B) Spezifische Transkriptionsfaktoren bei Herzinsuffizienz

Die Apoptose von Herzmuskelzellen als pathophysiologischer Mechanismus der progredienten Herzinsuffizienz war lange Zeit ein umstrittenes Konzept. Dies lag im Wesentlichen daran, dass in den verfügbaren Gewebeproben von Patienten nur eine äußerst geringe Zahl von Zellen die typischen Veränderungen der Apoptose (Kern-Fragmentierung, Verlust des Membranpotentials in Mitochondrien, Nachweis von Phosphatidylcholinen auf der Zelloberfläche durch Bindung von Annexin V) aufwiesen (Kang and Izumo, 2000). Statistisch signifikant errechnete Veränderungen zwischen Kontrollproben und Gewebeproben von herzinsuffizienten Patienten wirkten wenig überzeugend, da die hier beobachtete Rate an Apoptose innerhalb kürzester Zeit zu einem vollständigen Verlust des Organs geführt hätte (Nadal-Ginard et al., 2003). Neben anderen Arbeiten haben jedoch vor allem Untersuchungen an transgen veränderten Mäusen das Konzept bestätigt. So führt die herzspezifische Verminderung der Expression des Interleukin 6-Rezeptors, gp130, zu dem Phänotyp einer Herzinsuffizienz unter Druckbelastung. Hierfür ist die verminderte Aktivierung von Überlebenspfaden in den Herzmuskelzellen verantwortlich; das Gleichgewicht zwischen pro-apoptotischen Signalen sowie aktivierten Überlebenspfaden des normalen Myokards ist aufgehoben (Hirota et al., 1999).

Verschiedene Transkriptionsfaktoren werden über den gp130-Rezeptor aktiviert: GATA-4, STAT´s und NF-κB (Condorelli et al., 2002). Die Aktivierung dieser Faktoren ist offenbar essentiell für die Fähigkeit des Myokards auf molekularer Ebene einer erhöhten Belastung zu widerstehen. Wir haben daher die Hypothese getestet, dass die spezifische Inhibition des Transkriptionsfaktors NF-κB eine erhöhte Rate an Apoptose in isolierten Kardiomyozyten der neonatalen Ratte verursacht. Darüber hinaus wurde untersucht, inwieweit die Vorbehandlung mit Statinen die Apoptose von Kardiomyozyten unterdrücken kann.

Die Hypertrophie von Herzmuskelzellen trägt einerseits zur Kompensation des Verlustes an Kontraktionskraft nach Verlust von Herzgewebe bei, andererseits scheinen hypertrophierte Kardiomyozyten gegenüber andauernden Streßstimuli weniger resistent zu sein. Darüber hinaus ist die Dehnbarkeit des hypertrophierten Myokardgewebes herabgesetzt; dies führt zu einem vermindertem Einstrom von Blut in die linke Herzkammer und ist klinisch als sogenannte „diastolische Dysfunktion“ bekannt. Die Unterdrückung der kardialen Hypertrophie führt im

Tiermodell bei Druckbelastung des linken Ventrikels nicht zu einer vorgezogenen Dekompensation sondern verlängert im Gegenteil die Überlebensphase (Esposito et al., 2002). Die Unterdrückung der Kardiomyozytenhypertrophie erscheint daher als ein vielversprechendes Konzept in der medikamentösen Behandlung von herzinsuffizienzen Patienten. Von klinischer Seite ist bekannt, dass Antagonisten des β-Adrenorezeptors (sogenannte β-Blocker) die Hypertrophie von Kardiomyozyten unterdrücken können. Wir haben daher die Rolle dieses Rezeptors bei der Hypertrophie von isolierten Kardiomyozyten nach Hypoxie/Reoxygenierung untersucht.

1.) NF-κB als Überlebensfaktor bei der TNF-α induzierten Apoptose von Kardiomyozyten

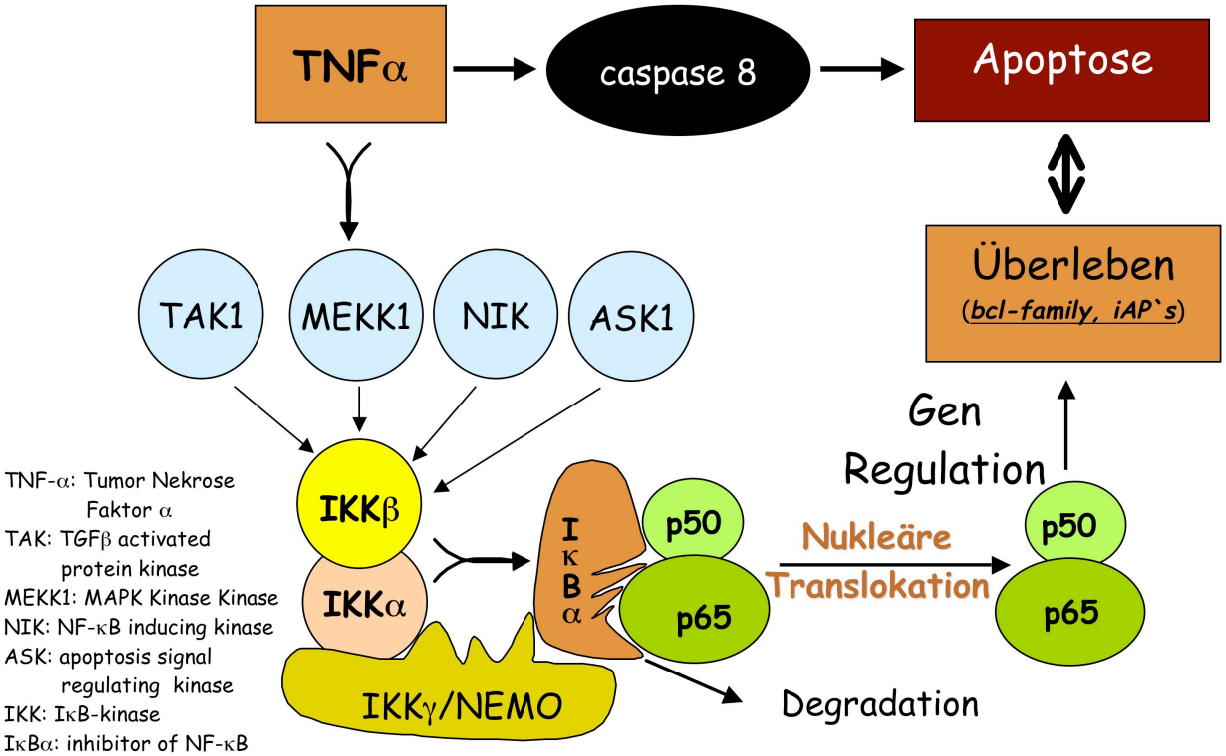
TNF-α wird sowohl systemisch als auch lokal im Rahmen von Stressreaktionen freigesetzt. Im Myokard ist sowohl bei Sepsis wie auch bei akuter Druckbelastung eine erhöhte Menge dieses Zytokins nachweisbar (Clerk et al., 1999; Irwin et al., 1999). Häufig enden diese Erkrankungen in einer Herzinsuffizienz; der molekulare Mechanismus ist noch ungenügend verstanden. Aus dem onkologischen Bereich ist bekannt, dass TNFα in Abhängigkeit des verwendeten Zellsystems sowohl pro-apoptotische Signale über die Caspase 8 wie auch anti-apoptotische Kaskaden über den NF-κB Pfad aktiviert (Wang et al., 1999). Kardiovaskulär wird die Aktivität des Transkriptionsfaktors NF-κB von einer Vielzahl anderer Faktoren beeinflusst, die im Rahmen akuter Streßsituationen freigesetzt werden. Eine Verschiebung der TNF-α induzierten Signaltransduktionswege hin zu einer pro-apoptotischen Aktivität wäre daher ein möglicher Mechanismus der Herzinsuffizienz. Es wurde daher die Hypothese überprüft, ob die spezifische NF-κB Inhibition zu einer gesteigerten Apoptose von primären Kardiomyozyten führt (Schema 3).

In einem ersten Schritt wurde überprüft, ob TNF-α zu einer gesteigerten Bindung von NF-κB Komplexen an die typische NF-κB DNA Bindungsstelle („Konsensus-site“) führt. Mit Hilfe von DNA Bindungsassays (EMSA) wurde nachgewiesen, dass 30 Minuten nach TNF-α Stimulation von primären Rattenkardiomyozyten eine gesteigerte DNA-Bindung an dieser Erkennungssequenz nachweisbar ist. Durch Zugabe von spezifischen Antikörpern ließen sich die klassischen rel-Proteine p50 und p65 als Bestandteile dieses NF-κB Heterodimers nachweisen

(Bergmann et al., 2001). Diese Bindung ließ sich durch Zugabe des Proteasom-inhibitors MG132 sowie durch adenovirale Überexpression der inhibitorischen Untereinheit IκBαΔN spezifisch unterdrücken. Mit Hilfe der im Durchflußzytometer bestimmten Bindung von Annexin V an Phosphatidylcholinreste der Zelloberfläche als frühem Marker der Apoptose konnte im nächsten Schritt nachgewiesen werden, dass Kardiomyozyten nach spezifischer Inhibition der NF-κB Aktivierung eine erhöhte Apoptose Rate aufweisen (Bergmann et al., 2001). Gleiche Ergebnisse konnten mit zwei anderen Methoden des Apoptosenachweises erzielt werden, der Messung fragmentierter DNA im subG1-peak nach Propidiumiodid Färbung unter Verwendung eines Durchflußzytometers sowie dem immunhistochemischen Nachweis im TUNEL-assay (Bergmann et al., 2001). Interessanterweise gelang es in dieser Arbeit nicht, dass für diesen NF-κB vermittelten Überlebenseffekt verantwortliche Protein zu identifizieren. Die aus Vorarbeiten bekannten NF-κB Zielproteine bcl-xL, bcl-2, iAP 1+2 und xiAP waren nach vollständiger Inhibition der NF-κB Aktivierung nicht vermindert (Bergmann et al., 2001) (Schema 3).

Die in dieser Arbeit beschriebenen Signaltransduktionswege (Schema 3) wurden durch die Ergebnisse einer anderen Arbeitsgruppe in vivo mit Hilfe eines Herzinfarktmodelles an einer transgenen Maus mit herzspezifischer NF-κB Inhibition bestätigt. Tatsächlich fand sich in den transgenen Tieren ein größeres Infarktareal als in den Kontrolltieren. Dieses Ergebnis bestätigt, daß NF-κB als Überlebensfaktor in Kardiomyozyten eine Rolle spielt (Misra et al., 2003). Die Autoren führen diesen Effekt auf eine transiente Reduktion von bcl-2 zurück.

Schema 3



TNF-α: Tumor Nekrose Faktor α
TAK: TGFβ activated protein kinase
MEKK1: MAPK Kinase Kinase
NIK: NF-κB inducing kinase
ASK: apoptosis signal regulating kinase
IKK: IκB-kinase
IκBα: inhibitor of NF-κB
iAP: inhibitor of apoptosis protein

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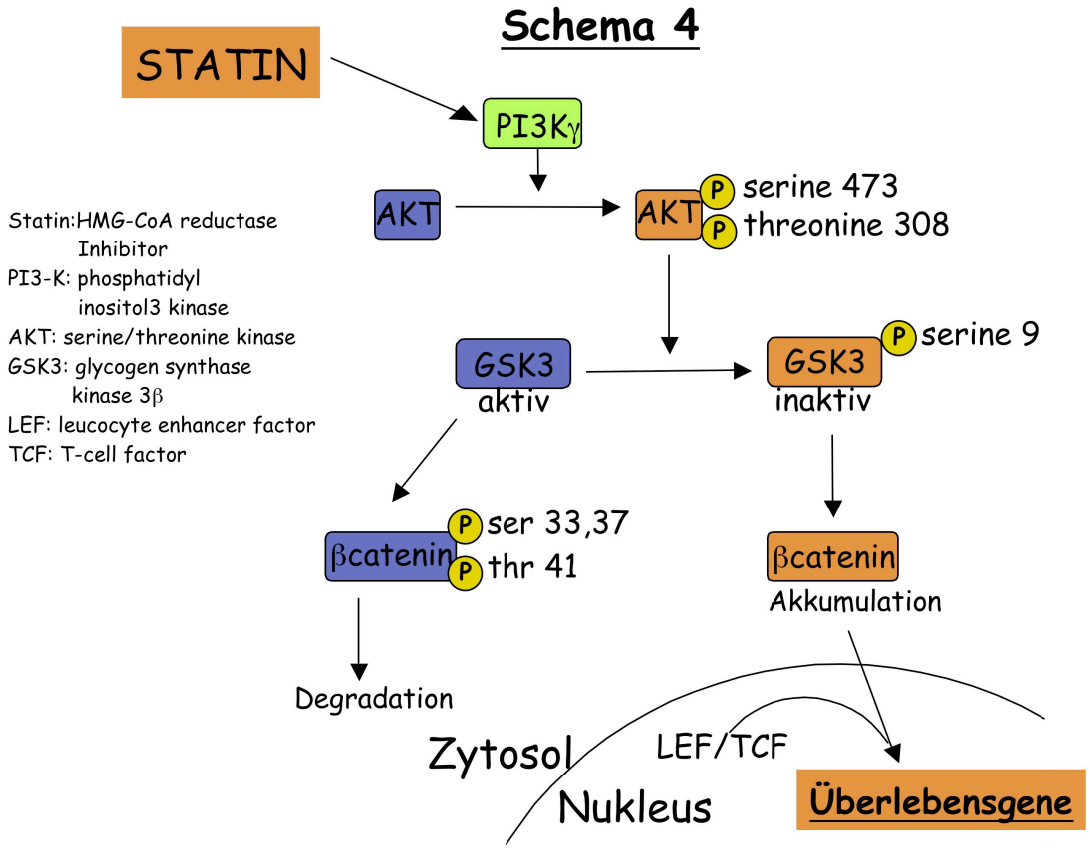
2.) Statine als Überlebensfaktoren bei der Apoptose von Kardiomyozyten

Die medikamentöse Senkung des Cholesterinspiegels hat sich auf der Basis einer Vielzahl von multizentrischen, randomisierten, klinischen Doppelblind-Studien als Eckpfeiler der modernen Arteriosklerosetherapie etabliert, da diese Therapie die Mortalität nach einem Erstereignis (Schlaganfall oder Herzinfarkt) und nach jüngeren Daten auch bei bestimmten Risikokonstellationen im Rahmen einer Primärprävention signifikant senkt (Group, 2002; Vaughan et al., 2000). Neuere Studien weisen auf eine Dosisabhängigkeit dieses Effektes hin: unter aggressiver Therapie bilden sich die typischen arteriosklerotischen Veränderungen zurück (Cannon et al., 2004). Der Effekt der Statine scheint jedoch interessanterweise nicht auf das vaskuläre Remodelling beschränkt zu sein. Kleinere Studien deuten darauf hin, dass Statine auch einen positiven Effekt auf die Anpassungsprozesse des Myokards nach Stressbelastung haben (Bellenger et al., 2002). Insbesondere die frühe, peri-interventionelle Gabe von Statinen scheint von Vorteil zu sein (Chan et al., 2002; Serruys et al., 2002; Walter et al., 2002).

Die Arbeitsgruppe hat daher die Hypothese überprüft, dass Statine in der Zellkultur direkt in Kardiomyozyten anti-apoptotische Signaltransduktionswege aktivieren (Schema 4). Im ersten Schritt wurde überprüft, ob Statine auch in Kardiomyozyten den Phosphatidyl-inositol 3 Kinase (PI-3 Kinase)/AKT Pfad aktivieren. Publizierte Voruntersuchungen in Endothelzellen hatten eine Aktivierung dieses Signalpfades im Zusammenhang mit Statinen erstmals beobachtet (Kureishi et al., 2000). Die Aktivierung der Serin/Threonin Kinase AKT kann wiederum die Apoptose in Kardiomyozyten unterdrücken (Matsui et al., 2002). Die ersten Untersuchungen bestätigten die Hypothese; es fand sich eine Phosphorylierung des Serinrestes 473 der AKT nach Stimulation primärer Rattenkardiomyozyten mit Statinen (Bergmann et al., 2004a). Darüber hinaus wurde eine Phosphorylierung des Serinrestes 9 der Glycogen Synthase Kinase 3 (GSK 3β), einer durch AKT regulierten Kinase, nachgewiesen. Die Phosphorylierung dieses Serinrestes führt zu einer Inaktivierung der Kinaseaktivität. Genetische Untersuchungen haben gezeigt, dass die Inaktivierung der GSK3β entscheidend für die AKT-vermittelte Überlebensaktivität sowie die Kardiomyozytenhypertrophie ist (Antos et al., 2002; Badorff et al., 2002; Hardt and Sadoshima, 2002).

Im nächsten Schritt wurde die Wirkung einer Statingabe in das Zellkulturmedium vor Hypoxie induzierter Apoptose untersucht. Die Aktivierung des PI-3-Kinase/AKT-Signalpfades nach Statingabe inhibierte die Caspase 3 als Effektor der Hypoxie-induzierten Apoptose. Gleiche Ergebnisse fanden sich bei Untersuchung der Kernfragmentierung als charakteristisches Zeichen der Apoptose sowohl in neonatalen Rattenkardiomyozyten als auch adulten Herzmuskelzellen. Um die für die Statingabe relevanten Signalmoleküle abwärts des AKT/GSK3β-Signalpfades zu identifizieren, wurde eine Reihe von Transkriptionsfaktoren untersucht. Es fand sich keine signifikante Aktivierung der Transkriptionsfaktoren NF-κB oder CREB. Ein weiteres Zielprotein der GSK3β, der Transkriptionsfaktor β-catenin, zeigte jedoch eine signifikante Stabilisierung 3h nach Statinstimulation von Kardiomyozyten. Dieser Effekt war erwartet, da die konstitutive Aktivität der GSK3β für die Phosphorylierung und anschließende Proteasom-vermittelte Degradation von β-catenin verantwortlich ist. Die Stabilisierung von β-catenin nach Statinstimulation konnte sowohl in neonatalen als auch adulten Rattenkardiomyozyten nachgewiesen werden. Darüber hinaus führte die Expression einer konstitutiv aktiven Mutante der GSK3β (GSK3βS9A) zu einer gesteigerten Apoptoserate der Kardiomyozyten. Die myokardiale Unterdrückung der GSK3β Kinaseaktivität nach Gabe von Statinen konnte auch in vivo nachgewiesen werden. Zusammenfassend legen diese Untersuchungen das Konzept nahe, dass die Gabe von Statinen durch Inhibition der Apoptose von Kardiomyozyten über die Aktivierung des PI3-Kinase/AKT/GSK3β/β-catenin Signalpfades einen positiven Effekt auf das linksventrikuläre Remodelling unter Stress ausübt (Schema 4). Dieser Statin-Effekt ist unabhängig von der pleiotropen Wirkung auf die Gefäßwand.

Schema 4



Statin:HMG-CoA reductase Inhibitor
PI3-K: phosphatidyl inositol3 kinase
AKT: serine/threonine kinase
GSK3: glycogen synthase kinase 3β
LEF: leucocyte enhancer factor
TCF: T-cell factor

M.W. Bergmann et al., J Mol Cell Cardiol 2004; 37:681-690

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Molecular and Cellular Cardiology 37 (2004) 681–690
Journal of Molecular and Cellular Cardiology
www.elsevier.com/locate/yjmcc

Original Article

Statins inhibit reoxygenation-induced cardiomyocyte apoptosis: role for glycogen synthase kinase 3β and transcription factor β-catenin

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Received 16 December 2003; received in revised form 4 May 2004; accepted 27 May 2004

Available online 31 July 2004

Abstract

Background. **–** Statins may improve left ventricular remodeling after myocardial infarction. We tested whether statins inhibit cardiomyocyte apoptosis through glycogen synthase kinase 3β (GSK3β) inactivation and evaluated activation of downstream transcription factors.

Methods/results. **–** Mevastatin and pravastatin activated serine/threonine kinase Akt in neonatal cardiomyocytes dose and time dependently with maximal activation at 15 min/10 μM. Caspase-3 activity was induced 2.73 ± 0.29-fold by 6 h of hypoxia followed by 18 h of reoxygenation. Pravastatin added at the beginning of the reoxygenation period reduced caspase-3 activation to 1.26 ± 0.06-fold compared to control cells ($P < 0.001$). Similar results were obtained for mevastatin (decreased to 1.98 ± 0.45-fold, $P < 0.05$). TUNEL staining of neonatal cardiomyocytes after 24 h reoxygenation and 4′,6′-diamidino-2-phenylindole staining of adult rat cardiomyocytes after 6 h H_2O_2 showed reduced cardiomyocyte apoptosis in the presence of statin. Analysis of signaling pathways downstream of Akt revealed phosphorylation of GSK3β. Transcription factor cAMP-responsive element binding (CREB) protein showed weak phosphorylation at serine 133; transcription factor NF-κB was not significantly activated after statin treatment as evaluated by EMSA. The GSK3β target protein β-catenin was stabilized at 3 h after statin treatment both in neonatal as well as adult rat cardiomyocytes. Transfection with constitutive active GSK3βS9A sensitized neonatal cardiomyocytes to hypoxia/reoxygenation-induced apoptosis as measured by annexin V/propidium iodide staining. Furthermore, myocardial protein extracts of mice revealed GSK3β inactivation after administration of pravastatin intraperitoneally.

Conclusions. **–** Statins inhibit cardiomyocyte apoptosis in association with GSK3β inactivation. Inactivation of GSK3β leads to stabilization of β-catenin in cardiomyocytes.

Keywords: Apoptosis; Glycogen synthase kinase 3β; β-catenin; Transcription factor; Hypoxia; Cardiomyocyte

1. Introduction

HMG-CoA reductase inhibitors (statins) may have salubrious effects on ventricular remodeling after experimental myocardial infarction [1–3]. The effect has been attributed to decreased granulocyte infiltration [3], increased endothelial nitric oxide synthase (eNOS) expression [1] and attenuated metalloproteinase activity [2]. However, whether the drugs act solely in the microcirculation or whether they might directly act on cardiomyocyte signaling pathways remains unclear. Factors that promote cardiomyocyte survival (anti-apoptosis) appear to function at least in part via the phosphatidylinositol-3′ (PI3)-kinase/Akt kinase cascade. Akt, also known as protein kinase B, is a serine/threonine protein kinase that can activate eNOS as a downstream target [4]. Statin-mediated Akt activation may stimulate angiogenesis via mobilization of endothelial progenitor cells [4,5].

Akt inhibits cardiomyocyte apoptosis downstream of insulin-like growth factor (IGF)-1 activation [6]. IGF-1 was shown to inactivate the bcl-2 family member, bad, via Akt and the nuclear transcription factor cAMP-response element binding protein (CREB) [7]. Inhibition of the Akt downstream kinase $p70^{S6}$ abrogates the protective effect of insulin on ischemia/reperfusion in isolated rat hearts [8]. Adenoviral

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doi:10.1016/j.yjmcc.2004.05.025

delivery of PI3-kinase or Akt inhibits apoptosis in hypoxic cardiomyocytes, both in vitro and in vivo [9,10]. Furthermore, resistance to hypoxia-induced cardiomyocyte apoptosis by preconditioning was shown to involve the glycogen synthase kinase 3β (GSK3β) [11].

Several transcription factors were shown to be regulated by GSK3β including CREB [12], NF-κB [13,14] and β-catenin [15]. All of these transcription factors have been implicated in cell survival downstream of GSK3β. We tested the hypothesis that statins might protect hypoxic cardiomyocytes via GSK3β. In addition, we sought to investigate which downstream transcription factors are regulated by GSK3β in cardiomyocytes.

2. Materials and methods

2.1. Cell culture

Cell culture reagents were purchased from Gibco-BRL unless otherwise stated. Two- to three-day-old Wistar rats (Schoenwalde, Germany) were killed and hearts excised. The digestion was performed as previously described with minor modifications [16]. The hearts were digested with 0.14 mg/ml collagenase II (Worthington Biochemical Corporation) and 1.2 mg/ml pancreatin (Sigma) at 37 °C and afterwards depleted of fibroblasts by percoll gradient centrifugation (Amersham Pharmacia, Germany). Cultures consistently contained >95% cardiomyocytes as revealed by α-sarcomeric actin (Sigma) staining. Isolated cardiomyocytes were cultured at 37 °C in a humidified atmosphere at 5% CO_2 in DMEM/F12 media containing 5% horse serum, 2 mM L-glutamine, 100 U/ml penicillin G, and 100 μM/ml streptomycin, 3 mM sodium pyruvat and 0.2% bovine serum albumin (Sigma), 0.1 mM ascorbate, insulin–transferrin–sodium–selenite (Sigma), cytosine β-D-arabinofuranoside (10 μM, Sigma) on culture plates coated with 1% gelatin and were serum-starved overnight prior to experiments.

Adult rat cardiomyocytes were isolated from 12- to 14-week-old male Wistar rats (Moellegard, Schoenwalde, Germany) as described before [17]. Briefly, excised hearts were sequentially perfused with Krebs–Henseleit buffer (KHB: 127 mM NaCl, 4.6 mM KCl 1.1 mM $MgSO_4$, 8.3 mM glucose, 25 mM $NaHCO_3$, 2 mM Na pyruvate, 10 mM creatine, 20 mM taurine) containing 1.7 mM $CaCl_2$, KHB containing 0.5% fatty acid-free bovine serum albumin (FAFBSA, Sigma) and KHB containing 0.04% collagenase (type II, Worthington Biochemical Corp.) and 0.23 % FAFBSA. After mechanical dissection, cardiomyocytes were washed in KHB with 0.5 % FAFBSA and then plated on laminin (Roche)-coated culture dishes. Cells were cultured in media M199 Earle (Biochrom AG) supplemented with 0.2% bovine albumin, 15 μg/ml insulin, 5 mM creatine, 2 mM carnithine, 5 mM taurine (Sigma), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco).

H9c2 rat cells were cultured at 37 °C in a humidified atmosphere at 5% CO_2 in DMEM/F12 media supplemented with 10% fetal calf serum (PAA), 2 mM L-glutamine, 100 U/ml penicillin G, and 100 μ/ml streptomycin and were also serum-starved prior to experiments.

2.2. Western blot and electrophoretic mobility shift assays

Approximately 2.7×10^6 cells were stimulated for the indicated times with pravastatin (Bristol-Myer Squibb) or mevastatin (Calbiochem). Afterwards, proteins were harvested in RIPA buffer for western blot analysis [16]. Phosphatase inhibitors were added to protein lysis buffer according to standard protocols (sodium fluorid 20 mM, sodium ortho-vanadate 100 μM). The PI3-kinase inhibitors LY294002 (10 μM, Calbiochem) and wortmannin (100 nM, Calbiochem) were added 30 min prior to statin treatment. SDS-PAGE/western blot was performed as previously described [16]. In brief, 12% polyacrylamide gels were used for electrophoresis. Proteins were transferred to either Hybond ECL nitrocellulose (Amersham) or PVDF membrane (Amersham). The primary antibodies against phospho-Akt serine 473 (Cell Signaling), total Akt (Cell Signaling), phospho-GSK3β serine 9 (Cell Signaling), β-catenin (BD Bioscience), total GSK3β (Santa Cruz), phospho-CREB (Cell Signaling), α-sarcomeric actin (Sigma), bcl-2 (Santa Cruz) and total CREB (Cell Signaling) were incubated overnight followed by HRP-labeled secondary antibodies (DAKO) for 2 h. Bands were detected by enhanced chemiluminescence (Amersham). Densitometric analysis was performed employing NIH imager. Values were normalized to total levels of Akt or GSK3β and are displayed as mean ± SEM.

For electrophoretic mobility shift assays, nuclear extracts were prepared as previously described, following 30 min pravastatin treatment. DNA/protein complexes were resolved on polyacrylamide gels followed by autoradiography [16].

2.3. Apoptosis assays

Apoptosis was induced by hypoxia followed by reoxygenation. For apoptosis assays, cells were placed in an oxygen regulated incubator (Labotect, Germany) for 6 h (0% O_2, 5% CO_2) followed by reoxygenation under standard cell culture conditions (21% O_2, 5% CO_2) for 18 h (caspase-3 assays) or 24 h (TUNEL assays). The inhibitors LY294002 (10 μM), wortmannin (100 nM) and L-mevalonate acid (100 μM, sigma), were added 30 min prior to pravastatin or mevastatin treatment, after the 6 h hypoxia incubation period.

For caspase-3 assays 10^5 cells were trypsinized after reoxygenation and incubated with the fluorogenic caspase-3 substrate D_2R according to the manufacturer instructions (Alexis). Briefly, harvested cells were incubated with 50 μM D_2R and 10 mM 2-mercaptoethanol in PBS for 10 min at 37 °C. After washing, cells were analyzed by FACS (FACS-Calibur, Becton Dickinson) employing the FL1-channel [18].

For TUNEL assays 200 000 cells were plated in chamber slides precoated with laminine (Roche). The assay was per-

formed according to the manufacturer instructions (ApopTAG Plus Fluorescein™, Intergene, USA). Briefly, cells were fixed after reoxygenation in 4% formaldehyde and permeabilized with 0.5% Triton-X-100. Slides were incubated with TdT terminal transferase and fluorescein-dUTP. Cells were co-stained with 4′,6′-diamidino-2-phenylindole (DAPI, Sigma).

For detection of apoptosis in adult rat cardiomyocytes, cells were seeded on glass cover slips coated with 10 μg/ml laminin (Roche) and cultured in serum-free medium. Apoptosis was induced by stimulation with 100 μM H_2O_2 for 6 h in the presence or absence of 10 μM mevastatin administered 30 min prior to stimulation. Cells were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde. Nuclei were stained with 4′,6′-diamidino-2-phenylindole (DAPI) (Sigma). Apoptotic nuclei appear fragmented, while intact adult rat cardiomyocytes typical stain two nuclei.

Annexin V/propidium iodide staining was performed after 18 h reoxygenation as recommended by the manufacturer (Nexins Research, The Netherlands). In brief, 10^5 cells were stained with propidium iodide and Annexin V–FITC solution for 10 min on ice in the dark and afterwards measured by flow cytometry.

2.4. Transfection

Cardiomyocytes were transfected employing the newly available nucleofector™ technique optimized for neonatal cardiomyocytes in collaboration with the manufacturer (Amaxa, Cologne, Germany). Briefly, 3×10^6 cells were resuspended in 100 μl of nucleofector™ solution, mixed with 3 μg of DNA and electroporated. After transfection, cells were Annexin V/propidium iodide stained and analyzed by FACS. Transfection efficiencies of control plasmid EGFP-N1 (Clontech) was consistently above 35%. Plasmids for GSK3β and GSK3βS9A were kindly provided by C. Badorff (Frankfurt, Germany) [19].

2.5. GSK3β kinase activity

Twelve-week-old BALB/c mice (Schoenwalde, Germany) were used in the present study. All animal experiments complied with the institutional and state *Guidelines for Animal Care*. BALB/c mice received intraperitoneal (i.p.) injections of 10 mg/kg BW pravastatin [2]. Hearts were excised after the indicated times and immediately frozen in liquid nitrogen before homogenization in supplemented RIPA-buffer. GSK3β kinase activity was assessed as described before [20]. In brief, 100 μg of heart extracts was immuoprecipitated with GSK3β antibody. The precipitate was then incubated with substrate glyogen synthase in the presence of ^{32P}ATP. After washing, ATP-bound substrate was quantified in a liquid scintillation counter.

2.6. Statistical analysis

Data are presented as mean ± SEM. Statistical analysis was performed with the Mann–Whitney-*U*-test using the statistics module of Graph Pad Prism.

3. Results

3.1. Statins activate Akt in neonatal cardiomyocytes and H9c2 cells

In this study, the effect of mevastatin and pravastatin on anti-apoptotic pathways of cardiomyocytes was investigated employing H9c2 cells and neonatal cardiomyocytes isolated from 2- 3-d-old Wistar rats. H9c2 is a cell line derived from rat cardiac ventricular embryonic cardiomyocytes. First we focused on the protein kinase Akt pathway. Akt activation has been linked to cardiomyocyte survival. Therefore we looked for the effect of statins on Akt phosphorylation.

Pravastatin activated Akt in both cell types, neonatal cardiomyocytes and H9c2 cells (Fig. 1). The maximal stimulation was found at 10 μM pravastatin treatment (Fig. 1A). Western blot analysis showed an increased serine 473 phosphorylation level of Akt of 1.55 ± 0.15-fold (Fig. 1D) which was similar to Akt activation induced by 10 ng/ml IGF-1 after 15 min (data not shown). Time-course analysis demonstrated maximal Akt activation by statins at 5–15 min in neonatal cardiomyocytes and at 15 min in H9c2 cells. The effect was transient, since at 60 min phospho-Akt levels were back to baseline (Fig. 1B). Furthermore, Akt activation 15 min after 10 μM pravastatin stimulation was blocked by the PI3-kinase inhibitors wortmannin (100 nM) and LY294002 (10 μM), documenting a PI3-kinase-dependent pathway (Fig. 1C,D). The experiments were repeated with mevastatin producing similar results (not shown).

3.2. Statins inhibit apoptosis

Since statins activate Akt in cardiomyocytes and the PI3-kinase/Akt pathway has been linked to cardiomyocyte survival we next investigated the effects of pravastatin and mevastatin on caspase-3 activation and apoptosis of neonatal cardiomyocytes following 6 h hypoxia with reoxygenation. Earlier studies showed that hypoxia followed by reoxygenation markedly induces apoptosis in cardiomyocytes [21]. Indeed, our experiments (Fig. 2A,B) showed that caspase-3 activation after 18 h reoxygenation was 2.73 ± 0.29-fold increased and was reduced by pravastatin and mevastatin treatment (10 μM) to 1.26 ± 0.06-fold, $P < 0.001$ or 1.98 ± 0.45 compared to control cells ($P < 0.05$). This effect was inhibited by the PI3-kinase inhibitor LY294002 (10 μM, increase to 2.56 ± 0.47-fold) and by L-mevalonate acid (100 μM, increase to 3.4 ± 0.9). These results show that statins attenuate caspase-3 activation and this effect is mediated by PI3-kinase. Moreover, the effect can be circumvented by L-mevalonate, which bypasses statin-mediated effects.

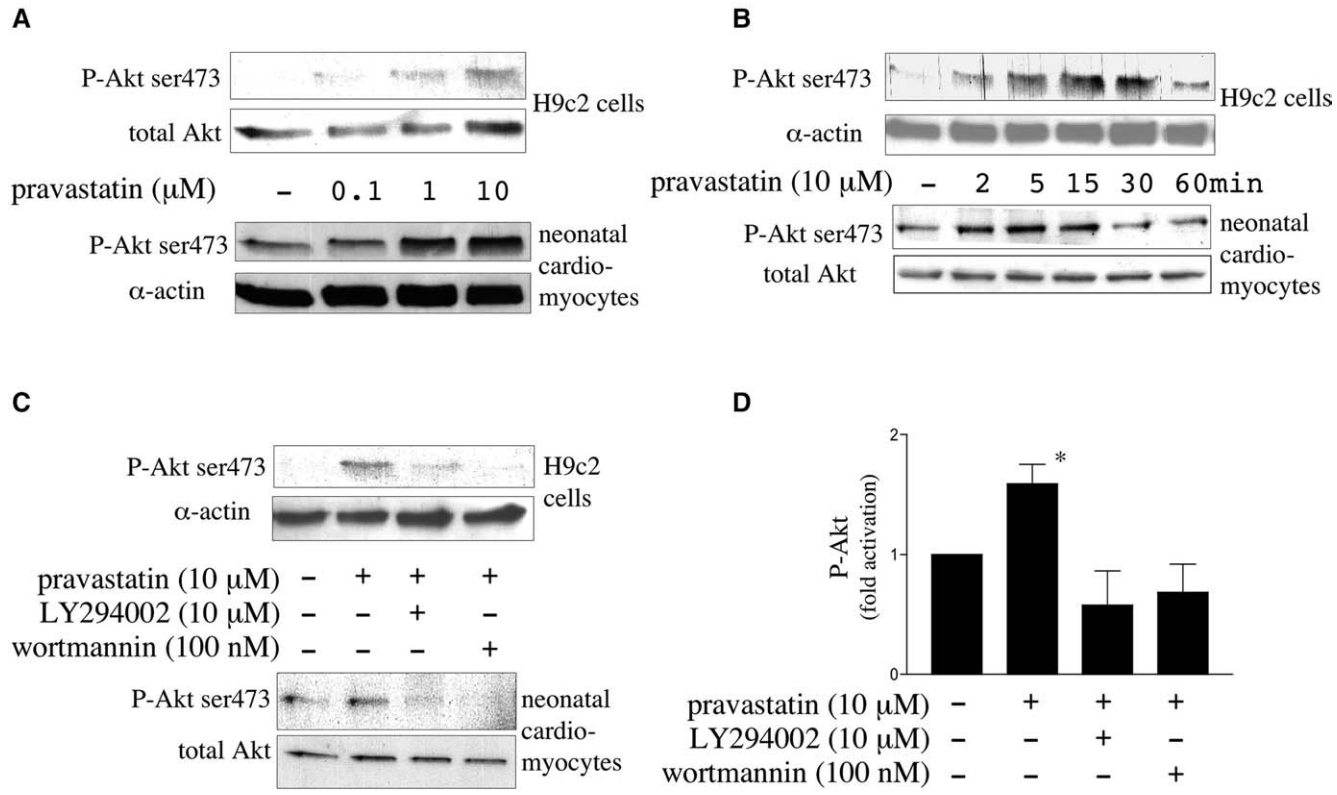

Fig. 1. Pravastatin activates Akt downstream of PI3-kinase in H9c2 cells and neonatal cardiomyocytes analyzed by western blot with antibodies against phospho-Akt (Ser473) and total Akt or α-sarcomeric actin. (A) Dose–response after 20 min pravastatin treatment. (B) Time-course analysis of Akt phosphorylation with 10 μM pravastatin treatment in H9c2 cells and primary rat neonatal cardiomyocytes. Results are representative for five independent experiments. (C) Effects of pharmacological inhibition of PI3-kinase by wortmannin (100 nM) and LY294002 (10 μM) on Akt activation after 15 min pravastatin (10 μM) treatment in both the cell types. The inhibitors were added 30 min before pravastatin treatment ($n = 4$). (D) Quantitative densitometric results of Akt activation after pravastatin treatment (10 μM) in neonatal cardiomyocytes with or without PI3-kinase inhibitors. Phospho-Akt levels were normalized to total Akt or α-sarcomeric actin. Activation levels were calculated compared to unstimulated samples. Data represent mean ± SEM of four independent experiments. *: $P < 0.05$ vs. unstimulated control.

TUNEL staining (Fig. 2C) demonstrated reduced apoptotic nuclei in cardiomyocytes after 6 h hypoxia and 24 h reoxygenation with pravastatin or mevastatin treatment (10 μM) during the reoxygenation period. Hypoxia followed by reoxygenation leads to an increase of apoptotic nuclei from 5.6% in the control sample to 36% apoptotic nuclei after treatment. Pravastatin and mevastatin reduced this to 21% and 22% apoptotic nuclei, respectively. Similarly, fragmented nuclei as detected by DAPI stain were reduced.

This latter method was also employed to assess the effect of mevastatin on H_2O_2-induced apoptosis of adult rat cardiomyocytes. While viable cardiomyocytes demonstrate intact staining of the typical two nuclei per cell (Fig. 4C), H_2O_2-induced fragmentation of nuclei (Fig. 4D). Adult rat cardiomyocytes pretreated with 10 μM mevastatin demonstrated reduced fragmentation of nuclei (Fig. 4D).

3.3. Statins regulate GSK3β and β-catenin

We next analyzed downstream pathways of Akt in cardiomyocytes. We included GSK3β and the transcription factors β-catenin, NF-κB and CREB in our investigations.

Mevastatin (10 μM) increased GSK3β phosphorylation 2.1 ± 0.42-fold after 30 min treatment ($P < 0.05$) (Fig. 3A,B). After 60 min treatment the phosphorylation level decreased to 1.47 ± 0.27-fold and increased again to 1.86 ± 0.56-fold after 3 h. At 18 h phosphorylation was still 1.24 ± 0.26-fold above control levels from untreated cells. This increase of GSK3β phosphorylation was blocked by PI3-kinase inhibition with wortmannin (100 nM) and LY294002 (10 μM) (data not shown). These results suggest that the Akt pathway converge at GSK3β and influence the activation of this kinase.

A merely detectable increase in β-catenin levels (Fig. 3C,D) was found after 15 min mevastatin (10 μM) treatment (1.35 ± 0.2-fold). This effect was transient since the β-catenin level went back to baseline (0.91 ± 0.08-fold) at 30 min after treatment. Interestingly, there was a larger effect on β-catenin stabilization observed at a later time point, starting at 90 min (1.22 ± 0.06-fold induction) with a maximum at 3 h (1.87 ± 0.28-fold induction). After 18 h the level went back again to baseline (1.05 ± 0.11-fold). A similar time course was observed in adult rat cardiomyocytes stimulated with mevastatin or the GSK3β kinase inhibitor, LiCl (Fig. 4A,B).

The transcription factor NF-κB was not significantly activated after statin treatment (electrophoretic mobility shift assay) whereas the transcription factor CREB showed weak

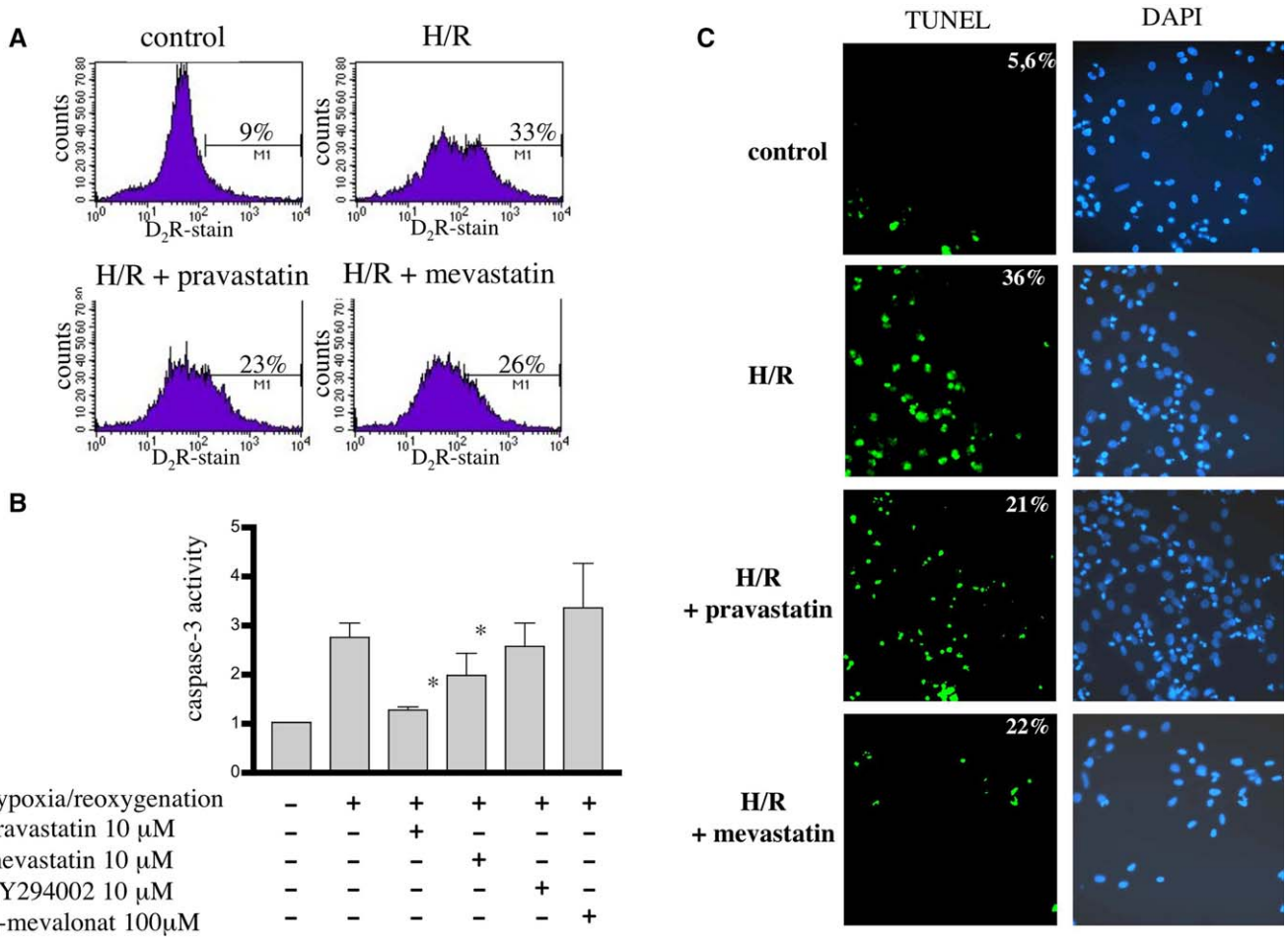

Fig. 2. Statins inhibit caspase-3 activation and apoptosis induced by hypoxia and reoxygenation of neonatal cardiomyocytes. (A,B) FACS analysis of caspase-3 activation by quantification of cleaved D_2R-substrate. Cells were exposed to 6 h of hypoxia followed by 18 h reoxygenation (H/R). Cells with activated caspase-3 were quantified in gate M1. Pravastatin (10 µM) or mevastatin (10 µM) were added at the beginning of the reoxygenation period (A). Quantitative results of independent experiments are presented as mean ± SEM (B, $*P < 0.001$ compared to hypoxia/reoxygenation, for pravastatin n equals seven independent experiments; for mevastatin n equals three independent experiments). LY294002 (10 µM) and L-mevalonate acid (100 µM) were added 30 min before pravastatin treatment. (C) TUNEL analysis after 6 h hypoxia and 24 h reoxygenation. Pravastatin (10 µM) or mevastatin (10 µM) were added at the beginning of the reoxygenation period. Nuclei appear blue (DAPI stain), TUNEL-positive nuclei stain green. Magnification 20×. Data represent apoptotic index (TUNEL-positive nuclei/DAPI-positive nuclei).

phosphorylation at serine 133 after mevastatin and pravastatin treatment in western blot (data not shown).

3.4. GSK3β inhibition increases apoptosis

Since our data suggested that GSK3β is important to statin-mediated cardiomyocyte protection, we next studied a GSK3βS9A plasmid with a mutated phosphorylation site in hypoxia/reoxygenation-treated cardiomyocytes. Transfection of control plasmid pcDNA3.1 or GSK3β wild-type plasmid had no significant effect on apoptosis at baseline or after hypoxia/reoxygenation. Cells transfected with GSK3βS9A demonstrated a slight increase in annexin V staining at baseline (25 ± 2.3%). In pcDNA3.1 transfected cells, 30.5 ± 1.4% of cells showed apoptosis after hypoxia/reoxygenation. In GSK3β wild-type transfected cells, similar levels of annexin V/propidium iodide-negative cells were found (32.7 ± 1.3%). In contrast, GSK3βS9A transfected cells showed a significant increase of apoptosis (41.5 ± 1.3%; Fig. 5A,B). These data support a role for GSK3β in statin-induced cardiomyocyte protection from hypoxia/reoxygenation-induced apoptosis.

3.5. In vivo statin-related effects on GSK3β activity

Finally, to test our findings in vivo, we treated BALB/c mice with pravastatin (10 mg/kg BW) by i.p. injection. The animals were killed and total protein extracts of the ventricles were analyzed for GSK3β phosphorylation over a time course of 3 h (Fig. 6A,B). In contrast to the in vitro experiments, GSK3β inactivation was sustained over the 3 h time course. We found that GSK3β phosphorylation was increased after 1 h (1.84 ± 0.06-fold) and 3 h (1.96 ± 0.25-fold). In parallel, GSK3β kinase activity (Fig. 6C) was reduced 2 h after i.p. injection of pravastatin (43 776 ± 950 cpm compared to 79 271 ± 15 111 cpm in control sample).

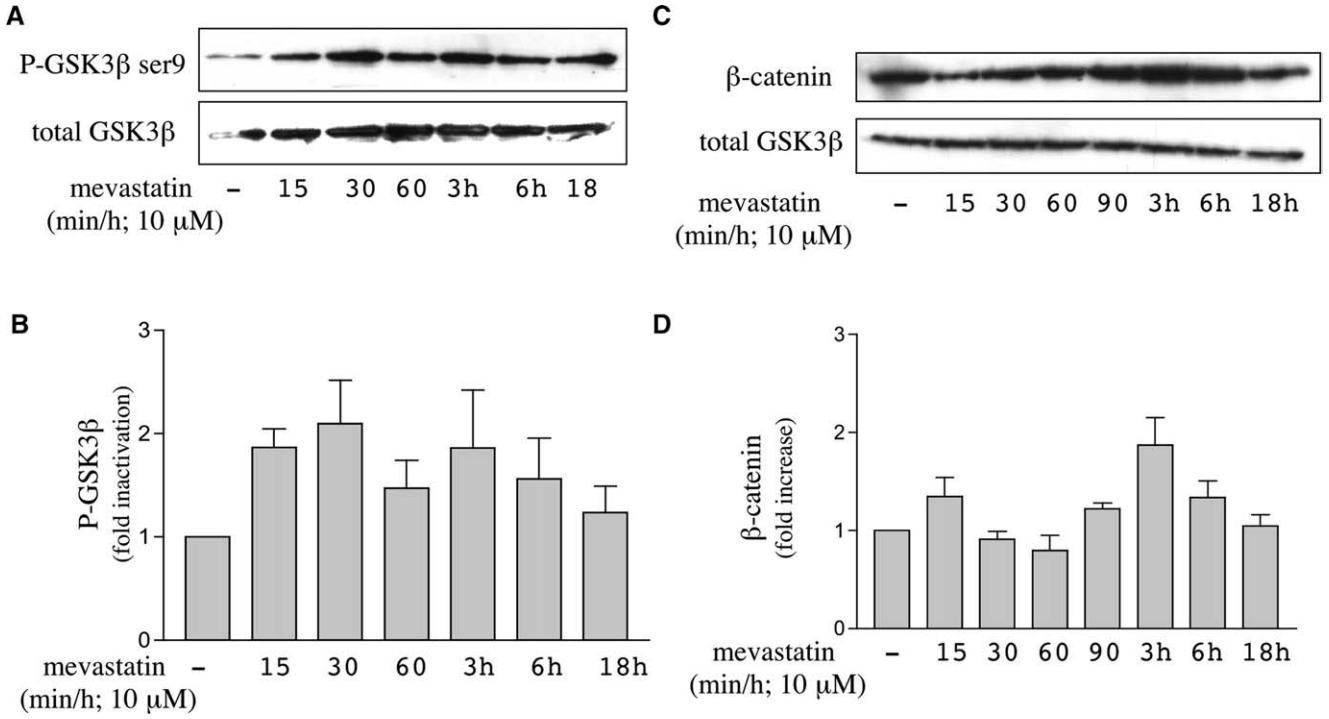

Fig. 3. Statins phosphorylate GSK3β and stabilize β-catenin in neonatal cardiomyocytes. Cells were stimulated with 10 μM mevastatin. (A) Time-course analysis of GSK3β activation by western blot using antibodies against phospho-GSK3β and total GSK3β. (C) Time dependent accumulation of β-catenin analyzed by western blot with antibodies against β-catenin and total GSK3β. (B,D) Quantitative densitometric results of phospho-GSK3β normalized to total GSK3β expressed as fold phosphorylation (B) and of β-catenin normalized to total GSK3β expressed as fold increase (D) compared to unstimulated samples ($n \geq 4$ for each time point).

4. Discussion

We found that statins protect cardiomyocytes from hypoxia/reoxygenation-induced apoptosis. The effect is associated with phosphorylation of Akt, GSK3β, and stabilization of transcription factor β-catenin. Inhibitors of PI3-kinase blocked the effects. We were able to extend these observations to whole organisms, when we demonstrated that pravastatin treatment leads to GSK3β phosphorylation and kinase inactivation in mouse hearts in vivo. Akt activation by statins has been shown earlier in human umbilical vein endothelial cells [4]. The dose–response observed in that study was similar to that we report here in cardiomyocytes. Recently, Akt activation by statins in peripheral blood-derived cells was shown to increase survival and differentiation of endothelial progenitor cells [5]. Akt activation was observed as early as 5 min after initial stimulation [22]. Our findings suggest that effects in cardiomyocytes are similar to those reported in endothelial cells and progenitor cells.

Cardiomyocyte apoptosis has been implicated in the development of heart failure. Conceivably, statins could protect from heart failure by inhibiting apoptosis. In an earlier study, left ventricular (LV) remodeling and survival were markedly improved by statin treatment [1,2]. Infarct size was not reduced in those studies, possibly because statins were started fairly late in the course of the model (d 7 after infarct). A recent study demonstrates reduced myocardial necrosis by rosuvastatin, a new water-soluble statin, given i.p. 6 h prior to ischemia/reperfusion injury in mice. This effect was ablated in eNOS deficient mice [23]. Similar data including a PI3-kinase-dependent pathway were reported with another potent statin, atorvastatin, administered at the onset of reperfusion similar to our in vitro model [24]. Treatment with fluvastatin administered 20 min prior to experimental infarction was also shown to reduce infarct size [25]. While these findings were largely attributed to eNOS downstream of Akt similar to earlier findings in neuronal necrosis after experimental occlusion of the middle cerebral artery in mice [26], the effect of statins on eNOS is only observed at doses higher than those used clinically [27]. Furthermore, statins were previously described to leave eNOS levels unaltered in isolated neonatal cardiomyocytes [4]. The Akt/GSK3β pathway described here is activated at lower statin doses and might therefore be relevant to the effect of statins on cardiac remodeling. This notion is also supported by the observation of decreased β-catenin levels in failing human hearts [28].

GSK3β is constitutively active in most cell types. Phosphorylation of serine residue nine by Akt inactivates this kinase in cardiomyocytes [19]. Protection of cardiomyocytes from apoptosis through preconditioning is dependent on inactivation of GSK3β by PI3-kinase-dependent phosphorylation [11]. Lack of GSK3β inactivation in mice deficient of Fas receptor leads to rapid LV dilatation and heart failure in pressure-overload-induced cardiac hypertrophy [19]. Our data indicate rapid inactivation of GSK3β by statins in cardiomyocytes. Over-expression of constitutively active GSK3βS9A sensitized cardiomyocytes to hypoxia/reoxygenation-induced apoptosis. Furthermore, hypoxia was re-

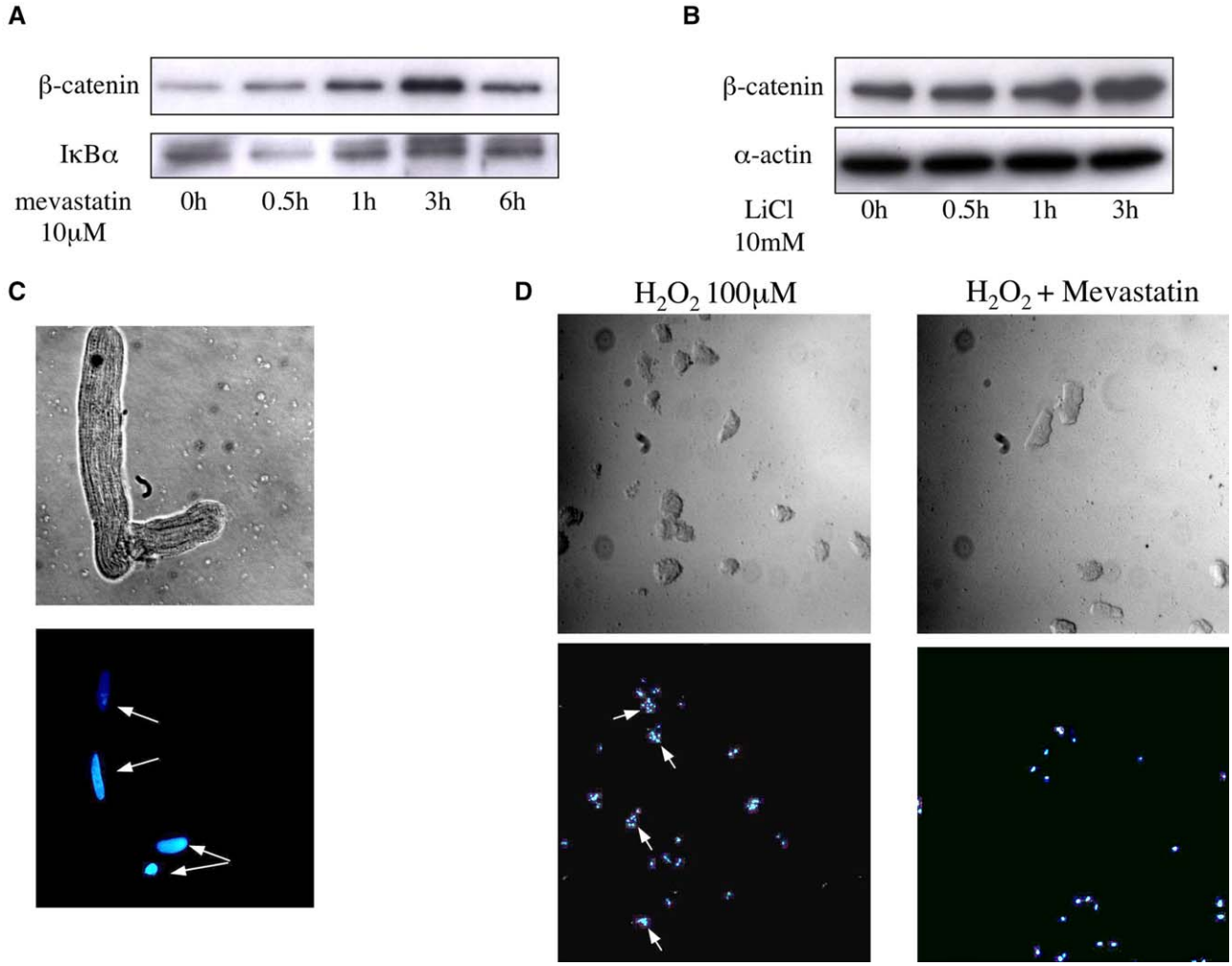

Fig. 4. Statins stabilize β-catenin levels in adult rat cardiomyocytes and inhibit H_2O_2-induced apoptosis. (A) Time-dependent accumulation of β-catenin levels in adult cardiomyocyte extracts as detected by western blot. IκBα levels served as loading control. (B) Time-course analysis of LiCl-induced β-catenin stabilization in adult rat cardiomyocytes. (C) DAPI-staining pattern of control adult cardiomyocytes at high resolution (40× magnification) demonstrating intact nuclei. (D) Effect of mevastatin 10 μM administered 30 min prior to H_2O_2 (100 μM) stimulation of apoptosis in adult rat cardiomyocytes. Apoptotic cells demonstrate typical fragmentation of nuclei (20× magnification). This field was taken exemplary for the whole slide. Similar data were obtained in at least two other experiments.

cently shown to induce apoptosis of vascular smooth muscle cells by activation of GSK3β [29]. These data support a model of statin-mediated GSK3β inactivation as the effector mediating reduced cardiomyocyte apoptosis.

The inhibitory effect of statins on caspase-3 might be of benefit in LV remodeling even independent of apoptosis due to reduced cleavage of vMLC1 (ventricular myosin light chain 1) [30]. Furthermore, statins were recently shown to attenuate oxidant-induced mitochondrial dysfunction in isolated cardiac myocytes [31]. This effect could be linked to a regulation of bcl-2 family members downstream of Akt as described earlier by our group [32]. The inhibitory effects of statins on cardiomyocyte apoptosis might also lead to stabilization of the infarct borderzone explaining the recently observed effect of statins on ventricular arrhythmias in ischemic heart disease [33].

The signaling pathway mediating statin-induced PI3-kinase, Akt, and GSK3β phosphorylation remains to be determined. Dominant-negative Ras-mutants were shown to abolish atorvastatin-induced Akt activation in endothelial cells, while inhibitors of Rho-kinase had no effect [27]. Statins may inhibit hypertrophy by downregulating Rac1- and RhoA-activity after long-term treatment [34,35]. Conversely, PI3-kinase activation has been shown to downregulate RhoA activation through Rac1 [36]. These data would argue in favor of statin-induced Ras activation upstream of PI3-kinase/Akt leading to RhoA-inhibition.

Several downstream targets of Akt have been described in terms of cell survival. Akt-mediated NF-κB activation was described as essential for cell survival [14]. We did not find NF-κB activation after Akt phosphorylation in cardiomyocytes. CREB was shown to mediate IGF-1-induced cell survival by the PI3-kinase/Akt/Erk-pathway [37]. However, we observed only a very modest phosphorylation of CREB after statin stimulation of cardiomyocytes. In addition, bcl-2 levels that are believed to be the effector of CREB-mediated cardiomyocyte survival [7], were unaltered (data not shown).

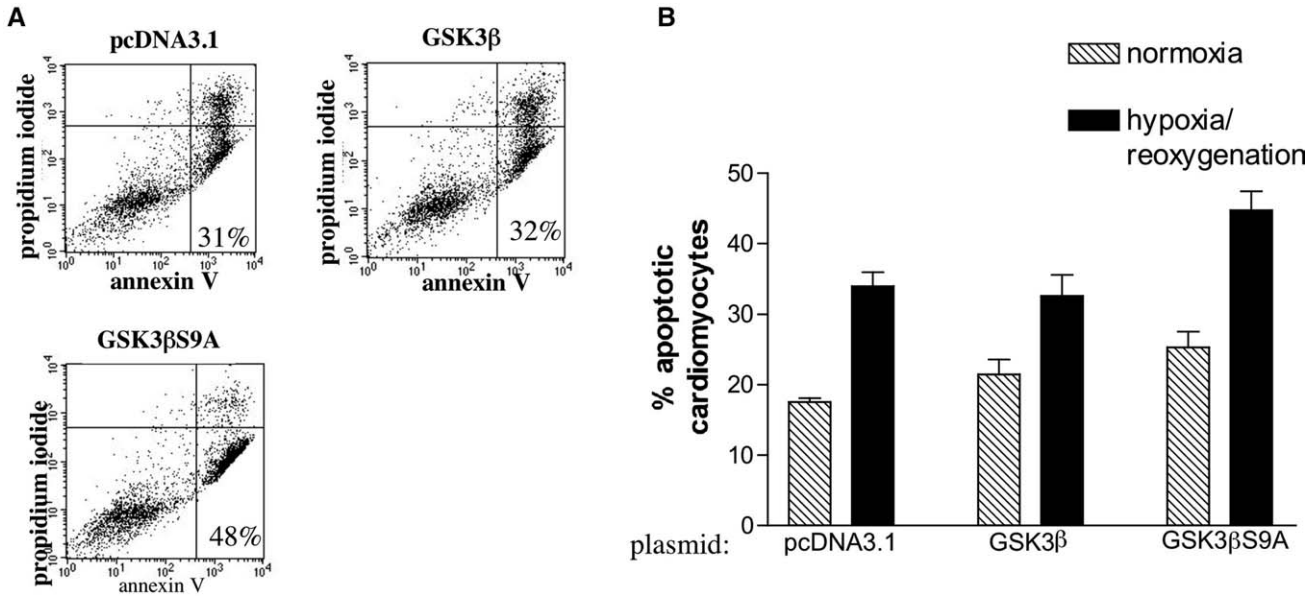

Fig. 5. Inhibition of GSK3β phosphorylation increases hypoxia/reoxygenation-induced apoptosis. Neonatal cardiomyocytes were transfected with 3 μg control plasmid (pcDNA3.1), GSK3β WT plasmid or mutated GSK3β S9A plasmid by nucleofector™ technology from AMAXA. (A) 12 h after reoxygenation, cells were harvested for annexin V–FITC (Fl1)/propidium iodide staining (Fl3, PI) and analyzed by FACS. (B) Data from four independent experiments concerning apoptotic cardiomyocytes (annexin V-positive/propidium iodide-negative cells; lower right quadrant of panel A) presented as mean ± SEM (*$P < 0.05$).

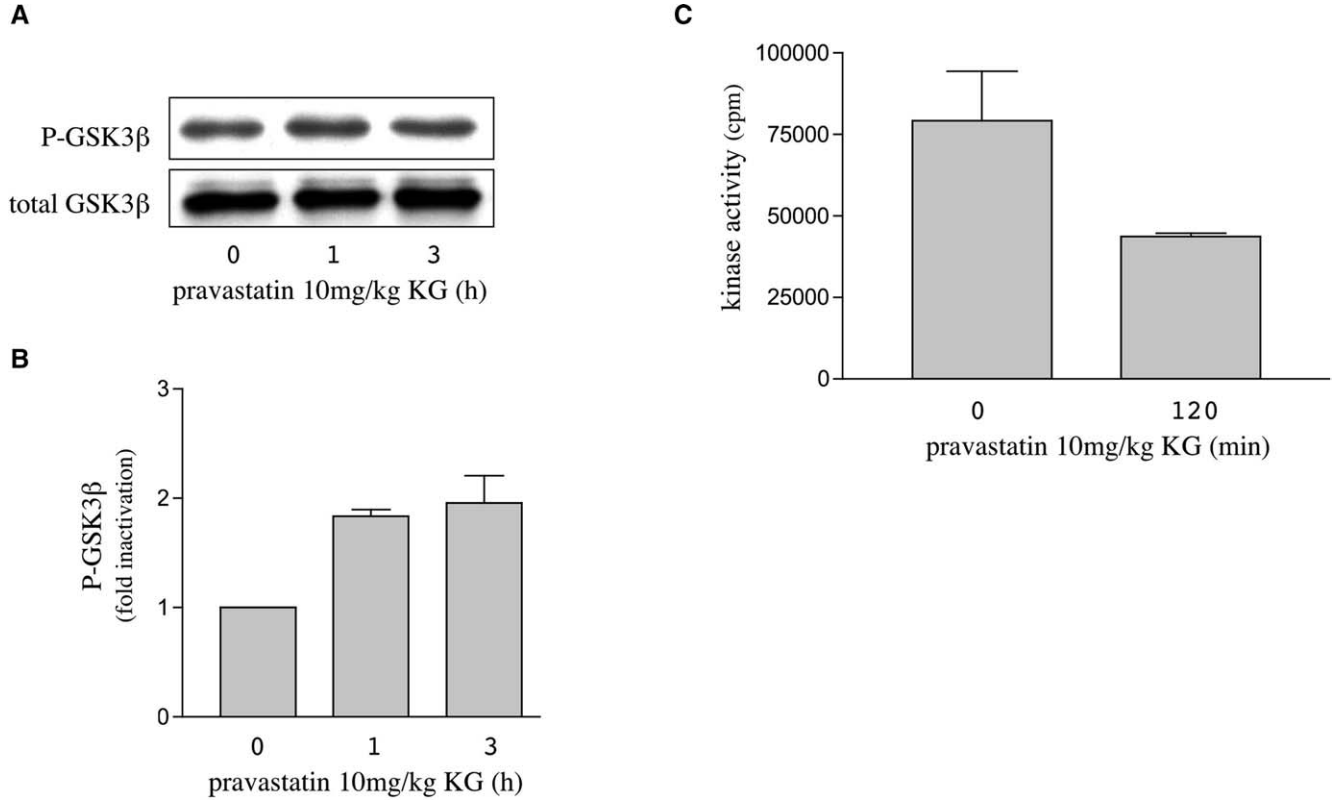

Fig. 6. Pravastatin phosphorylates GSK3β and inactivates kinase activity in hearts of BALBc mice. BALBc mice were injected with 10 mg/kg BW pravastatin i.p. and sacrificed at the indicated time points. Hearts were immediately removed and frozen in liquid nitrogen. (A,B) Phospho-GSK3β and total GSK3β protein levels were analyzed by western blot. Time-course analysis of in vivo GSK3β phosphorylation in protein extracts from ventricles after i.p. injection of pravastatin was performed (A). Data from three independent experiments for each time point were analyzed densitometrically (B). Phospho-GSK3β expression levels were normalized to total GSK3β levels. (C) In two independent experiments GSK3β kinase activity was measured in whole heart extracts from mice injected with pravastatin i.p. and harvested after 2 h. Data are shown as mean ± SEM.

Our data concerning the stabilization of β-catenin by statin treatment in cardiomyocytes are in line with recent publications describing a prominent role for the Akt/GSK3β pathway in ventricular β-catenin regulation, while the canonical Wnt/frizzled pathway was not involved in β-catenin stabilization after hypertrophic stimuli [15]. Interestingly, β-catenin is also essential for cardiomyocyte differentiation [38]. Enhanced differentiation of cardiac progenitor cells under statin treatment might therefore also contribute to improved ventricular remodeling and this pathway warrants further studies. In the context of apoptosis, β-catenin was recently shown to inhibit vascular smooth muscle cell apoptosis [39]. The neuroprotective effects of fibroblast growth factor 1 were also shown to depend on the GSK3β/β-catenin signaling pathway [40]. Therefore the increase in cellular β-catenin levels after cardiomyocyte statin treatment observed in this study may indeed account for the effect on cardiomyocyte survival.

Several recently published studies suggest an effect of statins on LV remodeling. Early statin therapy might limit reperfusion injury in the context of myocardial infarction [25]. Statins were shown to limit peri-procedural myocardial damage after coronary stenting in humans [41]. Independent of cardiomyocyte apoptosis, inhibition of caspase activation by statins might lead to reduced cleavage of vMLC1, thereby conserving ventricular function [30]. GSK3β appears to be a central signaling molecule for cardiomyocyte apoptosis and hypertrophy. In vivo studies identified a role concerning cardiomyocyte apoptosis [11] and hypertrophy [19,42]. Clinical studies evaluating the effect of statin therapy on LV remodeling in ischemic heart disease are warranted to further evaluate the physiologic role of statins in this context. Genetic analysis of β-catenin in adult cardiac remodeling is necessary to assess the relevance of the Akt/GSK3β/β-catenin pathway described here.

Acknowledgements

This study was supported by DFG grant BE2025/3-1 to M.W.B., a grant-in-aid from Bristol-Myers-Squibb, Germany and a grant from Grimmke-Stiftung, Germany. M.W.B. holds a Helmholtz fellowship. The authors thank B. Pohl, S. Feineis, and G. Welsch for expert technical assistance and F.C. Luft for critical reading of the manuscript.

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