

Inhibition of PI-3 kinase/Akt/mTOR, but not calcineurin signaling, reverses insulin-like growth factor I-induced protection against glucose toxicity in cardiomyocyte contractile function

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Abstract

Insulin-like growth factor-I (IGF-1) ameliorates cardiac dysfunction in diabetes although the mechanism of action remains poorly understood. This study examined the role of PI-3 kinase/Akt/mammalian target of rapamycin (mTOR) and calcineurin pathways in cardiac effects of IGF-1 against glucose toxicity. Adult rat ventricular myocytes were cultured for 8 h with either normal (NG, 5.5 mM) or high (HG, 25.5 mM) glucose, in the presence or absence of IGF-1 (10–500 nM), the PI-3 kinase/Akt inhibitor LY294002 (10 μ M), the mTOR inhibitor rapamycin (20 μ M) or the calcineurin inhibitors cyclosporin A (5 μ M) or FK506 (10 mg/l). Mechanical properties were evaluated using an IonOptix MyoCam system. HG depressed peak shortening (PS), reduced maximal velocity of shortening/relengthening (\pm dl/dt) and prolongs time-to-90% relengthening (TR₉₀), which were abolished by IGF-1 (100 and 500 nM). Interestingly, the IGF-1-elicited

protective effect against HG was nullified by either LY294002 or rapamycin, but not by cyclosporine A or FK506. None of the inhibitors affected cell mechanics. Western blot analysis indicated that HG and IGF-1 stimulated phosphorylation of Akt and mTOR. HG also activated p70s6k and suppressed GSK-3 β phosphorylation. However, the HG-induced alterations in phosphorylation of Akt, mTOR, p70s6k and GSK-3 β were significantly reversed by IGF-1. Protein expression of Akt, mTOR, p70s6k, GSK-3 β , SERCA2a and phospholamban was unaffected by HG, IGF-1 or rapamycin. Rapamycin significantly enhanced Akt phosphorylation whereas it inhibited mTOR phosphorylation. Collectively, our data suggest that IGF-1 may provide cardiac protection against glucose in part through a PI-3 kinase/Akt/mTOR/p70s6k-dependent and calcineurin-independent pathway.

Journal of Endocrinology (2005) **186**, 491–503

Introduction

Glucose toxicity contributes to development of diabetic cardiomyopathy characterized by systolic and diastolic dysfunctions independent of coronary macro- and micro-vascular diseases (Galderisi *et al.* 1991, Ren *et al.* 1997). Diastolic dysfunction is the most prominent cardiac abnormality and is characterized by prolonged relaxation and decreased compliance (Galderisi *et al.* 1991, Ren *et al.* 1997, Ren & Bode 2000). Several mechanisms are suggested for the pathogenesis of glucose-triggered cardiac dysfunction including prolonged action potential, impaired excitation–contraction coupling, loss of myofilament Ca²⁺ sensitivity, elevated oxidative stress, intracellular Ca²⁺ dysregulation and dampened insulin-like growth factor I (IGF-1) signaling (Cai & Kang 2001, Esberg & Ren 2003, Lagadic-Gossmann *et al.* 1996, Ren 2000, Duan *et al.* 2003). Most of these speculations have received

convincing support from clinical and experimental studies using antioxidant, gene delivery and IGF-1 as therapeutic remedies for cardiovascular complications in diabetes (Goo *et al.* 1996, Ren *et al.* 1999a, Kajstura *et al.* 2001, Trost *et al.* 2002, Norby *et al.* 2002, 2004). Nevertheless, the precise cellular signaling mechanism behind glucose toxicity-induced cardiac mechanical dysfunction and the therapeutic value of IGF-1 against hyperglycemia remains poorly understood.

The mammalian target of rapamycin (mTOR), or FKBP-12 rapamycin-associated protein, is considered a critical player for cell signaling pathways from translational machinery, cell growth and metabolism to survival/apoptosis in many cell types including ventricular myocytes. Phosphorylation of mTOR may be triggered by activation of its upstream molecules phosphatidylinositol 3 (PI-3) kinase and the serine/threonine kinase PKB/Akt (Hornberger *et al.* 2004, Oudit *et al.* 2004). Meanwhile,

increased levels of nutrients, growth factors such as insulin, IGF-1 and amino acids may also turn on the mTOR signaling cascade through translational and phosphorylation mechanisms (Tremblay & Marette 2001, James & Zomerdijk 2004). However, direct contribution of mTOR to cardiac contractile function has not been reported. It was recently documented that altered signaling mechanism of mTOR, its upstream signaling molecules PI-3 kinase/Akt or its downstream activation of p70s6 kinase (p70s6k) is present in diabetic and insulin resistant conditions and may attribute to diabetes-associated metabolic and growth defects (Duan *et al.* 2003, Huisamen 2003, Um *et al.* 2004, Briaud *et al.* 2005). However, a direct link between elevated glucose levels and altered signaling of the PI-3 kinase/Akt, mTOR and p70s6k cascade has not been depicted in the hearts. Thus, the aim of our present study was to investigate the role of PI-3 kinase/Akt/mTOR/p70s6k in glucose toxicity-induced cardiac mechanical defects and IGF-1-elicited therapeutic effect against glucose toxicity. Possible contribution of the cyclosporin-inhibitable phosphatase calcineurin in IGF-1-induced cardiac contractile response was also tested since the calcineurin pathway is known to be involved in cardiac hypertrophic response of IGF-1 (Miyashita *et al.* 2001). We also evaluated glycogen synthase kinase-3 β (GSK-3 β), a ubiquitously expressed serine/threonine kinase and a key downstream signaling molecule of Akt serving as a negative regulatory target for Akt and p70s6k (Hardt & Sadoshima 2002). GSK-3 β has been demonstrated to participate in a wide variety of biological effects including metabolism, gene transcription, protein translation, apoptosis, Ca²⁺ homeostasis and diastolic function (Hardt & Sadoshima 2002, Michael *et al.* 2004). We took advantage of a cardiac myocyte culture model developed in our lab, where diabetic cardiomyopathy may be phenotypically replicated in normal myocytes by culturing in a high glucose environment (Davidoff & Ren 1997, Esberg & Ren 2003, Ren *et al.* 2003). The abnormal excitation-contraction coupling is apparent in high glucose-treated myocytes reminiscent of those from *in vivo* diabetes (Davidoff & Ren 1997, Ren *et al.* 1997, 1999b, 2003).

Materials and Methods

Myocyte isolation

The experimental procedures used in this study were approved by the Institutional Animal Care and Use Committees at University of North Dakota (Grand Forks, ND, USA) and University of Wyoming (Laramie, WY, USA). All animal procedures were in accordance with the NIH animal care standards. In brief, adult male Sprague-Dawley rats weighing ~250 g were anesthetized with ketamine/xylazine (5:3, 1.32 mg/kg i.p.) before the hearts were rapidly removed and perfused (at 37 °C) with Krebs-Henseleit bicarbonate (KHB) buffer

(mM: NaCl 118, KCl 4.7, CaCl₂ 1.25, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 25, N-[2-hydro-ethyl]-piperazine-N'-[2-ethanesulfonic acid] (HEPES) 10, glucose 11.1, pH 7.4). Hearts were subsequently perfused with Ca²⁺-free KHB containing 223 U/ml collagenase II (Worthington Biochemical Corp., Freehold, NJ, USA). After perfusion, the left ventricle was removed, minced and the cells were filtered through a nylon mesh (300 μ m). Myocytes were washed with Ca²⁺-free KHB buffer to remove remnant enzyme, and extracellular Ca²⁺ was added incrementally and slowly back to 1.25 mM. Isolated myocytes were then maintained in a defined medium consisting of Medium 199 with Earle's salts containing HEPES (25 mM) and NaHCO₃ (25 mM), supplemented with albumin (2 mg/ml), L-carnitine (2 mM), creatine (5 mM), taurine (5 mM), insulin (100 nM), penicillin (100 U/ml), streptomycin (100 μ g/ml), and gentamicin (5 μ g/ml). This myocyte culture medium contained either normal (NG, 5.5 mM) or high (HG, 25.5 mM) glucose. The high extracellular glucose is comparable to serum glucose levels in diabetic rats (Ren & Bode 2000). A subset of each medium was also supplemented with either IGF-1 (10, 100 and 500 nM), the PI-3 kinase/Akt inhibitor LY294002 (10 μ M), the mTOR inhibitor rapamycin (20 μ M) and the calcineurin inhibitors cyclosporin A (5 μ M) or tacrolimus (FK506, 10 mg/L), which functions through binding of FK506 binding protein 12 (FKBP12). DMSO was used as the solvent for some pharmacological inhibitors (e.g., LY294002) with a final concentration <0.2% in cell medium, which exerted no discernable effect on myocyte mechanics over the 8-h incubation period (data not shown). Our earlier studies indicated that the high glucose-induced myocyte contractile response was unlikely due to changes in extracellular osmotic pressure since replacement of glucose with equal molar (20 mM) D-mannitol exerted little effect on myocyte mechanics (Ren *et al.* 1997, 2003). The cells were maintained for 8 h at 37 °C in a 100% humidity and 5% CO₂ incubator (Ren *et al.* 2003).

Cell shortening/relengthening

Mechanical properties of ventricular myocytes were assessed using a SoftEdge MyoCam system (IonOptix Corporation, Milton, MA, USA). In brief, left ventricular myocytes were placed in a Warner chamber mounted on the stage of an inverted microscope (Olympus, IX-70) and superfused (~1 ml/min at 25 °C) with a buffer containing (in mM): 131 NaCl, 4 KCl, 1 CaCl₂, 1 MgCl₂, 10 glucose, 10 HEPES, at pH 7.4. The cells were field stimulated with suprathreshold voltage at a frequency of 0.5 Hz, 8 msec duration, using a pair of platinum wires placed on opposite sides of the chamber connected to a FHC stimulator (Brunswick, NE, USA). The myocyte being studied was displayed on a computer monitor using an IonOptix MyoCam camera (IonOptix Corporation,

Milton, MA, USA). IonOptix SoftEdge software was used to capture changes in cell length during shortening and relengthening (Ren *et al.* 2003).

Western blot analysis

Proteins from NG or HG-cultured ventricular myocytes with or without IGF-1 or selective pharmacological inhibitor treatment were extracted as described (Ren *et al.* 2003). Briefly, cardiac myocytes were lysed by sonication (3×5 s, 10 W) over ice in 0.5 ml RIPA lysis buffer containing 150 mM NaCl, 0.25 deoxycholic acid, 1% NP-40, 1 mM EDTA, 50 mM Tris-HCl, pH7.4, sodium orthovanadate 2 mM, and 1% protease inhibitor cocktail, and then by centrifugation (12 000 g, 10 min) to remove the precipitated material. Protein concentration was determined in the supernatant containing the soluble proteins using Bradford assay. The soluble proteins (50 μ g/lane) were separated on 10% (Akt, pAkt, p70s6k, phosphor-p70s6k (pp70s6k), GSK-3 β , phosphor-GSK-3 β (pGSK-3 β), phospholamban (PLB), phosphoserine of PLB, phosphothreonine of PLB) or 7% (mTOR, phosphor-mTOR (pmTOR), sarco(endo)plasmic reticulum Ca^{2+} -ATPase (SERCA2a)) SDS-polyacrylamide gels in a minigel apparatus (Mini-PROTEAN II, Bio-Rad) and transferred to nitrocellulose membranes. The membranes were blocked with 5% milk in TBS-T, and then incubated with anti-Akt (1:1000), anti-pAkt (Thr308) (1:1000), anti-mTOR (1:1000), anti-pmTOR (Ser2448) (1:1000), anti-p70 S6 kinase (1:1000), anti-pp70 S6 kinase (1:1000), anti-GSK-3 β (1:000) and phospho-GSK-3 β (Ser9) (1:1000) antibodies (Cell Signaling Technology, Inc., Beverly, MA, USA), anti-SERCA2a antibody (1:2000, MA3-919, Affinity BioReagent, Denver, CO, USA), anti-PLB antibody (1:5000, 2D12 were kindly provided by Dr Steven Cala, Wayne State University School of Medicine, Detroit, MI, USA), and anti-phosphoserine of PLB (1:2000) and anti-phosphothreonine of PLB (1:2000) antibodies (Zymed Laboratory Inc. South San Francisco, CA, USA). The antigens were detected by the luminescence method (ECL Western blotting detection kit, Amersham) with peroxidase-linked anti-rabbit IgG (1:5000 dilution). After immunoblotting, the film was scanned and the intensity of immunoblot bands was detected with a Bio-Rad Calibrated Densitometer (Model: GS-800).

Statistics

Data are presented as means \pm S.E.M. Statistical significance was ascertained by analysis of variance (ANOVA). When an overall significance was determined, a Dunnetts *post hoc* analysis was incorporated. $P < 0.05$ was considered significant. Control groups (i.e., NG and HG cells) were always

recorded on the same day as experimental groups (i.e., with and without drugs) in order to control for any potential inter-culture variability.

Results

IGF-1 protects against HG-induced prolonged relengthening

Culturing myocytes with HG, IGF-1 or pharmacological inhibitors had no overt effect on cell phenotype. Cell shape and presence of distinct striations were similar among all experimental groups. The resting cell length was comparable between NG and HG-cultured myocytes. However, incubation of IGF-1 appears to lengthen the resting cell length although such response was not consistent among the concentration range tested (Fig. 1A and 3A). As reported earlier (Davidoff & Ren 1997, Esberg & Ren 2003), myocytes maintained in HG medium exhibit reduced PS and \pm dl/dt associated with prolonged TR_{90} and normal TPS compared with myocytes maintained in NG medium (Fig. 1–3). Interestingly, the reduction in PS, \pm dl/dt and prolongation in TR_{90} in HG myocytes was ablated by 100 nM and 500 nM IGF-1 supplementations. Lower IGF-1 concentration (10 nM) did not affect the HG-induced mechanical defects. IGF-1 (100 nM) did not affect the mechanical properties in NG cells (Fig. 1–3). These results suggest that IGF-1 may effectively reconcile the HG environment induced cardiomyocyte mechanical defects simulating those in diabetes (Davidoff & Ren 1997, Ren & Bode 2000).

Inhibition of PI-3 kinase/Akt or mTOR but not calcineurin ablated IGF-1-induced protection against HG

To examine the potential signaling pathway(s) underscoring IGF-1-induced protection against glucose toxicity, NG and HG-cultured myocytes were co-incubated with selective blockers of PI-3 kinase/Akt, mTOR and calcineurin. Data shown in Figs 1, 2 and 3 indicated that the IGF-1-induced protection against HG-elicited mechanical defects was abolished by the PI-3 kinase/Akt inhibitor LY294002 and the mTOR inhibitor rapamycin but not the calcineurin inhibitors cyclosporine A or FK506. None of the inhibitors themselves affected myocyte mechanics with the exception that rapamycin prolonged TPS in HG myocytes (Figs 1–3). These data suggested involvement of PI-3 kinase/Akt and/or mTOR but unlikely calcineurin in IGF-1-induced protection against glucose toxicity-induced cardiomyocyte mechanical defects.

Western blot analysis of Akt, mTOR and p70s6k

IGF-1-induced cardiac contractile response has been shown to be mediated through post-receptor signaling pathways such as PI-3 kinase, the serine-threonine kinase

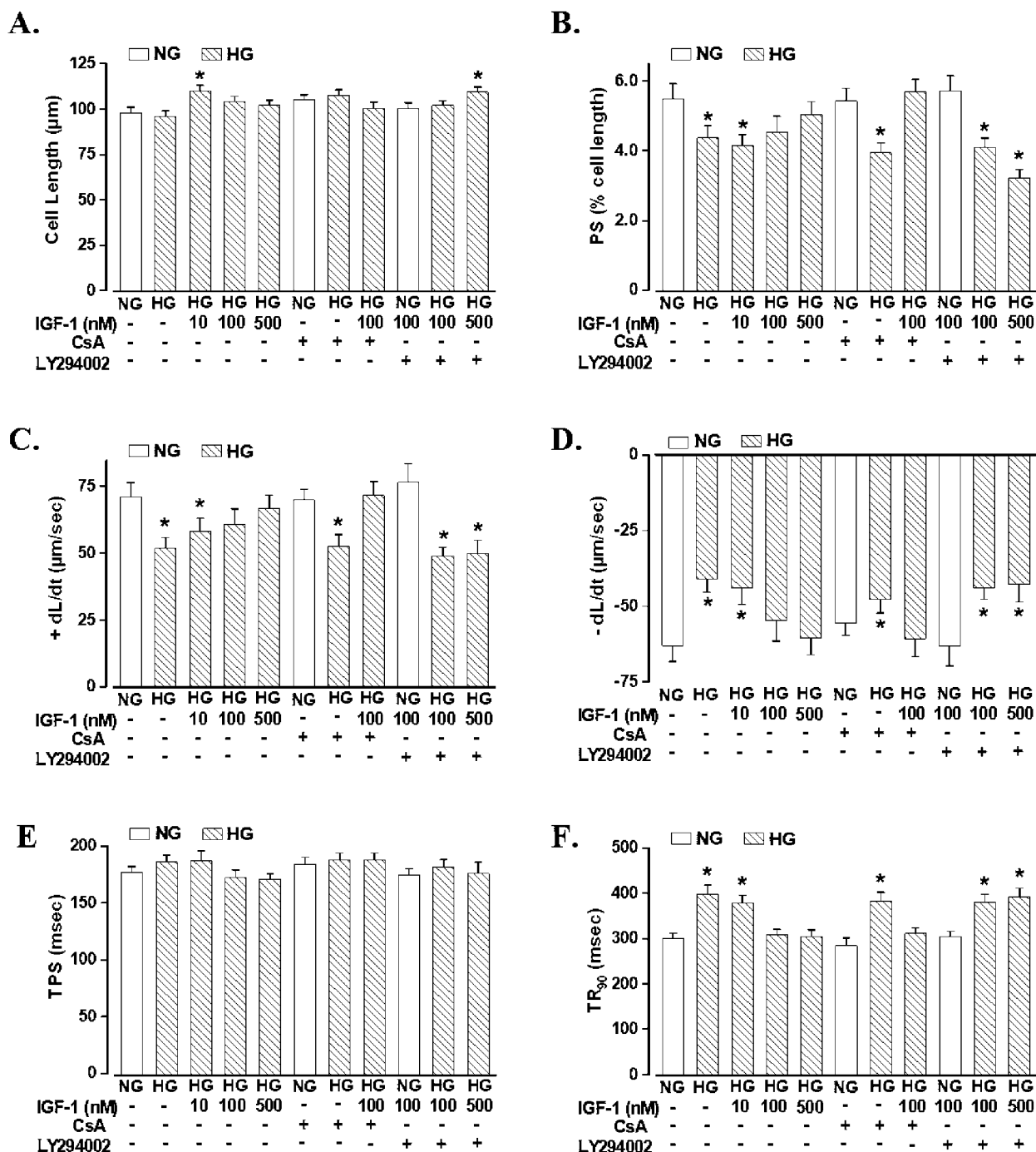


Figure 1 Contractile properties of left ventricular myocytes maintained for 8 h in medium containing either normal (NG, 5.5 mM) or high glucose (HG, 25.5 mM) supplemented with IGF-1 (10, 100 and 500 nM), the PI-3 kinase inhibitor LY294002 (10 µM) or the calcineurin inhibitor cyclosporin A (CsA, 5 µM). (A) Resting cell length (CL); (B) peak shortening (PS) normalized to CL; (C) maximal velocity of shortening (+ dL/dt); (D) maximal velocity of relengthening (- dL/dt); (E) time-to-PS (TPS); and (F) time-to-90% relengthening (TR₉₀). Means ± S.E.M., n=50–52 cells/group, *P<0.05 vs NG only group.

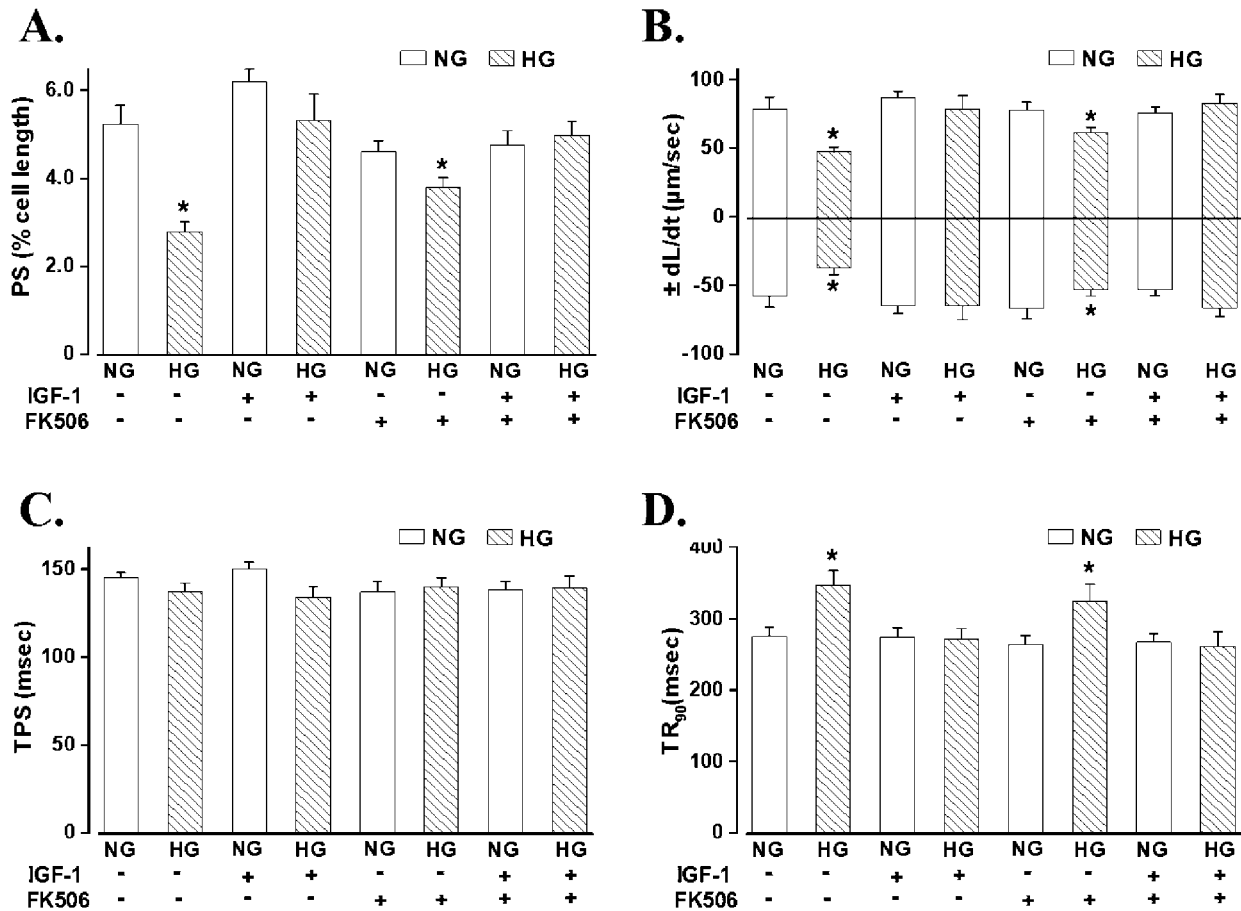


Figure 2 Contractile properties of left ventricular myocytes maintained for 8 h in NG or HG medium supplemented with IGF-1 (100 nM) or the calcineurin inhibitor FK506 (10 mg/l). (A) Peak shortening (PS) normalized to restign cell length (CL); (B) maximal velocity of shortening/relengthening (\pm dl/dt); (C) time-to-PS (TPS); and (D) time-to-90% relengthening (TR_{90}). Means \pm s.e.m., $n=60$ cells/group, * $P<0.05$ vs NG group.

Akt, mTOR and p70s6k (Ren 2000, Yamashita *et al.* 2001, Cui *et al.* 2003). This is supported by our mechanical data shown in Fig. 1 and Fig. 3. To further elucidate the role of Akt/mTOR/p70s6k under either NG or HG condition with or without IGF-1 addition, the protein expression of Akt, mTOR, p70s6k and activated forms of Akt, mTOR, p70s6k (pAkt, pmTOR, pp70s6k) were determined. Our immunostaining analysis revealed that while total Akt, total mTOR and total p70s6k levels were comparable among all cell groups (Fig. 4 and Fig. 5), the level of pAkt was both significantly and independently enhanced by HG and IGF-1. Paradoxically, IGF-1 significantly reduced HG-induced Akt phosphorylation. Inhibition of mTOR with rapamycin increased Akt phosphorylation in all groups tested (Fig. 4). Not surprisingly, IGF-1 stimulated the activation of mTOR shown as enhanced pmTOR in NG myocytes. HG significantly enhanced pmTOR and pp70s6k levels which may be

attenuated by IGF-1 (Fig. 4 and Fig. 5). Activation of mTOR was abolished by rapamycin, as expected (Fig. 4).

Western blot analysis of GSK-3 β , SERCA2a and PLB

Parallel to the mTOR/p70s6k pathway, the serine/threonine kinase GSK-3 β is another main downstream target for Akt activation (Hardt & Sadoshima 2002). We examined the expression of GSK-3 β and phosphorylation of GSK-3 β (phospho-GSK-3 β), along with essential Ca²⁺-regulating proteins SERCA2a, PLB and serine/threonine phosphorylation of PLB. Our data suggested that while the total GSK-3 β level was comparable among all cell groups, phosphorylation of GSK-3 β (phospho-GSK-3 β) was significantly reduced by HG which was ablated by IGF-1 (Fig. 5).

Protein expression of GSK-3 β , SERCA2a, PLB and phosphorylated PLB was unaffected by HG, IGF-1 or

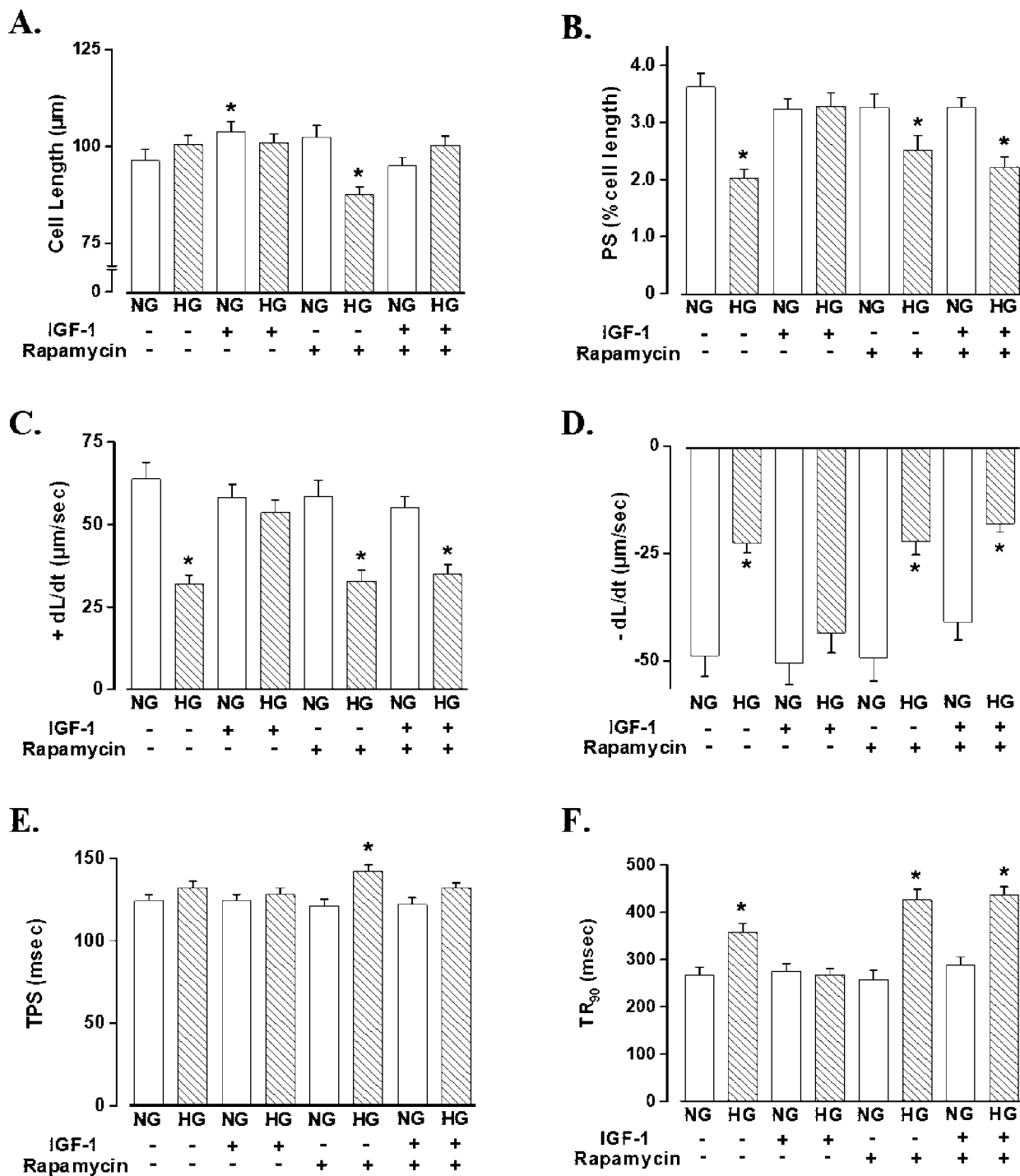


Figure 3 Contractile properties of left ventricular myocytes maintained for 8 h in NG or HG medium supplemented with IGF-1 (100 nM) or the mammalian target of rapamycin (mTOR) inhibitor rapamycin (20 μM). (A) Resting cell length (CL); (B) peak shortening (PS) normalized to resting cell length (CL); (C) maximal velocity of shortening (+ dL/dt); (D) maximal velocity of relengthening (- dL/dt); (E) time-to-PS (TPS); and (F) time-to-90% relengthening (TR₉₀). Means \pm s.e.m., $n=53-66$ cells/group, * $P<0.05$ vs NG only group.

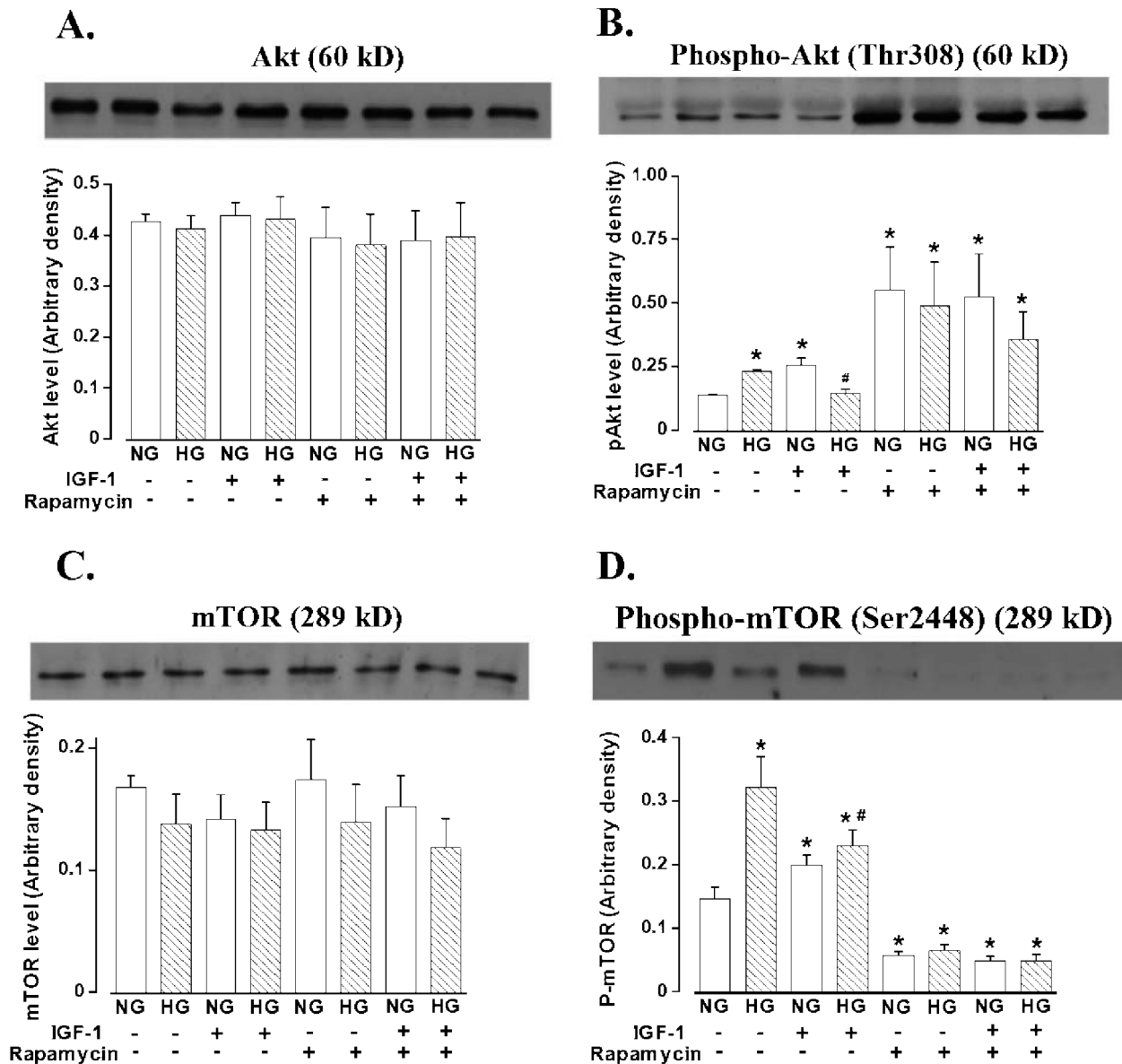


Figure 4 Protein abundance of Akt (A), pAkt (B), mammalian target of rapamycin (mTOR) (C) and p-mTOR (D) of myocytes maintained for 8 h in NG or HG medium supplemented with IGF-1 (100 nM) or the mTOR inhibitor rapamycin (20 μ M). Insets are actual gel blots using anti-Akt, anti-pAkt, anti-mTOR and anti-p-mTOR antibodies with the same order of cell groups shown in the x-axis labels. Means \pm s.e.m., $n=6-8$ cultures, * $P<0.05$ vs NG only group, # $P<0.05$ vs HG only group.

rapamycin (except that rapamycin itself slightly but significantly suppressed serine phosphorylation of PLB) (Fig. 6).

Discussion

Results from our current study indicated that IGF-1-elicited protective effect against glucose toxicity-induced cardiac contractile dysfunctions. The beneficial effect of

IGF-1 was mediated through a PI-3 kinase/Akt/mTOR/p70s6k-dependent mechanism, rather than a calcineurin-dependent pathway. Our western blot analysis further revealed that both high glucose and IGF-1 stimulated phosphorylation of Akt and mTOR, although the high glucose-stimulated upregulation of pAkt and pmTOR may be paradoxically attenuated by IGF-1. Our gel electrophoresis data demonstrated that p70s6k and GSK-3 β , both of which downstream signal molecules for PI-3 kinase/Akt, were activated and suppressed respectively, by

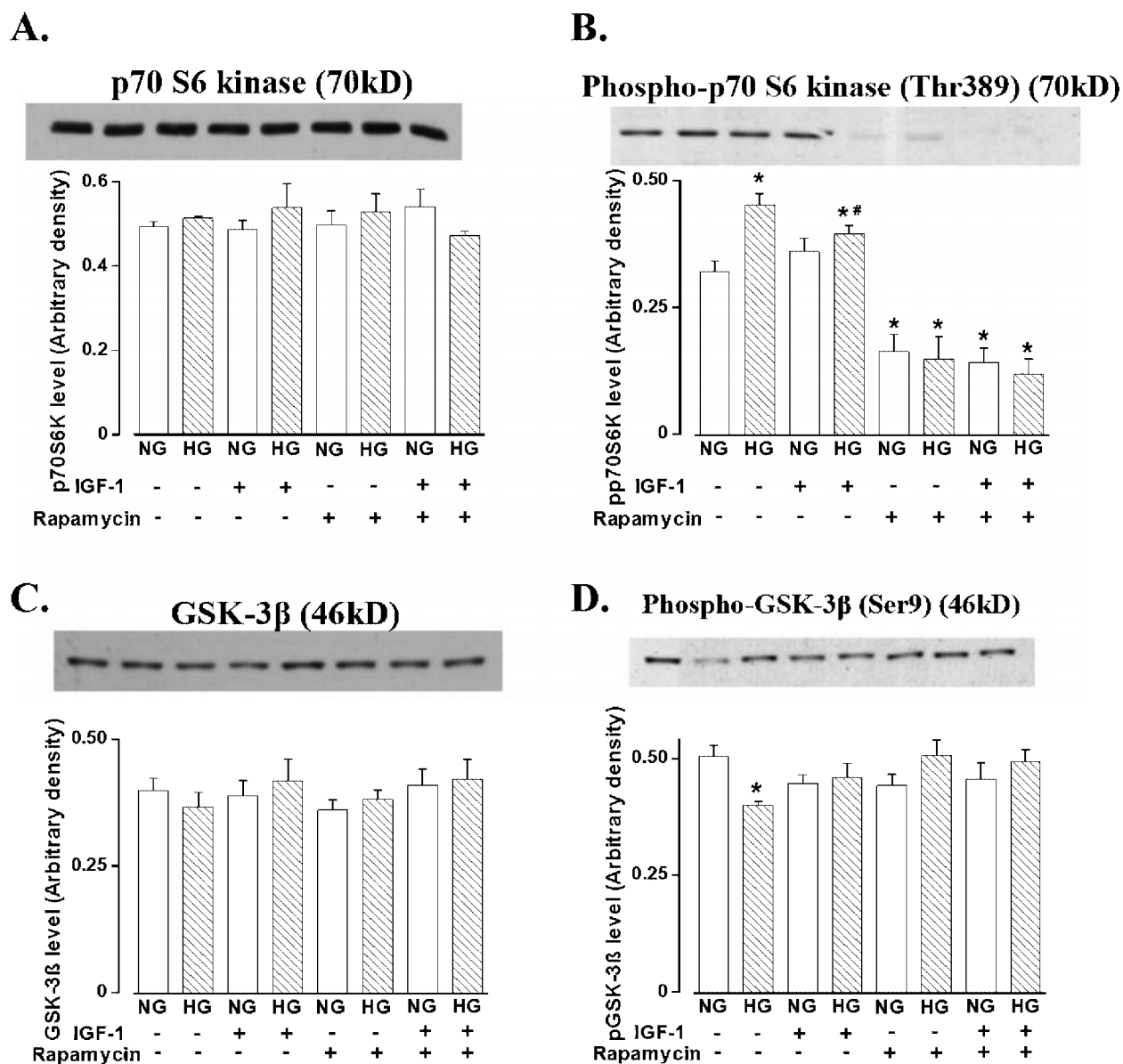


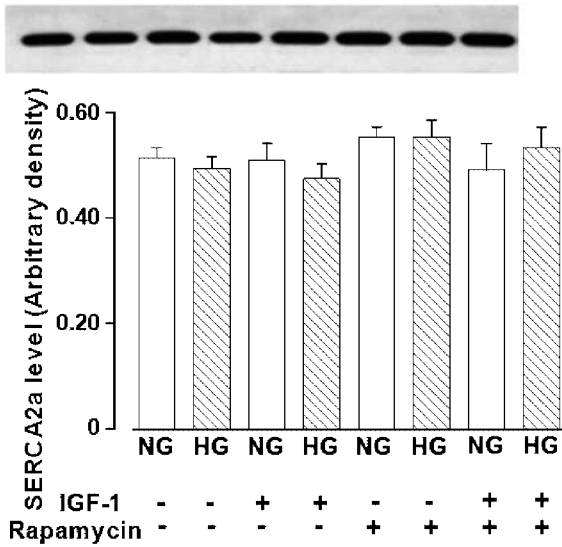
Figure 5 Protein abundance of p70s6k (A), phospho-p70s6k (B), GSK-3β (C) and phospho-GSK-3β (D) of ventricular myocytes maintained for 8 h in NG or HG medium supplemented with IGF-1 (100 nM) or the mammalian target of rapamycin (mTOR) inhibitor rapamycin (20 μM). Insets are actual gel blots using anti-p70s6k, anti-phospho-p70s6k, anti-GSK-3β and anti-phospho-GSK-3β antibodies with the same order of groups shown in the x-axis labels. Means ± S.E.M., $n=4$ cultures, * $P<0.05$ vs NG only group, # $P<0.05$ vs HG only group.

high glucose. Consistent with observations from Akt and mTOR phosphorylation, co-incubation of IGF-1 with ventricular myocytes reconciled the high glucose-induced alteration in p70s6k and GSK-3β phosphorylation. Collectively, our data indicated a possible feedback mechanism of IGF-1 on high glucose-induced activation of Akt and mTOR, resulting in an ultimate protection from IGF-1 against high glucose-induced changes in phosphorylation of p70s6k and GSK-3β. The unaltered

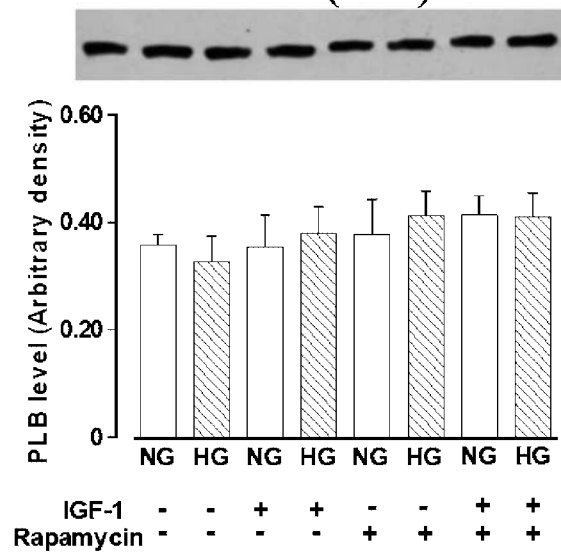
expression of SERCA2a and PLB in high glucose-treated cells (with or without IGF-1 or rapamycin) indicated that the adverse effects of glucose toxicity on myocyte mechanics are unlikely mediated through translational modification of these key Ca^{2+} regulatory proteins.

Hyperglycemia is probably the most important predisposing factor in diabetes-related cardiovascular complications and directly contributes to the onset of cardiac dysfunction in diabetes (Sowers & Epstein 1995,

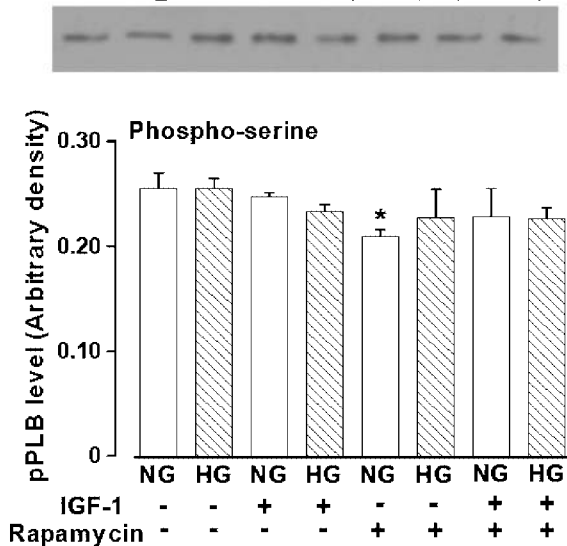
A. SERCA2a (110kD)



B. PLB (6kD)



C. Phospho-PLB (Ser) (6kD)



D. Phospho-PLB (Thr) (6kD)

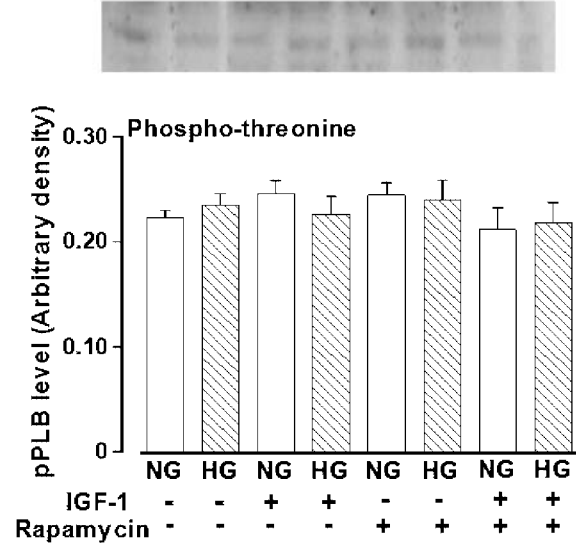


Figure 6 Protein abundance of SERCA2a (A), PLB (B), serine phosphorylation of PLB (C) and threonine phosphorylation of PLB (D) of myocytes maintained for 8 h in NG or HG medium supplemented with IGF-1 (100 nM) or the mammalian target of rapamycin (mTOR) inhibitor rapamycin (20 μM). Insets are actual gel blots using anti-SERCA2a, anti-PLB, anti-phosphoserine and anti-phosphothreonine antibodies with the same order of groups shown in the x-axis labels. Means ± s.e.m., n=4 cultures, *P<0.05 vs NG only group.

Ren *et al.* 1997, 1999b). Data from our present study confirmed our earlier observations in that ventricular myocytes maintained in high glucose medium exhibited depressed cardiac contractility, reduced maximal velocity of contraction and relaxation and prolonged duration of relaxation, all of which are reminiscent of diabetic

cardiomyopathy found in *in vivo* diabetes (Ren *et al.* 1997, Ren & Bode 2000). Prolonged relaxation and reduced cardiac contractility are hallmarks of diabetic cardiomyopathy and glucose toxicity-induced cardiomyocyte dysfunction (Davidoff & Ren 1997, Ren *et al.* 1999b, Esberg & Ren 2003). These glucose toxicity-induced

cardiac mechanical defects are believed to be mediated through a number of cellular events including impaired intracellular Ca^{2+} handling, altered excitation–contraction coupling and dysregulated cellular enzyme activity or gene expression in cardiac myocytes (Gupta & Wittenberg 1993, Smogorzewski *et al.* 1998, Esberg & Ren 2003, Ren *et al.* 2003). Our present study revealed that IGF-1 may improve the high glucose-induced cardiac mechanical dysfunction, consistent with *in vivo* findings from our own lab as well as other labs, using diabetic rodent models (Kajstura *et al.* 2001, Norby *et al.* 2002, 2004). IGF-1 is known to facilitate glucose metabolism, lower insulin levels, improve insulin sensitivity and lipid profile (Ren *et al.* 1999a, Samarel 2002), suggesting both physiological and therapeutic roles of the peptide. Several scenarios have been speculated for IGF-1-induced cardiac protection against stress such as diabetes in addition to its well established action on glucose transport and lipid metabolism. For example, IGF-1 may regulate membrane ionic channels responsible for cardiac excitation–contraction coupling (Solem & Thomas 1998). IGF-1 may also elicit cardiac protection against diabetes involving attenuated p53 function, angiotensin II production and angiotensin receptor activation (Kajstura *et al.* 2001). Our studies suggested that IGF-1 may rescue the diabetes-induced reduction in SERCA protein abundance and impaired β -adrenergic responsiveness (Norby *et al.* 2002, 2004). In addition, diabetic tissues including hearts usually display IGF-1 resistance (Rodgers *et al.* 1995, Kelley *et al.* 1999, Ren *et al.* 1999a, Ren 2000, Duan *et al.* 2003), making glucose clearing by IGF-1 largely dependent on cross-reaction with the insulin receptor, which requires higher levels of IGF-1 agonist.

Perhaps the most significant finding from our results is that the IGF-1-offered cardiac protection against glucose toxicity is mediated via a PI-3 kinase/Akt/mTOR/p70s6k-dependent rather than calcineurin-dependent pathway. Selective inhibitors of PI-3 kinase (LY294002) or mTOR (rapamycin) effectively ablated IGF-1-elicited cardiac protection against glucose-induced mechanical dysfunction in our cellular system. The PI-3 kinase/Akt/mTOR/p70s6k pathway was recently emerged as a modulator of insulin (and IGF-1)-mediated glucose metabolism (Tremblay & Marette 2001, Tremblay *et al.* 2005) as well as cardiac growth and survival (Hornberger *et al.* 2004, Oudit *et al.* 2004). Our immunoblot observation indicated that IGF-1 directly stimulated Akt (in NG cells) and mTOR activation (in NG and HG cells), which is consistent with the previous findings (Yamashita *et al.* 2001, Cui *et al.* 2003, Ren *et al.* 2003). However, it is somewhat surprising that IGF-1 failed to stimulate phosphorylation of p70s6k, downstream signal molecule for Akt/mTOR. On the other hand, our results depicted that 8 h of high glucose treatment directly stimulates phosphorylation of Akt, mTOR and p70s6k. The impact of glucose on phosphorylation of Akt in the

heart is somewhat controversial. Compromised Akt phosphorylation has been reported in both type 1 and type 2 diabetes as well as in cardiac myocytes exposed to a prolonged (24 h) high glucose environment (Huisamen 2003, Duan *et al.* 2003, Ren *et al.* 2003). Two scenarios may be considered for reduced Akt signaling in diabetes including ‘resistance’ to insulin-stimulated Akt activation and inhibition of Akt by elevated protein kinase C (PKC) activity in diabetes (Shiojima *et al.* 2002). However, recent evidence indicated that glucose-stimulated chronic Akt over-activation may contribute to ‘de-sensitization’ of Akt phosphorylation *en route* to insulin resistance (Ogihara *et al.* 2002). Over-phosphorylated Akt has been shown in cardiac tissues under both type 2 diabetes (Huisamen 2003) and hypertension (Ogihara *et al.* 2002). Hyperinsulinemia in insulin resistance and type 2 diabetes chronically activates Akt in the hearts, triggering a negative feedback loop to inhibit further Akt activation and excessive IRS-1 degradation (Haq *et al.* 2001). Thus, the activation status (acute vs chronic) of Akt is essential to cardiac function since acute Akt activation (such as via gene transfer) is known to regulate cell size, apoptosis, glucose metabolism, contraction and relaxation in cardiomyocytes (Latronico *et al.* 2004).

Probably the most puzzling findings from our study were that while both high glucose and IGF-1 stimulated phosphorylation of Akt and mTOR, IGF-1 itself significantly reduced the high glucose-induced activation of Akt, mTOR and p70s6k. Although we cannot provide a precise explanation of such findings at this time, several speculations may be made for this paradoxical phenomenon. First, it is possible that IGF-1-induced Akt phosphorylation is in the form of acute activation through IGF-1 or insulin receptor. On the other hand, high glucose-induced activation of Akt is in the form of chronic activation. While acute activation of PI-3 kinase/Akt has proven beneficial cardioprotective effects, the adverse consequences of chronic PI-3 kinase/Akt activation should be carefully elucidated. It was recently reported that chronic activation of Akt may lead to defective cardiac function (Nagoshi *et al.* 2005). Secondly, it is quite possible that activation of mTOR by IGF-1 may initiate a rapamycin-sensitive feedback phosphorylation of PI-3 kinase/Akt, which may diminish activated form Akt in high glucose environments (Fig. 7). This seems to be supported by the observation that rapamycin can overtly enhance the Akt phosphorylation in all groups. This is consistent with earlier reports that p70s6k may suppress the signaling through PI-3 kinase/Akt pathway, thus inhibition of p70s6k (through rapamycin) may boost Akt phosphorylation (Sarbasov *et al.* 2005). Inhibition of mTOR alone by rapamycin has also been demonstrated to stimulate Akt phosphorylation in a negative feed-back manner (Tremblay *et al.* 2005). Thirdly, inhibition of mTOR has been shown to markedly potentiate the ability of insulin to increase phosphorylation of Akt at the levels

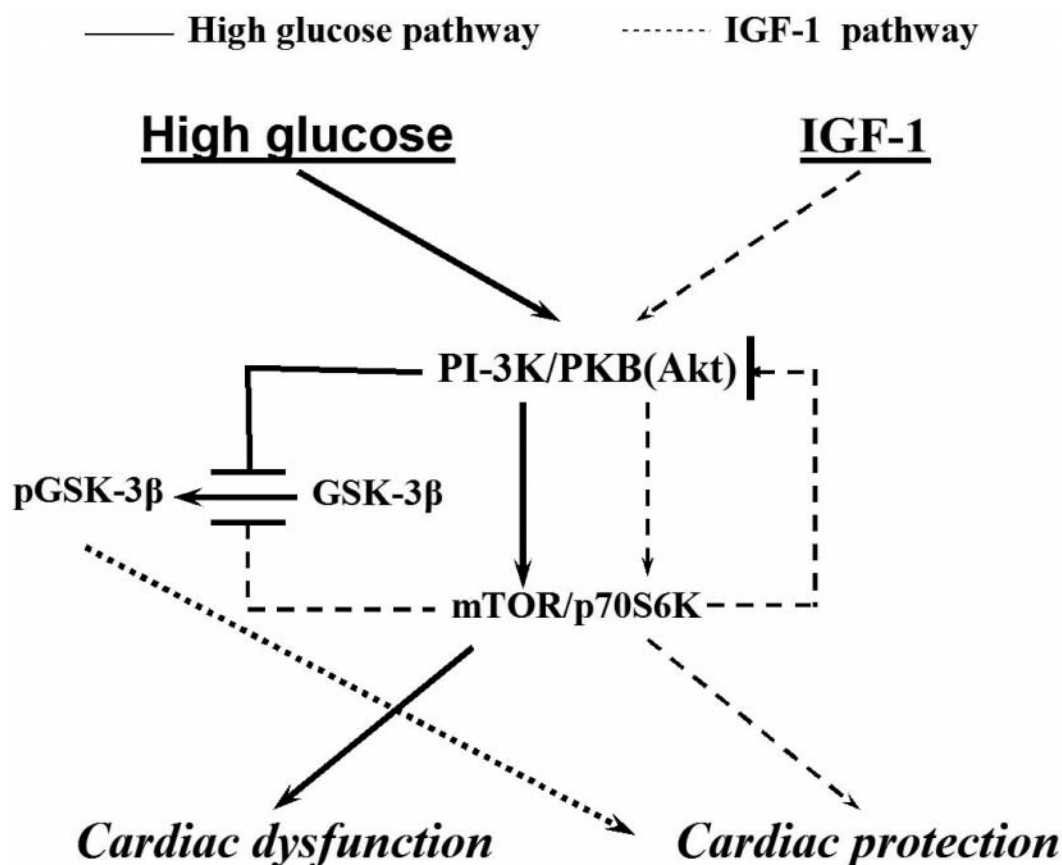


Figure 7 Schematic figure depicting the model of signaling mechanisms for high glucose (solid lines) and IGF-1 (dotted lines) in cardiac myocytes. Note the feedback inhibition (solid vertical line to the left of PI-3 kinase/Akt) of IGF-1-induced mammalian target of rapamycin (mTOR) activation at the level of PI-3 kinase/Akt (triggered by high glucose).

of plasma membrane and cytosol (Tremblay *et al.* 2005). This again, is consistent with the higher Akt phosphorylation in the presence of rapamycin. It is thus possible that activation of mTOR, offered by the insulin analogue IGF-1 may reduce the Akt phosphorylation (stimulated by high glucose). Last but not least, mice deficient for p70s6k (S6K1), an effector of Akt/mTOR, were shown to be hypoinsulinaemic, glucose intolerant and protected against age- and diet-induced obesity with enhanced insulin sensitivity (Um *et al.* 2004). The enhanced p70s6k phosphorylation under high glucose environment is consistent with enhanced Akt/mTOR phosphorylation in high glucose-treated cardiomyocytes and underscored the likelihood of a protective mechanism for IGF-1 since IGF-1 effectively alleviated high glucose-induced p70s6k phosphorylation.

GSK-3 β is a negative regulator of stress-induced cardiomyocyte hypertrophy and may serve to maintain normal cardiac growth, cell survival and contractile function (Michael *et al.* 2004). GSK-3 β is normally active in un-stimulated cells and is inhibited when cells are exposed

to either growth factors or the Wnt family of secreted glycoproteins. Our observation that the phosphorylation of GSK-3 β was depressed in high glucose-treated myocytes is consistent with the notion that this signal molecule is negatively regulated by Akt and p70s6k (both of which were activated by HG in our experimental setting) (Hardt & Sadoshima 2002, Michael *et al.* 2004). De-regulation of GSK-3 β through phosphorylation (in transgenic mice with wild-type GSK-3 β) leads to severe systolic and diastolic dysfunction and progressive heart failure accompanied with down-regulation of SERCA2a (Michael *et al.* 2004). Whether IGF-1-induced reversal against high glucose-suppressed GSK-3 β phosphorylation plays a role in IGF-1-offered cardiac protection is still elusive at this stage.

Taken together, our results indicate that the Akt/mTOR/p70s6k pathway is an important modulator of the signals involved in glucose toxicity-induced mechanical dysfunction in cardiac myocytes and seems to play an essential role in IGF-1-offered cardiac protection against glucose toxicity. Further investigation is warranted to

understand the precise mechanism underscoring the inter-relationship between the mTOR/p70s6k pathway and the PI-3 kinase/Akt pathway in the regulation of cardiomyocyte function and metabolism.

Acknowledgments

This work was supported by grants from the American Diabetes Association (07-RA-21), the American Heart Association Pacific Mountain Affiliate (#0355521Z), the National Institute of Aging (1R03 AG21324-01) and the University of Wyoming Faculty Grant-in-Aid to J R. IGF-1 was obtained from the National Hormone and Peptide Program, NIDDK and Dr Parlow (National Hormone and Peptide Program, NIDDK, NIH, Bethesda, MA, USA). The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received in final form 24 April 2005

Accepted 28 June 2005

Made available online as an Accepted Preprint

13 July 2005