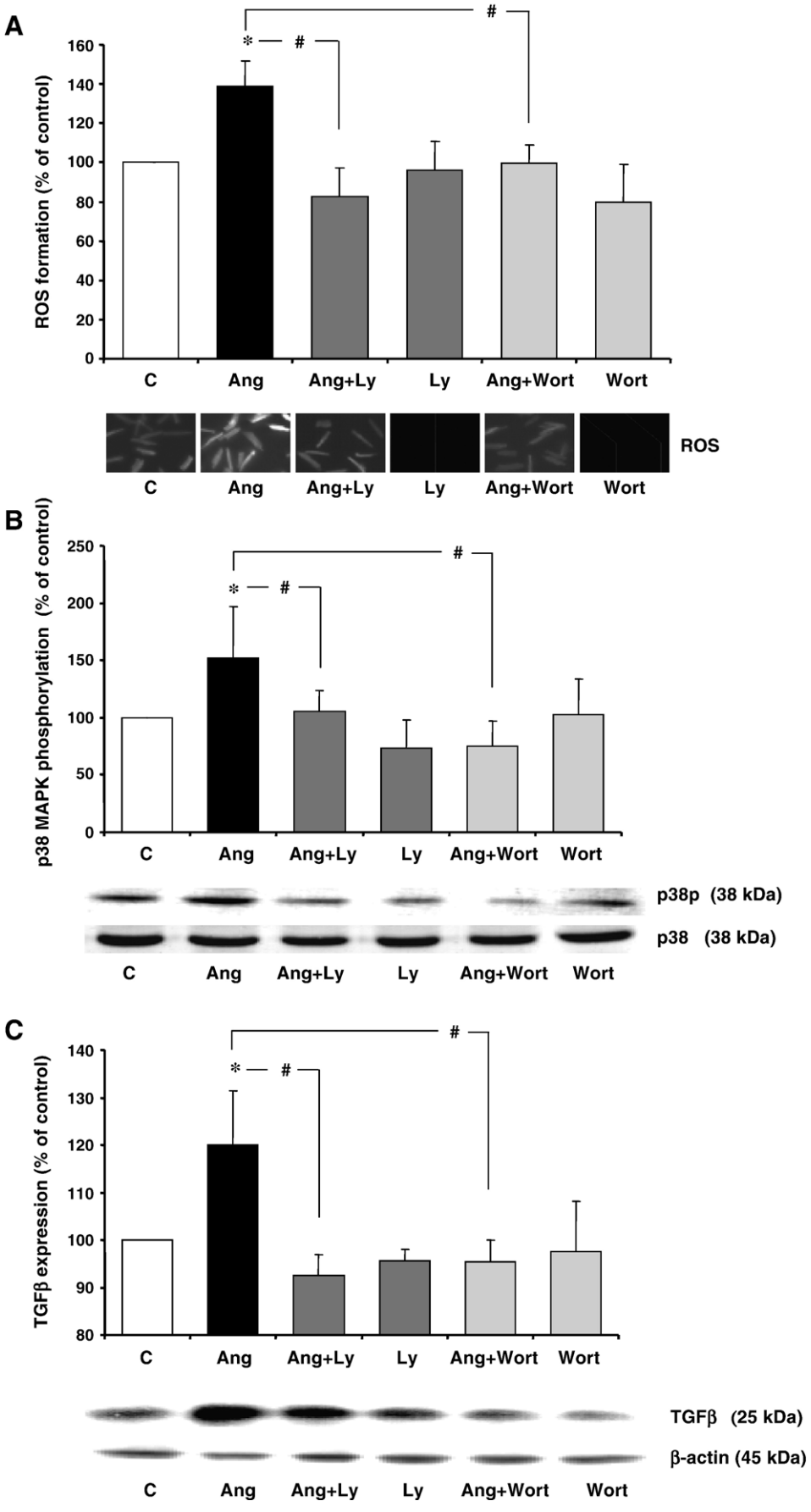


Fig. 1. Verification of the uptake of oligonulceotides (20-mer length) by cardiomyocytes. The uptake of oligonucleotides by cardiomyocytes was controlled by the use of FITC-labelled oligonucleotides. Cells were either cultured under control conditions (C) or trasfected with non-labelled oligonuclotides (oligo, 10 μg/ml) or with FITC-labelled oligonucleotides (oligo-FITC, 10 μg/ml) for 24 h. Afterwards the uptake was visualised by fluorescence microscopy at a wavelength of 484 nm.


A
ROS formation (% of control)
160
140
120
100
80
60
40
20
0
*
#
#
C
Ang
Ang+Ly
Ly
Ang+Wort
Wort
ROS
B
p38 MAPK phosphorylation (% of control)
250
200
150
100
50
0
*
#
#
C
Ang
Ang+Ly
Ly
Ang+Wort
Wort
p38p (38 kDa)
p38 (38 kDa)
C
Ang
Ang+Ly
Ly
Ang+Wort
Wort
C
TGFβ expression (% of control)
140
130
120
110
100
90
80
*
#
#
C
Ang
Ang+Ly
Ly
Ang+Wort
Wort
TGFβ (25 kDa)
β-actin (45 kDa)
C
Ang
Ang+Ly
Ly
Ang+Wort
Wort

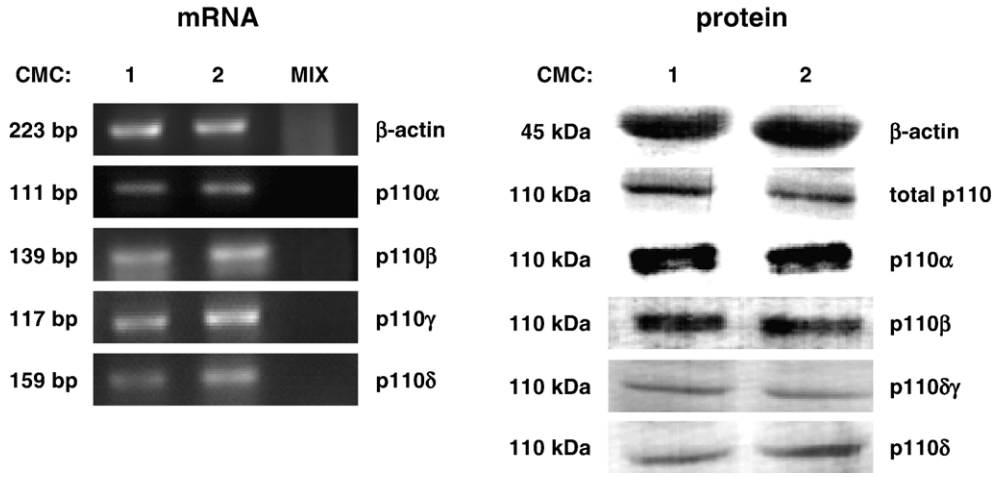


Fig. 3. Determination of mRNA and protein expression of the class I PI 3-kinase isoforms (p110α, β, γ, and δ) by RT-PCR or Western blotting. For RT-PCR, products were amplified at a temperature of 60 °C. Protein bands of the four isoforms were visualised with isoform-specific antibodies. As a loading control, β-actin is additionally shown. All four isoforms are expressed in cardiomyocytes (CMC: 1, 2) on the mRNA and protein level. Representative parts of PCR agarose-gels and SDS-gels are shown.

performed by one-way analysis of variance and use of the Student–Newman–Keuls test for post hoc analysis. In cases in which two groups were compared, conventional *t*-tests were performed. Differences with $p<0.05$ were regarded as statistically significant. All data analyses were performed using SPSS software, version 11.5.

3. Results

3.1. Influence of PI 3-kinase inhibition on the angiotensin II-induced increase in formation of radicals, phosphorylation of p38 MAPK, and expression of TGFβ

The present study investigated whether PI 3-kinase is involved in angiotensin II-mediated NAD(P)H oxidase activation and downstream mechanisms such as p38 MAPK phosphorylation and TGFβ expression. In order to examine the influence of PI 3-kinase on the aforementioned signalling cascade, we used Ly294002 (Ly, 100 μM) or wortmannin (Wort, 100 nM), two chemically unrelated inhibitors of PI 3-kinase with effects at different sites. Pretreatment of cardiomyocytes for 15 min with both inhibitors reduced the angiotensin II-induced formation of radicals (Fig. 2A). Inhibition of PI 3-kinase with Ly294002 and wortmannin also blocked the angiotensin II-induced phosphorylation of p38 MAPK (Fig. 2B) and TGFβ expression (Fig. 2C). In all experiments the inhibitors alone had no influence on basal ROS formation, p38 MAPK phosphorylation, or TGFβ expression (Fig. 2A–C).

3.2. Expression of PI 3-kinase isoforms in cardiomyocytes

Besides investigating the participation of PI 3-kinase in the angiotensin II-induced signalling pathway, this study examined the role of diverse PI 3-kinase isoforms in the aforementioned pathway. Therefore, we focused on the class I PI 3-kinase isoforms, because they are widely distributed and well known. In order to determine whether the four p110 subunit isoforms (α, β, γ, or δ) are expressed in cardiomyocytes, primers for RT-PCR were first created. The products were amplified at a temperature of 60 °C. Specific bands were visualised in the expected amount. As a control, β-actin was additionally examined. Second, the expression of the four isoforms in myocytes was determined with isoform-specific antibodies by SDS-gel electrophoresis and Western blotting. Fig. 3 demonstrates the expression of all four subunits of PI 3-kinase on an mRNA level as well as on a protein level in cardiomyocytes.

Fig. 2. Effect of PI 3-kinase inhibition on the angiotensin II-mediated signalling pathway. A) ROS formation: production of radicals was determined via DCF fluorescence. Representative fluorescence images are given. Production of radicals was measured 15 min under untreated test conditions. After pre-incubation with Ly294002 (Ly: 100 μM) or wortmannin (Wort: 100 nM) lasting 15 min, angiotensin II (Ang)-induced formation of radicals was measured for 30 min. Data are means±s.e.m. from $n=35$ different cells of six different preparations; * = $p<0.05$ vs. control; # = $p<0.05$ vs. angiotensin II. B) p38 MAPK phosphorylation: p38 MAPK activation was determined by the ratio of phosphorylated (p38p) to non-phosphorylated p38 MAPK (p38) after an incubation time of 45 min. Cells were stimulated with angiotensin II (Ang: 100 nM), angiotensin II plus Ly294002 (Ly: 100 μM) or angiotensin II plus wortmannin (Wort: 100 nM). Representative Western blots are shown. Data are means±s.e.m. from $n=5$ cultures; * = $p<0.05$ vs. control; # = $p<0.05$ vs. angiotensin II. C) TGFβ expression: TGFβ expression was determined by Western blotting after 24 h of angiotensin II incubation. Cells were stimulated with angiotensin II (Ang: 100 nM), angiotensin II plus Ly294002 (Ly: 100 μM) or angiotensin II plus wortmannin (Wort: 100 nM). TGFβ values were normalised to β-actin values. Data are means±s.e.m. from $n=5$ cultures; * = $p<0.05$ vs. control; # = $p<0.05$ vs. angiotensin II. Two representative Western blots are additionally shown. In all three cases, the two inhibitors alone had no effect on the examined parameters.

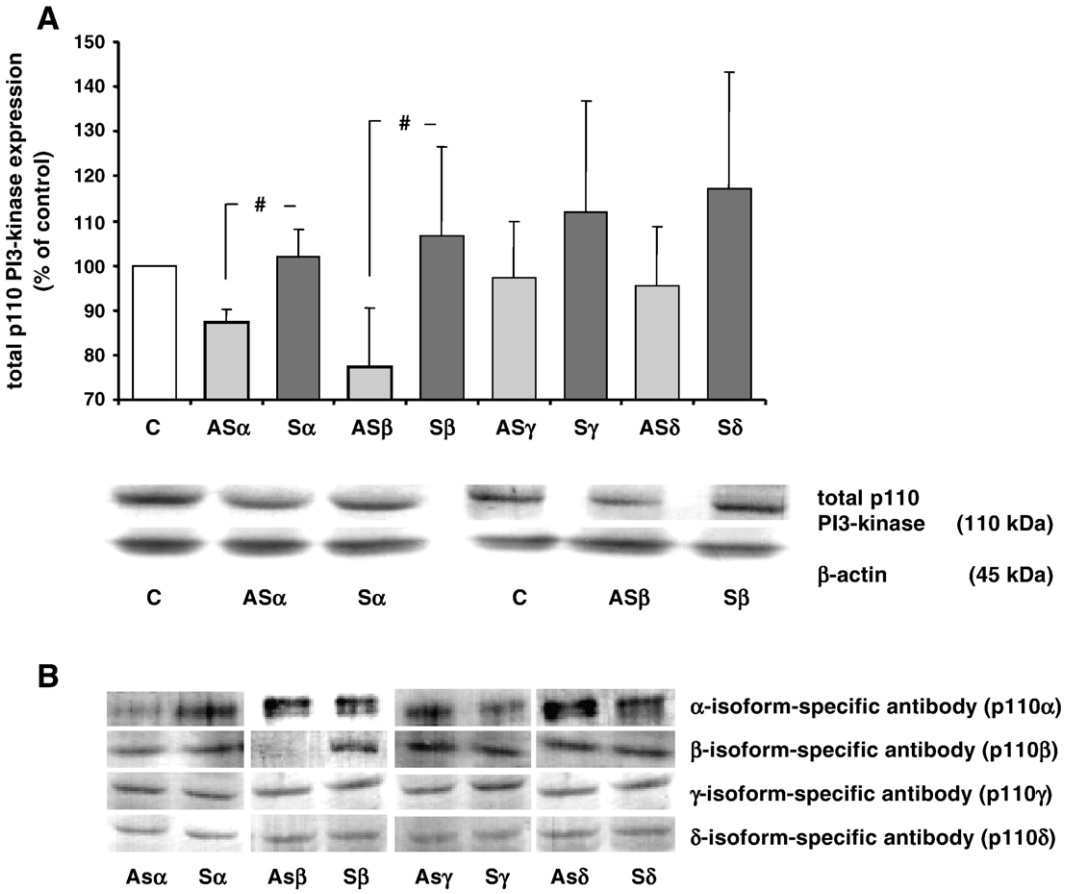
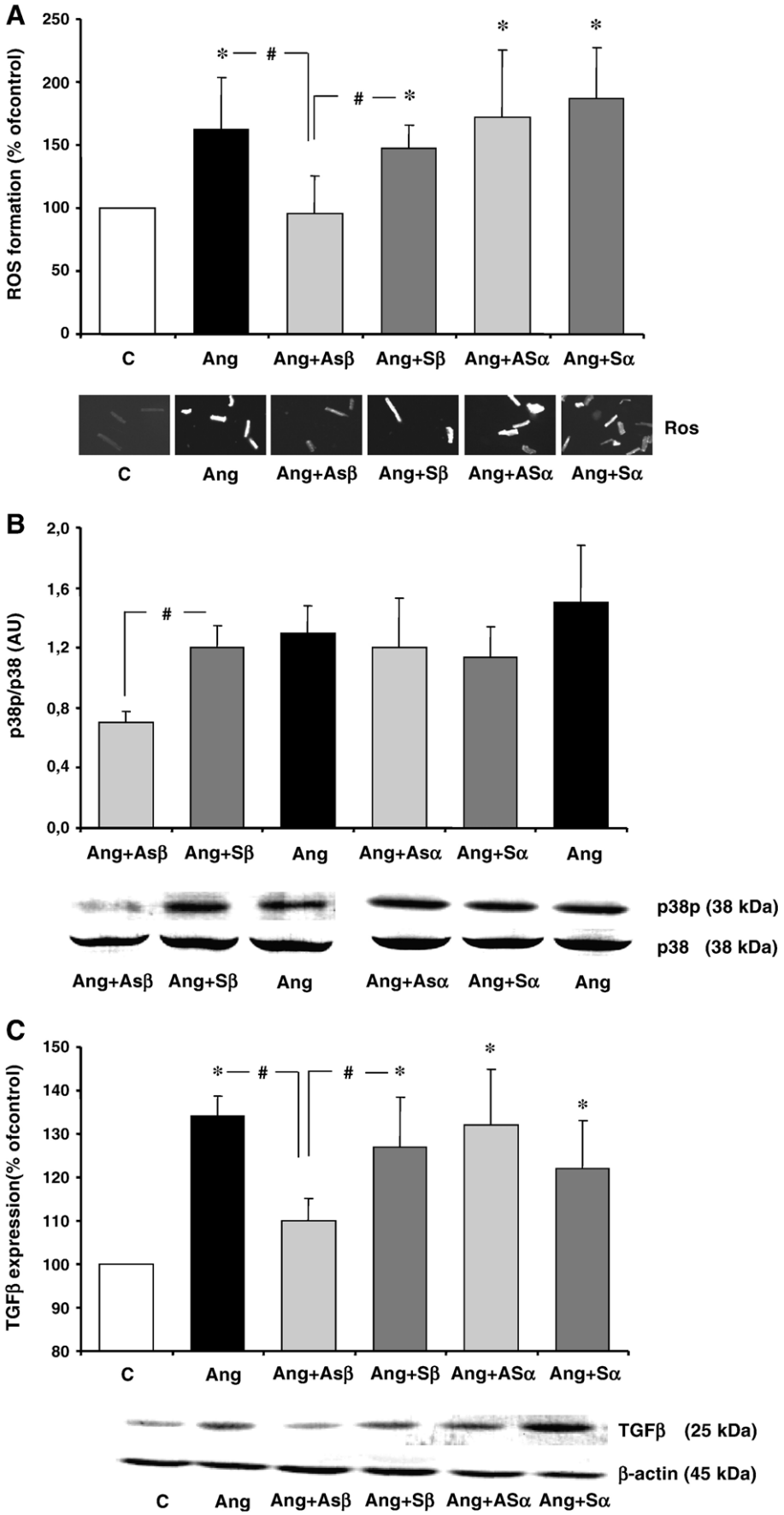


Fig. 4. Down-regulation of PI 3-kinase with antisense oligonucleotides against the diverse PI 3-kinase isoforms. A) On total PI 3-kinase level: quantitative analysis of Western blots are shown. Cells were transfected with sense (S, 10 μg/ml) or antisense (As, 10 μg/ml) oligonucleotides directed against one out of four isoforms as indicated for 24 h. Data are means ± s.e.m. using a non-isoform-specific p110 antibody from $n = 6$ cultures; $^{\#} = p < 0.05$ vs. the particular sense control. Four representative parts of Western blots for down-regulation of p110α and p110β are shown. B) On isoform-specific PI 3-kinase protein level: representative parts of Western blots are given on the diverse isoform-specific PI 3-kinase protein level (α-, β-, γ-, and δ-isoform-specific antibody). Cells were transfected with sense (S, 10 μg/ml) or antisense (As, 10 μg/ml) oligonucleotides directed against one out of four isoforms as indicated for 24 h. Afterwards down-regulation by antisense oliognucleotides was visualised with isoform-specific antibodies. Only antisense olionucleotides against p110α and p110β were able to down-regulate their respective PI 3-kinase isoform. Sense oligonucleotides were used as control. Antisense oligonucleotides against p110γ and p110δ had no effect on p110γ or p110δprotein level, respectively.

3.3. Impact of different PI 3-kinase isoform antisense oligonucleotides on total and isoform-specific PI 3-kinase expression

Sense and antisense oligonucleotides were created against p110α, p110β, p110γ, and p110δ isoforms. In order to test the efficiency of these oligonucleotides, we transfected cells with 10 μg/ml of each oligonucleotide for 24 h and subsequently determined total PI 3-kinase expression with a pan-specific PI 3-kinase antibody. Only antisense oligonucleotides against the isoforms p110α and p110β were

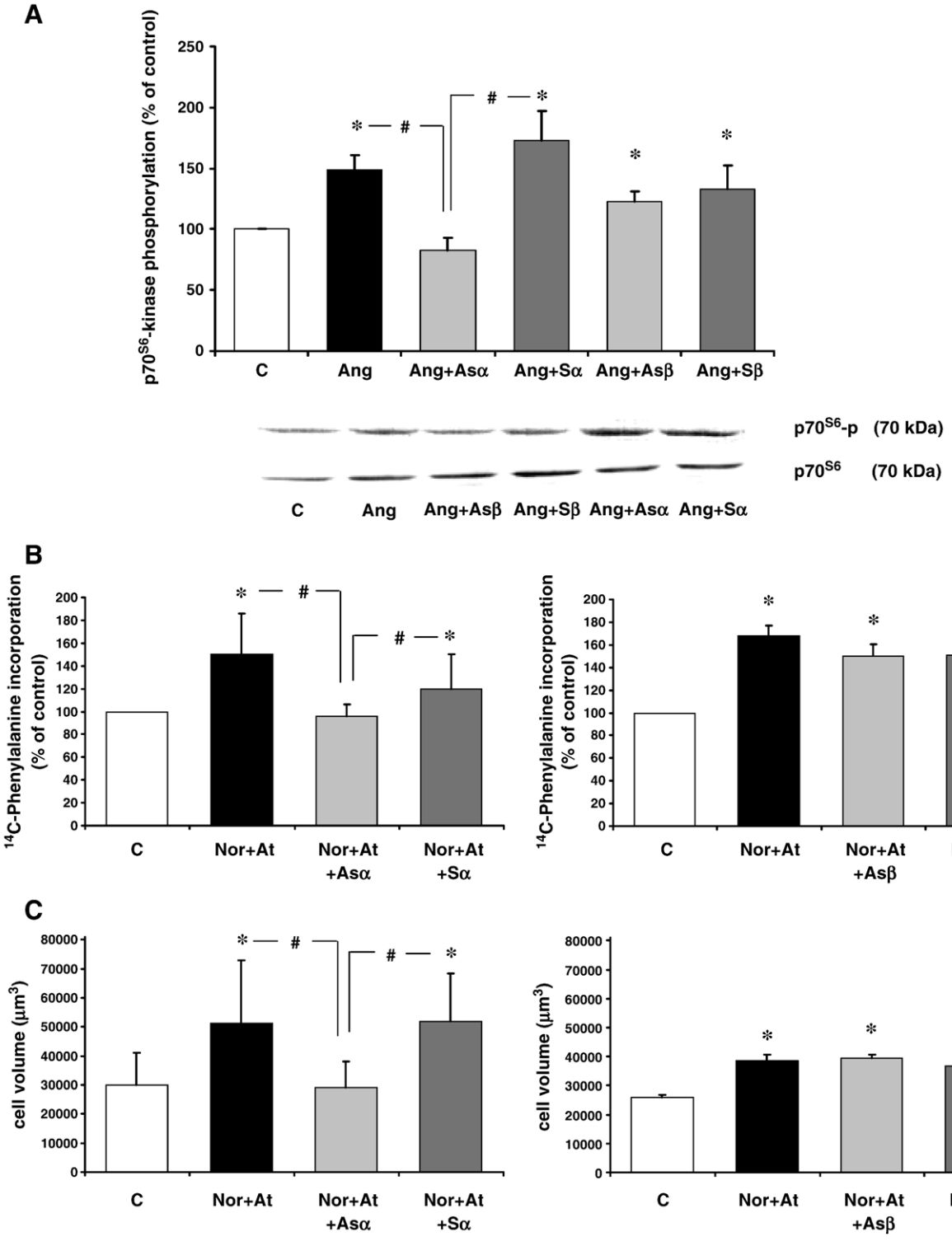
Fig. 5. Effects of down-regulation of p110α and p110β on the angiotensin II-induced signalling pathway. A) ROS formation: influence of transfection of cells with sense and antisense oligonucleotides against the PI 3-kinase isoform p110β and p110α on angiotensin II (Ang: 100 nM)-induced ROS formation. Sense oligonucleotides (S) served as control. Cells were transfected with these oligonucleotides (10 μg/ml) for 24 h. Afterwards they were stimulated with angiotensin II. Representative fluorescence images are shown for ROS formation 30 min after angiotensin II stimulation. Only antisense oligonucleotides (As) against p110β were able to block the angiotensin II-induced effect. Data are means ± s.e.m. from $n = 48$ different cells of eight different preparations; $* = p < 0.05$ vs. control; $^{\#} = p < 0.05$ vs. angiotensin II or vs. angiotensin II plus sense oligonucleotides against p110β. B) p38 MAPK phosphorylation: influence of transfection of cells with sense (S) and antisense oligonucleotides (As) against the PI 3-kinase isoforms p110β and p110α on angiotensin II (Ang: 100 nM)-induced p38 MAPK phosphorylation. Sense oligonucleotides served as control. Cells were transfected with these oligonucleotides (10 μg/ml) for 24 h. Afterwards, they were stimulated with angiotensin II for 45 min. p38 MAPK activation was determined by the ratio of phosphorylated (p38p) to non-phosphorylated (p38) p38 MAPK. Only antisense oligonucleotides against p110β reduced the angiotensin II-induced p38 MAPK phosphorylation. Four representative Western blots are shown. Data are means ± s.e.m. from $n = 5$ cultures; $^{\#} = p < 0.05$ vs. angiotensin II plus sense oligonucleotides against p110β. C) TGFβ expression: Influence of transfection of cells with sense (S) and antisense oligonucleotides (As) against the PI 3-kinase isoform p110β and p110α on angiotensin II (Ang: 100 nM)-induced TGFβ expression. Sense oligonucleotides served as control. Two representative Western blots for the effect of p110α and p110β are shown. Cells were transfected with these oligonucleotides (10 μg/ml) for 24 h. TGFβ expression was measured 24 h after angiotensin II stimulation and values were normalised to β-actin values. Data are means ± s.e.m. from $n = 6$ cultures; $* = p < 0.05$ vs. control; $^{\#} = p < 0.05$ vs. angiotensin II or vs. angiotensin II plus sense oligonucleotides against p110β.


A
ROS formation (% ofcontrol)
250
200
150
100
50
0
C
Ang
Ang+Asβ
Ang+Sβ
Ang+ASα
Ang+Sα
Ros
B
p38p/p38 (AU)
2,0
1,6
1,2
0,8
0,4
0,0
Ang+Asβ
Ang+Sβ
Ang
Ang+Asα
Ang+Sα
Ang
p38p (38 kDa)
p38 (38 kDa)
C
TGFβ expression(% ofcontrol)
150
140
130
120
110
100
90
80
TGFβ (25 kDa)
β-actin (45 kDa)

able to down-regulate the total PI 3-kinase expression significantly (by up to 30%). Sense oligonucleotides were used as control and had no effects (Fig. 4A). Antisense oligonucleotides against p110γ and p110δ showed no effect on total p110 PI 3-kinase expression within the time period of 24 h of transfection. To exclude unequal protein loading, PI 3-kinase values were normalised to β-actin values as an internal loading control. Significant effects of down-regulation of p110α and p110β could also be replicated by the use of isoform-specific antibodies (Fig. 4B).

3.4. The role of p110β on angiotensin II-induced ROS formation

Using the antisense technique we now had a tool to down-regulate the PI 3-kinase isoforms p110α and p110β.



Transfection of cardiomyocytes with antisense oligonucleotides (10 μg/ml) against PI 3-kinase p110β for 24 h reduced the angiotensin II-induced increase in ROS formation (after 30 min of angiotensin II stimulation) significantly. Sense oligonucleotides were used as control and did not show any effect. In addition, neither antisense oligonucleotides nor sense oligonucleotides against p110α had any effect on angiotensin II-induced ROS formation (Fig. 5A).

3.5. The role of p110β on angiotensin II-induced p38 MAPK phosphorylation

To investigate whether the p110β isoform is involved in downstream elements of the angiotensin II-dependent signalling pathway, experiments concerning p38 MAPK phosphorylation were performed. Thus, we transfected cells with antisense oligonucleotides (10 μg/ml) against p110β for 24 h and additionally stimulated with angiotensin II for 45 min. As a control, cells were also transfected with sense oligonucleotides. Only antisense oligonucleotides directed against the p110β isoform were able to reduce the angiotensin II-induced phosphorylation of p38 MAPK. Transfection of cardiomyocytes with antisense or sense oligonucleotides (10 μg/ml) against PI 3-kinase p110α for 24 h also had no effect on the angiotensin II-induced increase in phosphorylation of p38 MAPK (Fig. 5B).

3.6. The role of p110β on angiotensin II-induced TGFβ expression

In order to examine the influence of down-regulation of p110β on the final step of the angiotensin II-induced signalling pathway, cells were transfected with antisense oligonucleotides (10 μg/ml) against PI 3-kinase p110β for 24 h, and 24 h later TGFβ expression was measured. This procedure reduced TGFβ expression significantly. Sense oligonucleotides were used as control and did not show any effect. Furthermore, neither antisense oligonucleotides nor sense oligonucleotides against p110α had any effect on angiotensin II-induced TGFβ expression (Fig. 5C).

3.7. The role of p110α on α-adrenoceptor-mediated increase in $p70^{S6}$-kinase phosphorylation

In order to determine which PI 3-kinase isoform is involved α-adrenoceptor-mediated hypertrophic growth, cells were transfected with sense and antisense oligonucleotides (10 μg/ml) against p110β and p110α for 24 h, and adrenoceptors were additionally stimulated with norepinephrine (1 μM). As norepinephrine is a agonist of both α- and β-adrenoceptors, β-adrenoceptor stimulation was blocked simultaneously by using the selective β-adrenoceptor antagonist atenolol (10 μM). α-Adrenoceptor stimulation for 30 min increased the phosphorylation of $p70^{S6}$-kinase, a signalling molecule involved in the α-adrenoceptor-mediated pathway. Transfection of cells with antisense oligonucleotides against p110α was able to block the α-adrenoceptor-mediated effect. Sense oligonucleotides for p110α were used as control and had no effect. Neither sense nor antisense oligonucleotides against p110β were able to reduce the phosphorylation of $p70^{S6}$-kinase (Fig. 6A).

3.8. The role of p110α in α-adrenoceptor-mediated increase in hypertrophic growth

In the same set of experiments, transfection of cells with antisense oligonucleotides (10 μg/ml) against the p110α isoform attenuated the α-adrenoceptor-induced hypertrophic growth of cardiomyocytes. The hypertrophic growth was measured first by ^{14}C-phenylalanine incorporation (Fig. 6B) and second by direct measurement of the cell volume (Fig. 6C) of myocytes. Both methods showed a significant increase in hypertrophic growth of cardiomyocytes by α-adrenoceptor stimulation and a decrease after transfection with antisense oligonucleotides against p110α. p110α sense oligonucleotides again had no effect (Fig. 6B, C). Neither sense nor antisense oligonucleotides against p110β had any effect on α-adrenoceptor-mediated hypertrophic growth (Fig. 6B, C).

4. Discussion

Myocardial induction of TGFβ is of particular interest in cardiac biology because this cytokine plays a crucial

Fig. 6. Effects of down-regulation of p110α and p110β on the α-adrenoceptor-mediated signalling pathway. A) $p70^{S6}$-kinase phosphorylation: influence of transfection of cells with p110α and p110β oligonucleotides on the amount of $p70^{S6}$-kinase phosphorylation in the presence of norepinephrine (Nor, 1 μM) plus atenolol (At, 10 μM) for 30 min. Only transfection of cells with antisense (As) oligonucleotides against p110α (10 μg/ml) attenuates the increase in $p70^{S6}$-kinase phosphorylation after α-adrenoceptor stimulation for 24 h. Sense (S) oligonucleotides against p110α as well as antisense and sense oligonucleotides against the isoform p110β (10 μg/ml) had no effect. Data are means ± s.e.m. from $n=5$ cultures, * = $p<0.05$ vs. control; # = $p<0.05$ vs. norepinephrine plus atenolol or vs. norepinephrine plus atenolol plus sense oligonucleotides against p110α. B) ^{14}C-Phenylalanine incorporation: Influence of transfection of cells with p110α and p110β oligonucleotides on norepinephrine (Nor, 1 μM) plus atenolol (At, 10 μM)-induced amount of protein synthesis. Only transfection of cells with antisense (As) oligonucleotides against p110α (10 μg/ml) attenuates the increase in cell mass after α-adrenoceptor stimulation for 24 h. Sense (S) oligonucleotides against p110α as well as antisense and sense oligonucleotides against the isoform p110β (10 μg/ml) had no effect. Data are means ± s.e.m. from $n=6$ cultures, * = $p<0.05$ vs. control; # = $p<0.05$ vs. norepinephrine plus atenolol or norepinephrine plus atenolol plus sense oligonucleotides against p110α. C) Cell volume: influence of norepinephrine (Nor, 1 μM) plus atenolol (At, 10 μM) on cell volume. Transfection of cells with antisense oligonucleotides against p110α attenuates the increase in cell mass after α-adrenoceptor stimulation for 24 h. Sense oligonucleotides against p110α as well as sense and antisense oligonucleotides against p110β had no effect. Data are means ± s.e.m. from $n=84$ different cells of 3 different preparations; * = $p<0.05$ vs. control, # = $p<0.05$ vs. norepinephrine plus atenolol alone or vs. norepinephrine plus atenolol plus sense oligonucleotides against p110α.

role in the transition from compensated to de-compensated hypertrophy. In addition, it is known that TGFβ modulates other signalling pathways like that of the β-adrenoceptor [15]. Previous studies demonstrated that the expression of TGFβ is directly mediated by angiotensin II in vivo and in vitro [4]. In order to improve treatment protocols to block the transition of hypertrophy to heart failure, it is important to completely understand the intracellular signalling steps necessary for the angiotensin II-mediated effects on ventricular cardiomyocytes. Earlier studies by our laboratory detected important molecules involved: for example, ROS generated by NAD(P)H oxidase, activation of p38 MAPK, protein kinase C, and the transcription factor AP1 [4]. This study investigated the mechanisms by which angiotensin II activates the formation of ROS through NAD(P)H oxidase in adult ventricular cardiomyocytes with the aim of deciphering the mechanism by which G-protein-coupled intracellular signalling pathways lead to different endpoints while utilizing the same molecules.

Our data clearly implicate PI 3-kinase activation in angiotensin II-induced ROS generation. These data confirm previous experiments on a variety of different cells, including vascular smooth muscle cells [16]. The involvement of PI 3-kinase in the formation of radicals is not consistent for different tissues or cells. There are different isoforms of NAD(P)H oxidases known. The common theory on phagocyte oxygen radical production states that the NAD(P)H oxidase is assembled in the plasma membrane, releasing superoxide and hydrogen peroxide into the extracellular milieu. In contrast, smooth muscle cells, cardiomyocytes, and stem cells, for example, exhibit a type of NAD(P)H oxidase that releases radicals inside the cells. In macrophages (radicals outside), ROS formation is not inhibited by wortmannin, an inhibitor of PI 3-kinase [17]. There seems to be a PI 3-kinase-dependent intracellular release of radicals, whereas the extracellular release is independent of PI 3-kinase [18]. Such a mechanism is consisted with our findings that PI 3-kinase regulates those NAD(P)H oxidases (the smooth muscle types) that release radicals inside the cells, as is the case for cardiomyocytes.

As PI 3-kinase is a multifunctional molecule involved in various signalling pathways, the question arises whether there is any isoform-specificity for the angiotensin II-mediated pathway and the α-adrenoceptor-mediated pathway, where PI 3-kinase is also involved [13]. In the first pathway, PI 3-kinase activation leads to a ROS-dependent activation of p38 MAPK, and in the second pathway, it is part of a strong hypertrophic growth response that is p38 MAPK independent [9]. In the literature there are some indications from knockout and transgenic studies for the functional specialization of class I isoforms. Knockout of p110α and p110β results in embryonic death [19,20]. p110α knockouts demonstrate complete failure of cell proliferation. This isoform has also been implicated in the regulation of cell size, with cardiac-specific expression of a constitutively active or dominant negative form of p110α resulting in mice with increased or decreased myocyte size, respectively. Other studies demonstrate an involvement of p110α in the physiological hypertrophy of knockout mice [21]. The isoform p110γ was also demonstrated in knockout studies to have effects, generating mice with a compromised immune system and reduced inflammatory responses. Okkenhaug and Vanhaesebroeck [22] also reported an elegant study with p110δ knockout mice, showing impaired B- and T-cell responses and implicating a role for that isoform in immune responses. However, it must be noted that knockout studies can be complicated by alteration of expression of one subunit affecting the expression of others. Moreover, it seems difficult to compare rats and mice concerning some aspects of intracellular signalling [23]. In genetically hypertensive rats, for example, p110δ is associated with pathological hypertension and altered arterial hypercontractility [24]. Kessler et al. [11] postulated a role of p110β in diversification of cardiac insulin signalling. Because of these extremes, we used the antisense technique in order to investigate the significance of diverse PI 3-kinase isoforms in our model of cultured adult rat cardiomyocytes. Use of phosphorothioated antisense oligonucleotides is a common and effective way to down-regulate protein expression. Our own studies with radioactively labelled oligonucleotides agree with that from Miller and Das [25] and showed that 1 to 2% (100–200 ng) of the exogenously added oligonucleotides were taken up by cardiac myocytes, with a maximum after 16 h of incubation. The efficiency of transfection of cells with oligonucleotides was additionally checked by FITC-labelled oligonucleotides. This measured small quantity of antisense oligonucleotides directed against one out of four different isoforms expressed in cardiomyocytes is sufficient to significantly down-regulate total PI 3-kinase expression by up to 30%. Transfection of cells with antisense oligonucleotides against the different isoforms then allowed us to directly determine the involvement of the individual isoforms. Our findings indicated that the PI 3-kinase isoform p110β is directly involved in the angiotensin II-mediated signalling pathway, as opposed to the PI 3-kinase isoform p110α, which is involved in the hypertrophic growth of myocytes. In particular, p110β seems to be associated with G-protein-coupled receptors that consist of β/γ subunits [26]. These data agree with those of Anderson and Jackson [7], who described a preferred involvement of p110α in cell growth and an involvement of p110β in other aspects of cell signalling.

4.1. Concluding remarks

In summary, our study describes for the first time PI 3-kinase p110β as an important intracellular molecule involved in the angiotensin II-mediated generation of radicals in adult cardiomyocytes. This pathway is one of

the critical events in the transition from stable hypertrophy to heart failure. The initial step after stimulation of angiotensin II receptors is the induction of PI 3-kinase activity and of NAD(P)H oxidase. Furthermore, this study helps to clarify the question of why PI 3-kinase, a multifunctional enzyme, leads to hypertrophic growth in the case of α-adrenoceptor stimulation and to an induction of TGFβ expression the in case of angiotensin II stimulation. We were able to link the p110β isoform of PI 3-kinase to the angiotensin II-dependent pathway and the p110α isoform of PI 3-kinase to the α-adrenoceptor-dependent stimulation of cardiac hypertrophy and heart failure.

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Lack of Endothelial Nitric Oxide Synthase–Derived Nitric Oxide Formation Favors Hypertrophy in Adult Ventricular Cardiomyocytes

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Abstract—Reduced activity and expression of endothelial NO synthase (eNOS) is observed in cardiomyocytes from pressure-overloaded hearts with heart failure. The present study was aimed to investigate whether reduced eNOS-derived NO production contributes to the hypertrophic growth and phenotype of these cardiomyocytes. Cultured ventricular cardiomyocytes from adult rats were exposed to Nω-nitro-L-arginine (L-NNA) to inhibit global NO formation, and cultured cardiomyocytes derived from eNOS-deficient mice were used as a model of genetic knockout of eNOS. Cell growth, formation of oxygen-derived radicals (reactive oxygen species [ROS]), activation of p38 mitogen-activated protein (MAP) kinase phosphorylation, and cytokine expression in cardiomyocytes were investigated. L-NNA caused a concentration-dependent acceleration of the rate of protein synthesis and an increase in cell size. This effect was sensitive to p38 MAP kinase inhibition or antioxidants. L-NNA induced a rapid increase in ROS formation, subsequent activation of p38 MAP kinase, and p38 MAP kinase–dependent increases in the expression of transforming growth factor-β and tumor necrosis factor-α. Similar changes (increased ROS formation, p38 MAP kinase phosphorylation, and cytokine induction) were also observed in cardiomyocytes derived from eNOS+/+ mice when exposed to L-NNA. Cardiomyocytes from eNOS−/− mice displayed higher p38 MAP kinase phosphorylation and cytokine expression under basal conditions, but neither these 2 parameters nor ROS formation were increased in the presence of L-NNA. In conclusion, our data support the hypothesis that reduced eNOS activity in cardiomyocytes contributes to the onset of myocardial hypertrophy and increased cytokine expression, which are involved in the transition to heart failure. **(*Hypertension*. 2007;49:193-200.)**

Key Words: myocardial hypertrophy ■ cytokine ■ TNF-α ■ TGF-β

NO, initially identified as the endothelium-derived relaxation factor, is a key regulator of blood vessel tone. Endothelial cells generate NO by the endothelial isoform of NO synthase ([eNOS] encoded by the NOS-3 gene). eNOS-deficient mice develop a hypertensive phenotype and moderate cardiac hypertrophy.[1] eNOS is also expressed in nonendothelial cells in concert with other NOS isoforms. In adult ventricular cardiomyocytes, eNOS and the neuronal NOS (nNOS encoded by the NOS-1 gene) are constitutively expressed. eNOS is located at the sarcolemmal and T-tubular caveolae where it is associated with caveolin-3. The activity of eNOS modifies β-adrenoceptor signaling.[2,3] nNOS seems to be localized at the sarcoplasmic reticulum, where it interferes with the activity of calcium-handling proteins.[4] Interestingly, eNOS undergoes downregulation in cardiomyocytes under conditions of hypertrophic cardiomyopathy and heart failure, but nNOS undergoes upregulation.[5–7]

Under experimental conditions, inhibition of endogenous NO formation induces myocardial hypertrophy, but this is independent of its hypertensive effect.[8] The underlying mechanisms by which reduced NO formation contributes to myocardial hypertrophy are unknown. In the present study we hypothesized that an inhibition of eNOS-derived NO disturbs the balance between NO and NAD(P)H oxidase–generated reactive oxygen species (ROS). As a possible signal transduction pathway, we focused on p38 MAP kinase activation and the previously identified p38 MAP kinase–dependent increase in protein synthesis and cytokine expression.[9,10] To rule out possible interactions of other neurohumoral factors, we performed the experiments on isolated and cultured cardiomyocytes from adult rat hearts and from mice with a genetic knockout of eNOS.

Methods

Cell Isolation and Cultivation

Ventricular cardiomyocytes were isolated from 200- to 250-g male Wistar rats as described before in great detail.[11] Hearts were excised

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from anesthetized rats and immediately connected to a Langendorff perfusion system. Hearts were perfused with calcium-free Tyrode solution and collagenase for 20 minutes, then the ventricles were separated from the hearts and minced. Finally, the cell suspension was filtered through a Nylon mesh, and the remaining cell pellet was resuspended in Tyrode solution by a stepwise increase of the calcium concentration to 1.2 mmol/L. Then the cells were plated on cell culture dishes precoated with 4% (vol/vol) FCS and washed to remove round and nonattached cells after 4 hours. The basal culture medium (CCT) was modified medium 199 including Earl's salts, 2 mmol/L of carnitine, 5 mmol/L of creatine, and 5 mmol/L of taurine. To prevent contaminations, 100 IU/mL of penicillin and 100 μg/mL of streptomycin were added. Cytosine-β-D-arabinofuranoside (10 μmol/L) was added to inhibit growth of nonmyocytes. Where indicated, the procedure was performed in an identical way using hearts from spontaneously hypertensive rats.

Experiments on isolated ventricular cardiomyocytes from mice were performed in a similar way. Some modifications were performed to adapt the isolation protocol to mice as described before in detail.[12] These modifications are related to the stepwise increase in calcium and the plating procedure. For mouse myocytes, laminin instead of FCS was used as an attachment substrate. Animal handling conformed with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication 85-23).

Protein Synthesis and Cell Sizes

The rate of protein synthesis was determined by exposing the cultures to L-^{14}C-phenylalanine (0.1 μCi/mL) for 24 hours. Incorporation of radioactivity into acid-insoluble cell mass was determined as described before[13] and normalized to the precursor pool (acid soluble cell mass). Myocyte size was determined on microphotographs by a charge-coupled device camera as described previously.[14]

Immunoblotting

Cells were stimulated as described in the Results section. At the end of the incubation period, cells were lysed in lysis buffer. After sodium dodecyl sulfate–gel electrophoresis, proteins were transferred onto reinforced nitrocellulose by semidry blotting. The membranes were saturated with 2% (wt/vol) BSA and incubated with the first antibody for 2 hours. After the membranes were washed, alkaline phosphatase–labeled goat anti rabbit-IgG antibodies were added for another 2 hours. Bands were visualized by alkaline phosphatase activity.

Real-Time RT-PCR

The mRNA expression of transforming growth factor (TGF)-β_1 and tumor necrosis factor (TNF)-α were quantified via real-time RT-PCR (see Reference [15] for further details of the RT-PCR protocol) using iQ SYBR Green supermix (BioRad). Hypoxanthine-phosphoribosyl-transferase was used as a housekeeping gene to normalize sample contents.

Detection of ROS

ROS generation in cells was assessed using the probe 2,7-dichlorofluorescein (DCF). The membrane-permeable diacetate from the dye (reduced DCF, DCF-DA, 10 μmol/L) was added to the cultures. Fluorescence intensity was measured in $\leq$10 different cells per preparation. Fluorescence was analyzed using a fluorescence microscope combined with a video imaging system.

Quantification of TNF-α Release

The release of TNF-α into the supernatant of cultured cardiomyocytes was investigated as described before.[16] Ten-microliter samples of the supernatant were used for a bioassay that used the cytotoxic effect of TNF-α on the cell line WEHI. The assay revealed a linear correlation between 0.1 and 10 pg/mL of TNF-α.

Statistical Analysis

Results are expressed as mean$\pm$SEM. Differences were analyzed by 1-way ANOVA, followed by Student–Neumann–Keul posthoc analysis. A value of $P<0.05$ was regarded as significant.

Results

Influence of NOS Inhibition on Protein Synthesis of Rat Cardiomyocytes

To investigate whether endogenous NO production in ventricular rat cardiomyocytes is part of the regulatory process controlling protein turnover and cell size, we determined the rate of protein synthesis in the presence or the absence of N$^{\omega}$-nitro-L-arginine (L-NNA), an inhibitor of constitutively expressed NOS, during 24-hour incubations. The influence of L-NNA on protein synthesis was compared with that of L-arginine, the natural substrate of NOS. In the presence of L-NNA, the rate of protein synthesis increased in a concentration-dependent way (Figure 1A). The effect of NO inhibition on protein synthesis reached the level of significance at 10 and 100 μmol/L. In all of the subsequent experiments, we used a concentration of 100 μmol/L of L-NNA. Addition of L-arginine (from 10 nmol/L to 100 μmol/L), however, had no influence on the basal rate of protein synthesis (Figure 1A). Figure 1B gives a representative example of cell sizes after 24 hours. L-NNA–treated rat cells (100 μmol/L) developed an increase in cell width, but cell lengths remained unchanged. On average, the calculated cell volumes increased from 18 788$\pm$782 to 28 385$\pm$1 173 μm^3 in the presence of L-NNA ($P<0.05$ n=120 cells). The cross-sectional area increased from 27.23$\pm$1.08 to 42.46$\pm$1.75 μm^2 ($P<0.05$; n=120 cells). These experiments suggest that NO inhibition increases protein synthesis and induces a concentric phenotype of hypertrophy.

Inhibition of p38 MAP Kinase Activation Inhibits the Prohypertrophic Effect Evoked by L-NNA on Rat Cardiomyocytes

In the next set of experiments we blocked the p38 MAP kinase activation by addition of SB202190, the activation of the p42/p44 MAP kinase pathway by addition of PD98059, and the activation of the protein kinase C (PKC) pathway by addition of bisindolylmaleimide (also known as Gö5850). The concentrations used in this study are based on our previously performed concentration-response curves in the same system.[10,17] In the present study we reconfirmed our previous findings that none of the 3 inhibitors alone attenuates the basal rate of protein synthesis (Figure 2). However, inhibition of either p38 MAP kinase or PKC activation, but not that of p42/p44 MAP kinase activation, attenuated the prohypertrophic stimulation caused by L-NNA (Figure 2). Inhibition of p38 MAP kinase did not attenuate the prohypertrophic stimulation of α-adrenoceptor stimulation, but PKC inhibition attenuated the prohypertophic effects of L-NNA and phenylephrine (Figure 2). These data led us conclude that the activation of the stress-activated p38 MAP kinase is essential for the prohypertrophic effect of NOS inhibition.

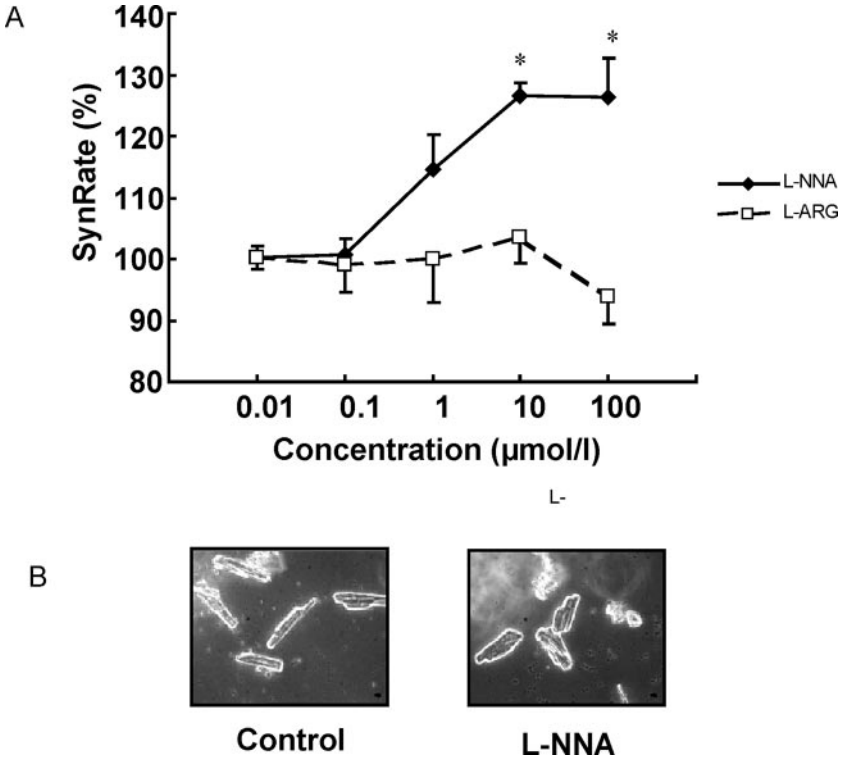

Figure 1. Effect of L-NNA on the hypertrophic growth of rat cardiomyocytes. (A) Effect of increased concentrations of L-NNA or L-arginine on the rate of protein synthesis determined as incorporation of ^{14}C-phenylalanine into cell protein. Data are mean±SEM from n=6 cultures. *$P<0.05$ vs L-arginine at each concentration. (B) Representative images of cardiomyocytes cultured in the presence or the absence of L-NNA (100 µmol/L) for 24 hours.

Inhibition of NO Formation and Generation of ROS in Rat Cardiomyocytes

As a first step to investigate possible upstream pathways involved in the aforementioned p38 MAP kinase–dependent effect of NOS inhibition on cell growth, we investigated the effect of NOS inhibition on ROS production. The fluorescence signal increased during a 15-minute time period constantly because of basal generation of ROS. As soon as L-NNA was added, the fluorescence signal increased more rapidly compared with basal production (Figure 3). On average, the fluorescence signal was 35±2% higher in L-NNA–treated cells compared nontreated cells after 30 minutes ($P<0.05$; 18 to 30 cells each). In contrast, no increase of the fluorescence signal was observed when the cells were analyzed in the presence of 4-amino-5-aminoethylaminopentyl-N-nitroguanidine, an nNOS-specific inhibitor (−6±7%; not significant versus untreated cells; n=18 to 22 cells each). The increase in ROS formation caused by L-NNA was attenuated in the presence of diphenylenium iodide (10 µmol/L) and apocynin (5 µmol/L): L-NNA at +43±9%; L-NNA+diphenylenium iodide at +15±12% ($P<0.05$ versus L-NNA); and L-NNA+apocynin at −10±14% ($P<0.05$ versus L-NNA).

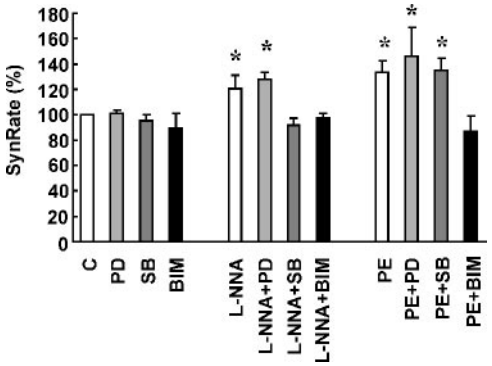

Figure 2. Effect of inhibition of p42/p44 MAP kinase, p38 MAP kinase, and PKC activation on the prohypertrophic effect of L-NNA. Rat cells were treated with phenylephrine ([PE] 10 µmol/L), L-NNA (100 µmol/L), PD98059 ([PD] 10 µmol/L), SB202190 ([SB] 1 µmol/L), bisindolylmaleimide ([BIM] 5 µmol/L), or combinations of these agents for 24 hours. Protein synthesis was determined as incorporation of ^{14}C-phenylalanine into cell protein. Data are mean±SEM from n=6 cultures. *$P<0.05$ vs untreated controls (C).

The causal relationship between ROS generation in the presence of L-NNA and the prohypertrophic stimulus evoked by L-NNA was investigated in additional experiments in which the increase in cell sizes because of L-NNA was attenuated by the presence of vitamin C (100 µmol/L) or N-acetyl cysteine (1 mmol/L), which were used as antioxidants. The concentrations used for these 2 antioxidants have recently been used to attenuate the angiotensin II- and ROS-dependent p38 MAP kinase activation in the same system.[9] Representative single cell graphs are shown as online supplements (available at http://hyper.ahajournal.org). On average, in this set of experiments, the cell volume of cardiomyocytes increased by 21.6±2.2% in the presence of L-NNA compared with untreated control cultures ($P<0.05$; n=53 cells) but not in the presence of either vitamin C (+1.8±2.4%, not significant versus control; n=43 cells) or N-acetyl cysteine (+4.2±2.1%, not significant; n=51 cells). These data confirm a causal relationship between L-NNA–dependent increases in ROS generation and their contribution to cell growth.

Inhibition of NOS Activity and p38 MAP Kinase Activation in Rat Cardiomyocytes

As the experiments in the present study indicate that NOS inhibition leads to an acceleration of ROS production and a

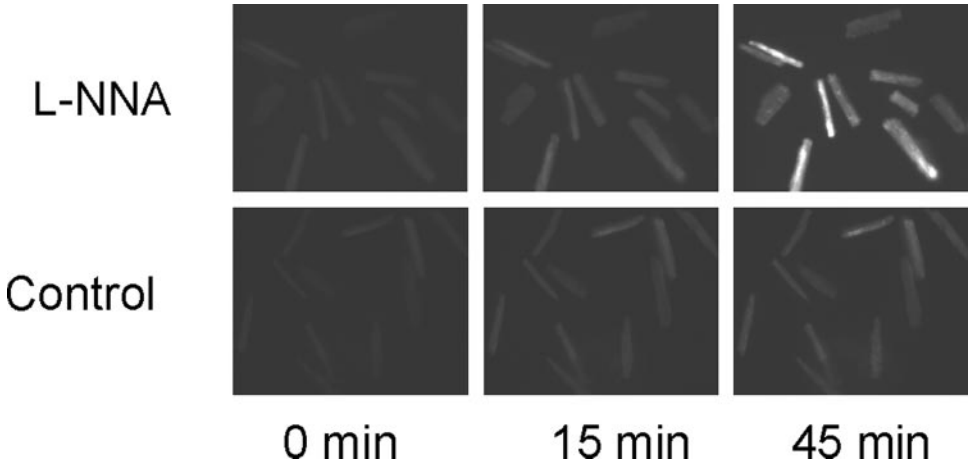

Figure 3. Effect of L-NNA (100 μmol/L) on oxygen-derived radical formation in cultured cardiomyocytes. Rat cells were loaded with dihydroxy-2,7-dichlorofluorescin, and the fluorescence indicates the production of radicals. Cells were incubated in the absence of L-NNA to monitor basal production for 15 minutes. L-NNA then was added where indicated and the cells were monitored again after 45 minutes.

p38 MAP kinase–dependent prohypertrophic stimulation, we now examined whether NOS inhibition indeed activates p38 MAP kinase. In the presence of L-NNA, p38 MAP kinase phosphorylation was significantly increased (Figure 4A). On average, the ratio of the phosphorylated to the nonphosphorylated form of p38 MAP kinase increased by 71.4±15.8% in a time-dependent manner (Figure 4B). L-NNA may activate p38 MAP kinase via ROS generation or by its cGMP-lowering effect. Selective inhibition of soluble guanylyl cyclase in cardiomyocytes by 1-H-[1,2,4]oxaldiazolo[4,3-a]quinoxalin-1-1 (ODQ) produced less p38 MAP kinase phosphorylation compared with the values achieved by L-NNA (Figure 5).

L-NNA and Cytokine Expression in Rat Cardiomyocytes

In addition to the L-NNA–dependent increase in cell growth, as indicated by a higher rate of protein synthesis and an increase in cell sizes, L-NNA–induced cytokine expression is another p38 MAP kinase–dependent phenomenon in cardiomyocytes accompanying prohypertrophic stimulation. The functional relevance of the observation that NOS inhibition leads to p38 MAP kinase activation is highlighted by the effects on cytokine expression. L-NNA significantly increased cellular TGF-β_1 expression in adult rat ventricular cardiomyocytes (Figure 6A). On average, TGF-β_1 expression in cardiomyocytes increased by 35.7±6.2%, and this effect was significantly attenuated in the presence of SB202190 to 13.7±3.3% ($P<0.05$ versus L-NNA; n=4). In parallel, the mean TNF-α release into the supernatant was elevated (Figure 6B).

Comparison of the Results to Cardiomyocytes Isolated From eNOS$^{-/-}$ Mice

The aforementioned experiments described the effect of global NOS inhibition on basal protein synthesis, ROS generation, p38

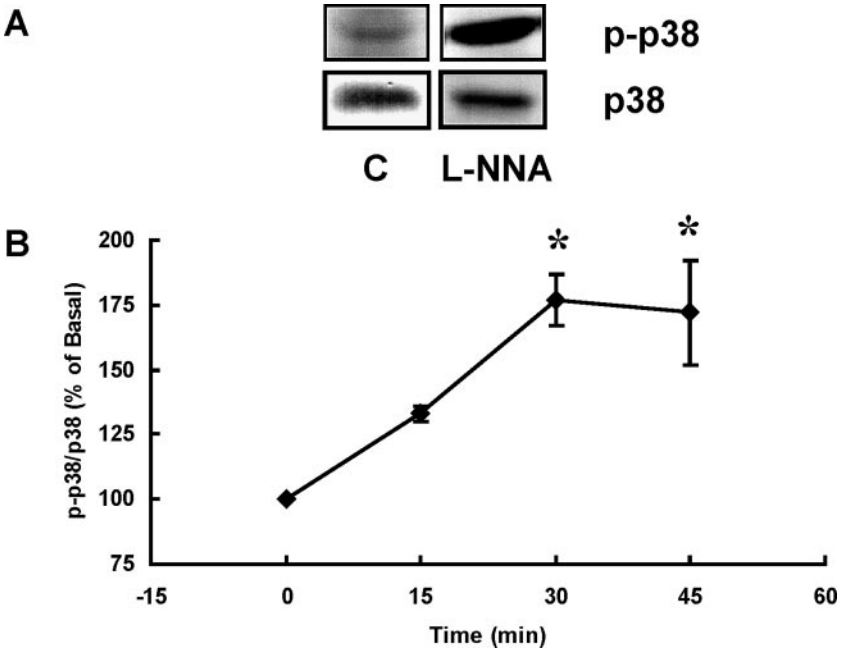

Figure 4. Effects of L-NNA (100 μmol/L) on p38 MAP kinase phosphorylation. (A) Representative bands of phosphorylated p38 MAP kinase (p-p38) and nonphosphorylated p38 MAP kinase (p38). (B) Rat cells were incubated in the presence of L-NNA for ≤45 minutes. p38 MAP kinase phosphorylation is expressed as the ratio of p-p38/p38 MAP kinase. Data are mean±SEM from n=6 cultures. *$P<0.05$ vs 0 min.

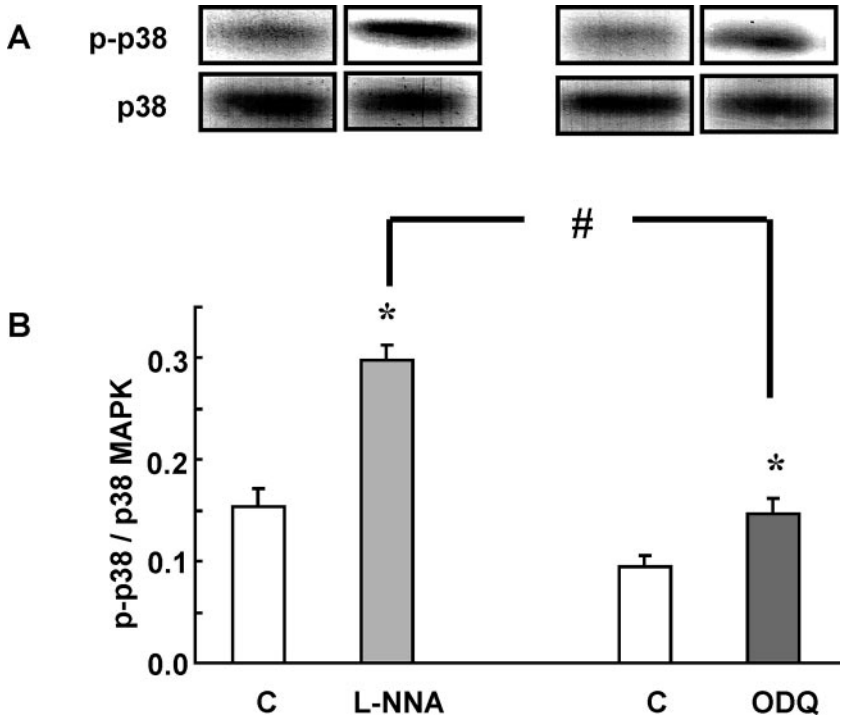

Figure 5. Comparison of the effects of L-NNA (100 μmol/L) and ODQ (10 μmol/L) on p38 MAP kinase phosphorylation. (A) Representative bands of phosphorylated p38 MAP kinase (p-p38) and nonphosphorylated p38 MAP kinase (p38) of rat cardiomyocytes cultured under control conditions or in the presence of L-NNA or ODQ. (B) Quantitative analysis of these experiments. Data are expressed as the ratio of p-p38 MAP kinase and normalized to basal phosphorylation before treatment. Data are mean±SEM from n=4 cultures. *$P<0.05$ vs untreated controls. #$P<0.05$ vs L-NNA.

MAP kinase phosphorylation, and cytokine expression on rat ventricular cardiomyocytes. The lack of effect of the nNOS inhibitor (determined by ROS generation) suggests that the observed effects are specifically linked to eNOS-derived NO. To test this hypothesis, we investigated whether cardiomyocytes isolated from eNOS$^{-/-}$ mice develop a comparable phenotype. Ventricular cardiomyocytes were isolated from eNOS$^{-/-}$ mice or their corresponding eNOS$^{+/+}$ littermates at the age of 12 to 16 weeks. At this time, hearts derived from eNOS$^{-/-}$ mice had developed a mild form of hypertrophy, as indicated by an increased heart weight/body weight ratio: 7.16±0.55 mg/g in eNOS$^{+/+}$ mice and 9.70±0.41 mg/g in eNOS$^{-/-}$ mice ($P<0.05$; n=6). Lack of eNOS expression in cardiomyocytes from eNOS$^{-/-}$ mice was verified by immunoblot (Figure 7A). This went along with an increased TGF-β_1 expression and increased phosphorylation of p38 MAP kinase. On average, the amount of phosphorylated p38 MAP kinase in isolated adult ventricular cardiomyocytes from eNOS$^{-/-}$ mice was ≈4-fold higher than that in isolated cardiomyocytes from eNOS$^{-/-}$ mice (Figure 7B).

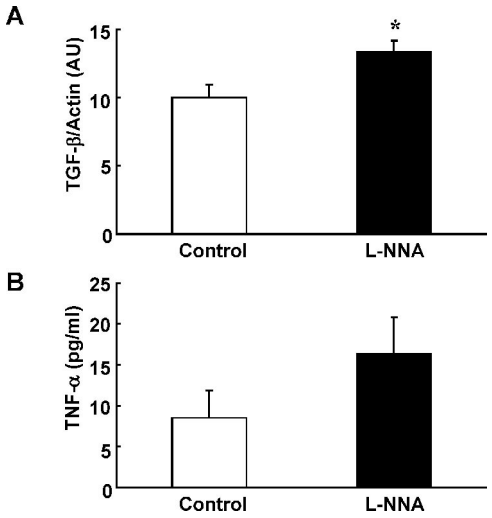

Figure 6. Effect of L-NNA (100 μmol/L) on TGF-β_1 and TNF-α production. Rat cells were exposed to L-NNA for 24 hours. Expression of TGF-β_1 protein was analyzed by immunoblots normalized to actin expression. TNF-α was analyzed as release into the supernatant. Data are mean±SEM from n=4 cultures. *$P<0.05$ vs control.

We next examined whether NOS inhibition in cardiomyocytes derived from eNOS$^{-/-}$ mice increases ROS formation. L-NNA increased ROS formation in cardiomyocytes derived from eNOS$^{+/+}$ mice but not in those derived from eNOS$^{-/-}$ mice (Figure 8A). In contrast, angiotensin II (100 nmol/L) increased ROS production in both cell cultures by either 41.0±5.6% (eNOS$^{+/+}$ mice) or 26.8±7.6% (eNOS$^{-/-}$ mice). As expected, L-NNA activated p38 MAP kinase in cardiomyocytes derived from eNOS$^{+/+}$ mice but not in that derived from eNOS$^{-/-}$ mice (Figure 8B).

Finally, we tested whether the lack of ROS formation in cardiomyocytes derived from eNOS$^{-/-}$ mice is caused by the mild hypertrophic phenotype itself. For this purpose we isolated cardiomyocytes from spontaneously hypertensive rats (SHRs). Cells were isolated from 6-month-old rats, because at this time the hearts from SHRs had developed a hypertrophic phenotype as expressed by a significantly higher heart weight/body weight ratio (6.76±1.11 versus 4.01±0.23 mg/g; n=8; $P<0.05$). L-NNA caused a 34±9% increase in the fluorescence signal because of ROS in cardiomyocytes derived from SHRs (n=16 to 22 cells; each $P<0.05$ versus untreated controls).

Discussion

In the present study, the effect of chronic (24 hour) inhibition of NO production on basal protein synthesis, ROS formation,

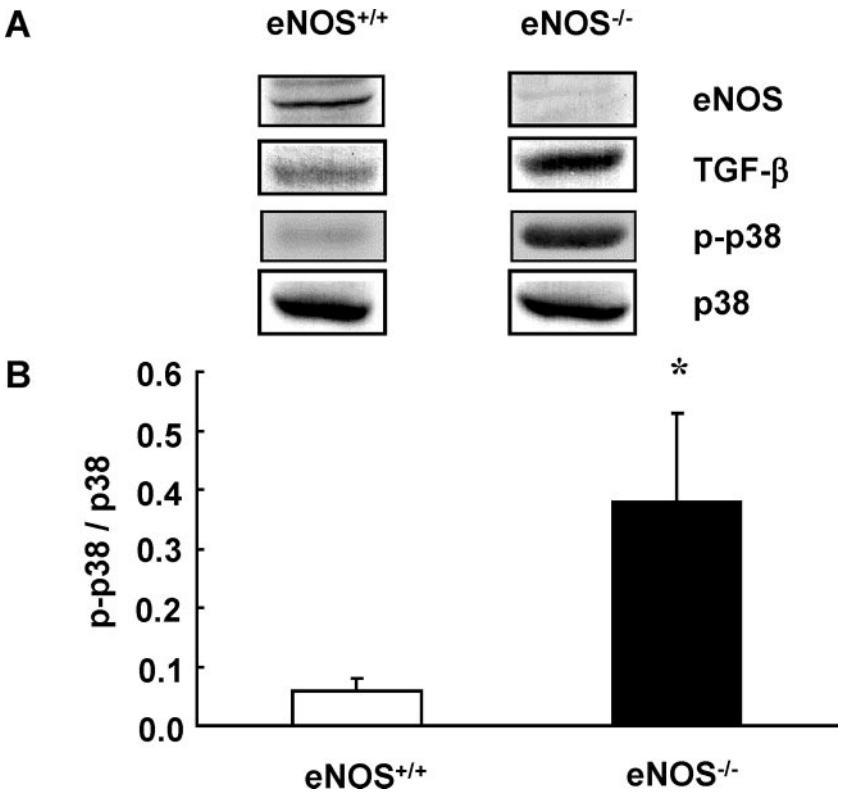

Figure 7. p38 MAP kinase phosphorylation and TGF-β_1 expression in isolated ventricular cardiomyocytes from eNOS$^{-/-}$ mice and eNOS$^{+/+}$ mice. (A) Representative immunoblots indicating the amount of eNOS protein, TGF-β_1 protein, phosphorylated p38 MAP kinase (p-p38), and total p38 MAP kinase (p38). (B) Quantitative analysis of p38 MAP kinase phosphorylation. Data are mean±SEM from n=4 cultures. *$P<0.05$ vs eNOS$^{+/+}$.

p38 MAP kinase phosphorylation, and cytokine expression was investigated. The main findings of this study are that an inhibition of eNOS-derived NO formation in rat ventricular cardiomyocytes induces cell growth, ROS formation, and p38 MAP kinase activation and increases cytokine expression. Similar results were obtained with isolated cardiomyocytes from eNOS$^{+/+}$ mice treated with L-NNA and cardiomyocytes from eNOS$^{-/-}$ mice under basal conditions. This suggests a similar contribution of eNOS-derived NO to the pathways under investigation in both species. We conclude that reduced eNOS-derived NO formation in cardiomyocytes contributes to the ongoing process of hypertrophic cardiomyopathy. As mentioned in the introduction, reduced eNOS expression and function is a common finding in pressure-overloaded hearts.[5–7] The observed effect of L-NNA on cytokine expression may be linked to processes involved in the transition of hypertrophy to heart failure. The question arises from our work of whether this relationship holds true under in vivo conditions and in the case of pathophysiological stages of hypertrophy. Experiments in which animals were fed with N^G-nitro-L-arginine methyl ester and the blood pressure was normalized with hydralazine seem to support our hypothesis.[8] One may argue that hydralazine acts via its antioxidative effect, but even this explanation is in agreement with our findings in which ROS formation was identified as a major trigger.

The effect of arginine derivates on basal NO production and cGMP content in isolated cardiomyocytes has been established previously.[18–20] It is well known that an elevation of cellular NO/cGMP levels negatively regulates the rate of protein synthesis.[21] In principle, the observed effect of eNOS inhibition on protein synthesis may be explained by the cGMP-lowering effect of such a treatment rather than by its direct effects on ROS production. However, our finding than an addition of antioxidants to the cells diminished the prohypertrophic effect of L-NNA is not in line with this possibility. Moreover, an inhibition of soluble guanylyl cyclase in the cells by ODQ did not produce such a strong p38 MAP kinase phosphorylation as that evoked by L-NNA. For these reasons we believe that the mechanism by which L-NNA increases protein synthesis is mainly independent from its effect on basal cGMP levels but linked to the ROS-dependent p38 MAP kinase activation. The observed inhibition of cell growth by addition of antioxidants is consistent with this.

Our data suggest a participation of p38 MAP kinase in the regulation of protein synthesis under such conditions but not that of p42/p44 MAP kinase. L-NNA does not activate p42/p44 MAP kinase, and our data confirmed the results of this study previously.[22] p38 MAP kinase activation alone is not sufficient to stimulate the rate of protein synthesis, but under osmotic stress or reconstitution of mechanical activity, a p38 MAP kinase–dependent prohypertrophic effect was observed.[10] Under such conditions, ROS formation is increased.[23,24] Therefore, under all of the conditions that went along with an induction of ROS formation, p38 MAP kinase–dependent steps are involved in the prohypertrophic effect. Other prohypertrophic stimuli are nevertheless independent of p38 MAP kinase activation.[25] We conclude from these results that p38 MAP kinase activation requires coactivation of further signaling steps to elicit hypertrophy. Such coactivated pathways may also be linked to ROS formation.

The strong myocardial p38 MAP kinase activation in cardiomyocytes from eNOS−/− mice found in this study is

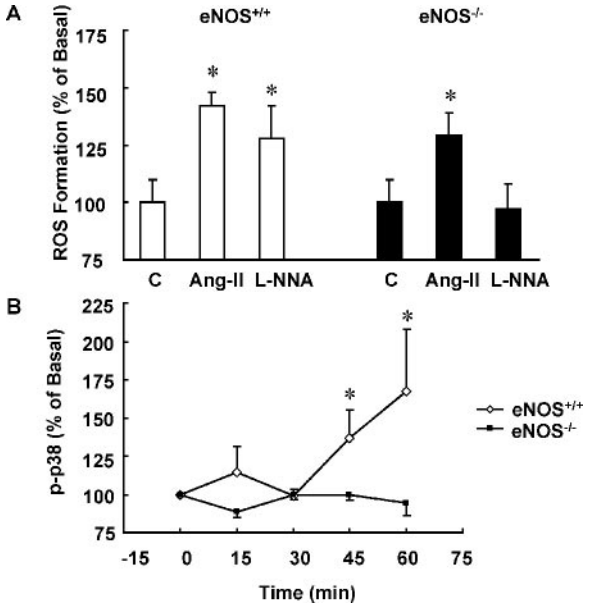

Figure 8. Effects of L-NNA (100 μmol/L) on ROS formation and p38 MAP kinase phosphorylation of cardiomyocytes derived from eNOS$^{+/+}$ or eNOS$^{-/-}$ mice. (A) ROS formation as determined by dihydroxy-2,7-dichlorofluorescin fluorescence in cardiomyocytes from eNOS$^{+/+}$ mice (□) or eNOS$^{-/-}$ mice (■). Cells were treated with angiotensin II ([Ang-II] 100 nmol/L) or L-NNA for 30 minutes. Data are normalized to untreated control cells. Data are mean±SEM from n=4 to 8 cells. (B) Time-dependent induction of p38 MAP kinase phosphorylation by L-NNA in cardiomyocytes derived from eNOS$^{+/+}$ mice (filled symbols) or eNOS$^{-/-}$ mice (open symbols). Data are mean±SEM from n=4 cultures. *P<0.05 vs eNOS+/+.

in contrast to the results of a former study.[26] However, in this study, a very high expression of atrial natriuretic factor (ANF) was found, and ANF counterbalances the prohypertrophic effect of eNOS deficiency.[26] In our hands, eNOS$^{-/-}$ mice developed a moderate hypertrophy in accordance with the observations made by Huang et al.[1] A possible explanation for the difference may be a different level of ANF expression in the different strains. Finally, we found a coupling of the p38 MAP kinase–dependent prohypertrophic effect caused by L-NNA to PKC activation. Such a coupling of ROS formation and PKC activation has been described before and is related to either PKC-δ or PKC-ε.[27,28] Both isoforms have been identified recently as responsible for the prohypertrophic growth response of adult ventricular rat cardiomyocytes.[29]

In a previous study we found that ROS-dependent p38 MAP kinase activation by angiotensin II is linked to an excessive induction of TGF-β_1. In SHRs, NOS inhibition causes end organ damage that could be blocked by p38 MAP kinase inhibition.[30] We demonstrated in this study that NOS inhibition can increase ROS formation in cardiomyocytes from SHRs. In salt-sensitive rats, eNOS expression decreased when the animals were fed with an 8% NaCl diet for 5 weeks. Re-expression of eNOS by treatment with betaxolol reduced myocardial hypertrophy, p38 MAP kinase phosphorylation, and TGF-β_1 expression in these animals.[31] Such an induction of TGF-β_1 is commonly found in all studies in which cardiac NO formation is inhibited, irrespective of whether this goes along with hypertrophic growth or not and independent of the effects on blood pressure.[32] In addition to these studies on rats, blood pressure reduction in eNOS$^{-/-}$ mice was not sufficient to inhibit the onset of fibrosis and hypertrophy in these animals.[33] It is in line with these findings that in rats undergoing protal hypertension associated with biliary cirrhosis, eNOS expression is upregulated but no fibrosis occurs even in the presence of hypertrophy.[34]

Our data strongly indicate a key role for p38 MAP kinase activation in the signaling events provoked by inhibition of NO formation. We did not further analyze the p38 MAP kinase isoform responsible for this effect. Rat cardiomyocytes express the main isoforms p38 MAP kinase-α and p38 MAP kinase-β.[35] Isoproterenol activates p38 MAP kinase in rat cardiomyocytes.[10] Recently, a p38 MAP kinase-α inhibitor has been used to inhibit the isoproterenol-dependent loss of cardiac function in rats.[36] Therefore, one may speculate that the p38 MAP kinase-α isoform is the dominant isoform responsible for the effects shown here.

Perspectives

Is there a clinical relevance for our study? Downregulation of eNOS or reduced activity of eNOS has been shown before in patients with hypertensive hypertrophy, specifically in the elderly.[37–39] Therefore, such an imbalance seems to contribute to the cardiac phenotype and hypertrophic responsiveness. We are aware of the fact that alterations in the NO system are not restricted to myocytes under such conditions. Vascular complications and cross-activities between endothelial cells and myocytes may also contribute. On the other hand, long-lasting application of low doses of NOS inhibitors leads to myocardial hypertrophy and fibrosis.[40] Collectively, these data suggest that an endogenous NO production in cardiomyocytes contributes to the multiple alterations in the ageing, hypertensive heart. Restoring NO formation to its normal, restricted level in cardiomyocytes should, therefore, be an aim of all treatment strategies.

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Disclosures

None.

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