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# Elevated glucose activates protein synthesis in cultured cardiac myocytes

Wen Yeshao[a,*], Jiali Gu[a], Xianghong Peng[a], Angus C. Nairn[b], Jerry L. Nadler[a]

[a]*Division of Endocrinology and Metabolism, Department of Internal Medicine, University of Virginia Health Science Center, Charlottesville, VA 22908, USA*
[b]*Department of Psychiatry, Yale University School of Medicine, New Haven, CT 06508, USA*

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**Abstract**

Diabetes mellitus results in chronic hyperglycemia, a serious metabolic disorder associated with a markedly increased risk of cardiovascular disease. However, the effects of high glucose (HG) on cardiac myocyte growth have not been fully clarified. In this study, the effect of glucose on cardiac myocyte growth was examined using leucine incorporation as an index of protein synthesis. High glucose (HG, 25 mmol/L) increased leucine incorporation (167% ± 0.2% over normal glucose, n = 4, $P < .01$) compared with a physiological glucose concentration (5.5 mmol/L, normal glucose). The HG-induced increase in leucine incorporation was time- and dose-dependent and was not due to osmotic changes because 25 mmol/L mannitol did not change leucine incorporation. High glucose also significantly reduced elongation factor 2 phosphorylation, an effect known to result in increased protein synthesis at the elongation step. Western blot analysis showed that HG-activated protein kinase B (PKB), also called Akt (PKB/Akt), at 18 hours. High glucose–induced leucine incorporation was attenuated with phosphatidylinositol 3-kinase (PI3K) inhibition using wortmannin and LY294002 and by rapamycin, a mammalian target of rapamycin (mTOR) inhibitor, 72%, 64%, and 65% ($P < .05$), respectively. High glucose also activated extracellular signal–regulated kinase 1/2 activity with peak stimulation at 5 minutes. In addition, PD98059, an inhibitor of mitogen-activated protein kinase kinase, attenuated HG-induced leucine incorporation. These data show for the first time that elevated glucose increases protein synthesis in cardiac myocytes. The increase appears to be mediated by activation of PI3K-PKB/Akt and/or PI3K-mTOR as well as extracellular signal–regulated kinase 1/2. These results provide new evidence for a direct effect of glucose independent of insulin on cardiac myocyte growth.


## 1. Introduction

Diabetes mellitus is a serious metabolic disorder associated with a markedly increased risk of cardiovascular diseases. Chronic hyperglycemia is a factor that induces certain biochemical changes associated with cell growth in diabetes mellitus. High glucose (HG) increases cell growth in a variety of cell types [1-4] including vascular smooth muscle cells [5]. Glucose has an additive effect with insulin on cell proliferation [6] and enhances angiotensin II (Ang II)–induced janus-activated kinase/STAT signaling in vascular smooth muscle cells [7]. High glucose increases diacylglycerol mass and activates protein kinase C [8] and c-*fos* and c-*jun* RNA and proteins in cultured mesangial cells [9]. In contrast, there is very limited information on HG effects in cardiac cells. High glucose has been shown to play an enhanced role in aldosterone-induced cardiomyocyte hypertrophy [10] and HG alone enhances cardiac fibroblast proliferation [11]. A recent report showed that HG had no effect on leucine incorporation vs normal glucose (NG) treatment in rat neonatal myocytes [12]. It has been well established that protein translation can be divided into two phases: initiation and elongation. There are data showing that HG increases phosphorylation of a binding protein of eukaryotic initiation factor 4E (eIF-4E), eIF-4E–binding protein 1 (4E-BP1, also known as PHAS-1), in islet cells [13,14]. The phosphorylation of 4E-BP1 induces its dissociation from eIF-4E and allows the factor to participate in translation initiation. Evidence has also shown that HG reduces phosphorylation of elongation factor 2 (EF-2), which is an essential step involved in extension of the polypeptide chain and in elongation phase of protein translation [15]. Therefore, in this study, we elucidated whether HG regulates protein synthesis in neonatal rat cardiomyocytes.

Our data show that HG alone reduces phosphorylation of EF-2 compared with cardiomyocytes in NG. We further demonstrate that HG induces increases in leucine incorpo-

* Corresponding author. Tel.: +1 434 243 4859; fax: +1 434 924 9730.
*E-mail address:* yw4w@virginia.edu (W. Yeshao).

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ration and activates protein kinase B (Akt/PKB) and extracellular signal–regulated kinase 1/2 (ERK1/2) in myocytes. These results suggest that HG regulates protein synthesis in cultured neonatal rat cardiac myocytes by multiple signaling pathways.

## 2. Methods

### 2.1. Cardiac myocyte isolation and culture

Neonatal rat cardiac myocytes were isolated from 2-day old Sprague-Dawley rat hearts based on the protocol in Reference [16] with modification. Hearts were removed following the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. The hearts were rinsed with calcium- and magnesium-free Hanks' Balanced Salt Solution (HBSS). They were minced and digested with collagenase in Leibovitz L15 medium and then digested with trypsin in calcium- and glucose-free HBSS. The dissociated cardiac cells were recovered step-by-step with addition of calcium chloride and finally suspended in growth medium (DMEM/F-12 [1:1] containing 5% horse serum, 20 mmol/L *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] [HEPES], 0.2% bovine serum albumin [BSA], 3 mmol/L pyruvic acid, 100 μmol/L ascorbic acid, 4 μg/mL transferrin, and 3.7 nmol/L sodium selenate). The dissociated cells were preplated for 1 hour, during which period the nonmyocytes attached readily to the bottom of the culture dish. The resultant suspension of myocytes was plated onto 0.2% gelatin-coated, 12-well culture plate with about 0.4 million cells per well. 5-Bromo-2′-deoxyuridine (100 μmol/L, Sigma, St Louis, Mo) was added to prevent proliferation of nonmyocytes. After the myocytes were allowed to attach for 18 to 24 hours, the medium was changed into serum-free DMEM medium containing all 18 types of essential amino acids including leucine (0.8 mmol/L), the same concentration of HEPES, BSA, pyruvate, ascorbic acid, 5-bromo-2′-deoxyuridine, transferrin, and selenium. All experiments were conducted after 72 hours of depletion. Using this isolation method, we routinely obtained myocyte-rich cultures of greater than 90% myocytes, as assessed by microscopic observation of cell beating and by immunofluorescence staining with a monoclonal antibody against sarcomeric α-actin. The animal protocol was approved by the Animal Care and Use Committee, University of Virginia.

### 2.2. Cell treatment with glucose and inhibitors

After 3 days of depletion, cells were then treated with 1 mL depletion DMEM medium containing 18 types of essential amino acid including leucine (0.8 mmol/L), 20 mmol/L HEPES, 0.2% BSA, 3 mmol/L pyruvic acid, 100 μmol/L ascorbic acid, 4 μg/mL transferrin, 3.7 nmol/L sodium selenate, and normal (5.5 mmol/L) or high (25 mmol/L) D-glucose for indicated time. Phosphatidylinositol 3-kinase (PI3K) inhibitors, wortmannin (100 nmol/L) [17] and LY294002 (2 μmol/L) [18], an inhibitor of mTOR, rapamycin (100 nmol/L) [19], and an inhibitor of mitogen-activated protein kinase kinase (MEK) [20], PD98059 (25 μmol/L) [21], were used (Biomol Research Laboratories, Pa). Cells were preincubated with inhibitors for 30 minutes and then treated with NG or HG along with inhibitors.

### 2.3. Elongation factor 2 phosphorylation measurement

Elongation factor 2 protein phosphorylation was measured using Western blot with phospho-specific antibodies generously provided by Dr Angus Nairn of Yale University, New Haven, Conn, as described in Reference [15]. Fifty micrograms of protein was resolved through 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred to a polyvinylidene difluoride (PVDF) membrane. After blocking and incubation with the primary antibody and a horseradish peroxidase–coupled goat anti-rabbit antibody, the protein bands are visualized with enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Piscataway, NJ). The same blot was stripped and reprobed with nonphosphorylated forms of antibody (Santa Cruz Biotech Inc, Santa Cruz, Calif). Autoradiograms of protein kinase activity were analyzed with AlphaImager 2200 Documentation and Analysis System (Alpha Innotech Corporation, San Leandra, Calif). Measurements were made in the linear range and the values were expressed as the ratio of the density of phosphorylated protein band over nonphosphorylated band, and the results were finally expressed as the fold of elevated glucose condition over NG condition.

### 2.4. Measurement of protein synthesis using leucine incorporation

Equal numbers of cardiac myocytes (400 000 cells per well) were seeded in each well of 12-well plates. Cells were treated with 1 mL serum-free DMEM medium containing all 18 types of essential amino acids including leucine (0.8 mmol/L), 20 mmol/L HEPES, 0.2% BSA, 3 mmol/L pyruvic acid, 100 μmol/L ascorbic acid, 4 μg/mL transferrin, 3.7 nmol/L sodium selenate, and normal (5.5 mmol/L) or high (25 mmol/L) D-glucose, NG, or HG medium supplemented with 2 μCi/mL of [$^3$H] leucine (170 Ci [6.38 TBq]/mmol, NEN Life Sciences, Boston, Mass) for 24 hours as described in Reference [22]. The medium was aspirated and cells were rapidly washed twice with 1 mL cold HBSS solution and once with 1 mL 10% trichloroacetic acid (TCA) and incubated in 1 mL fresh 10% TCA at 4°C for 30 minutes. The TCA insoluble material was solubilized in 0.1 N NaOH at 24°C for 2 hours. $^3$H-leucine incorporation was determined with liquid scintillation spectrometry. The scintillation counting was started after 2 hours of dark adaptation and the counting time was 10 minutes per vial. Cardiac myocytes after 3 days no longer divide, and because an equal number of isolated cells were seeded in each well, it is assumed that the cell number was very closely matched between wells. To increase the confidence of the findings, 4 wells of cells were

used for each treatment condition. The results were calculated as the fold of leucine incorporation in HG condition over that in NG condition. Finally, the results were shown as mean ± SEM from at least 3 separated experiments using different batches of isolated cells.

### 2.5. PKB/Akt, $p70^{s6k}$, and ERK1/2 activity measurement

PKB/Akt, ERK1/2, and $p70^{s6k}$ activity was measured using Western blot with phospho-specific antibodies (Santa Cruz). After treatment, cells were lysed in lysis buffer. Fifty micrograms of protein was resolved through 12% SDS-PAGE and subsequently transferred to a PVDF membrane. After blocking and incubation with the primary antibody, a horseradish peroxidase–coupled goat anti-rabbit secondary antibody was added. The protein bands are visualized with enhanced chemiluminescence detection system (Amersham Pharmacia Biotech). The same blot was stripped and reprobed with nonphosphorylated forms of the antibody. Autoradiograms of protein kinase activity were analyzed (AlphaImager 2200 Documentation and Analysis System, Alpha Innotech Corporation). The integrated density value of phosphorylated protein band was subtracted by blank integrated density value with the same size of area adjacent to the phosphorylated protein band. Measurements were made in the linear range and the values are expressed as the ratio of the density of phosphorylated protein band over nonphosphorylated band and finally expressed as the fold of elevated glucose condition over NG condition.

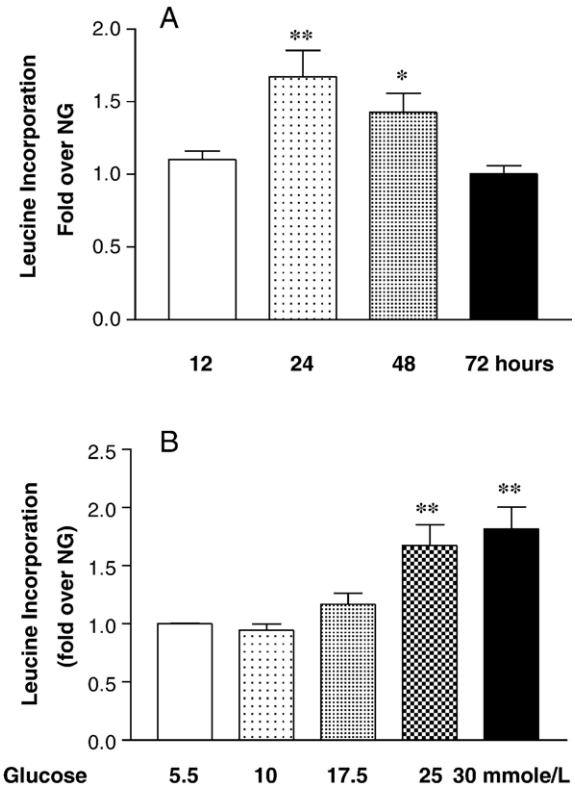


Fig. 1. The time course and dose-dependent effects of HG-induced leucine incorporation. Equal numbers of cardiac myocytes were seeded and grown on 12-well plates in growth medium with 5% horse serum for 24 hours. Cells were depleted in 1 mL serum-free DMEM medium containing all 18 essential amino acids including leucine (0.8 mmol/L), 20 mmol/L HEPES, 0.2% BSA, 3 mmol/L pyruvic acid, 100 μmol/L ascorbic acid, 4 μg/mL transferrin, 3.7 nmol/L sodium selenate, and NG (5.5 mmol/L) for 3 days. A, Myocytes were treated with 1 mL same depletion medium containing 25 (HG medium) or 5.5 mmol/L glucose (NG medium) supplemented with 2 μCi/mL $^3$H-leucine for indicated hours. B, Myocytes were treated with 1 mL same medium containing 5.5, 10, 17.5, 25, or 30 mmol/L glucose supplemented with 2 μCi/mL $^3$H-leucine for 24 hours. Cells were washed and treated for determination of $^3$H-leucine incorporation as described in Methods. Four wells of cells were for each treatment condition. Leucine incorporation was calculated as fold of incorporation under HG condition over that under NG condition. The results are expressed as mean ± SEM from 4 separate experiments using different batches of cells. $^{**}P < .01$ vs NG; $^{*}P < .02$ vs NG.

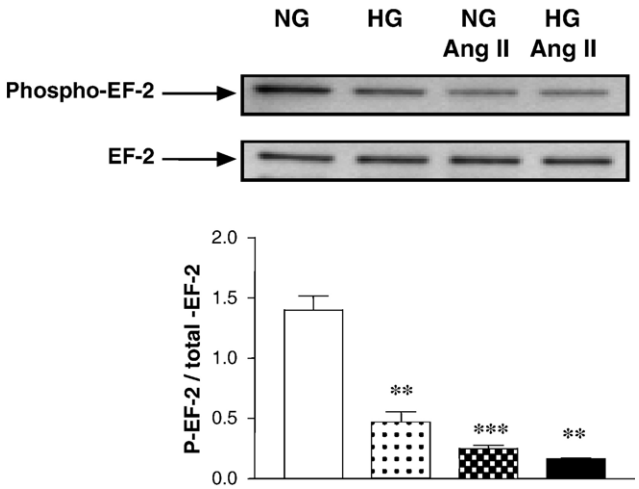


Fig. 2. High glucose reduces EF-2 protein phosphorylation in neonatal rat cardiomyocytes. Myocytes were seeded in 60-mm plates with 3 million cells each and were grown, depleted in the same condition as described in Fig. 1. Myocytes were then treated with NG, HG, or Ang II for 5 minutes. Elongation factor 2 phosphorylation activity was measured using Western blot with phospho-specific antibody against phosphorylated EF-2 protein (top panel). The PVDF membrane was stripped and blotted with a total EF-2 antibody (bottom panel). Densitometric quantification of phosphorylation levels is shown at the bottom of the figure, and data were expressed as the ratio of the density of phosphorylated band over total EF-2. Each point is an average (mean ± SE) of 3 separate experiments.

### 2.6. Data analysis

The results are expressed as mean ± SEM from at least 3 separate experiments using different batches of cells. Analysis of variance with Dunnett or Tukey-Kramer multiple comparison tests were used to analyze the data.

## 3. Results

### 3.1. High glucose increases leucine incorporation in cardiac myocytes

Isolated cardiac myocytes were placed in depletion medium (serum-free, NG [5.5 mmol/L D-glucose]) for 3 days and cells were then treated with depletion medium with normal or 25 mmol/L D-glucose. Fig. 1A shows the time course of HG-induced leucine incorporation. The

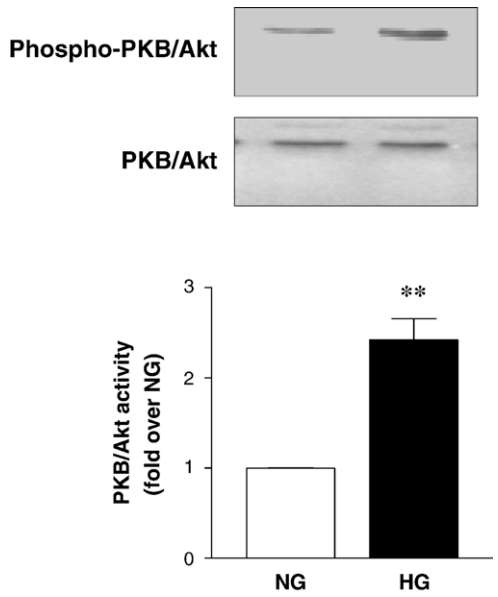


Fig. 3. High glucose activates PKB/Akt activity. Myocytes were seeded in 60-mm plates with 3 million cells each and were grown, depleted in the same condition as described in Fig. 1. Myocytes were then treated with NG or HG for 18 hours. Cells were lysed and proteins were extracted. Fifty micrograms of protein was resolved through 12% SDS-PAGE and subsequently transferred to a PVDF membrane. After blocking, the membrane was incubated with phospho-specific anti-Akt(Ser473) antibody. The incubation with the second antibody and the visualization were described in Methods. The membrane was stripped for blotting with a nonphosphoprotein antibody. The upper panel illustrates a representative Western blotting of phosphorylated or nonphosphorylated PKB protein bands. The lower panel illustrates the quantification of the PKB protein bands by scanning densitometry. Each bar is the mean ± SEM of 3 separate experiments. $^{**}P < .01$ vs NG.

leucine incorporation reached a peak at 24 hours (4820 ± 284 cpm [HG]; 1.67-fold over NG, 2889 ± 198 cpm; $P < .01$) and this effect was sustained to 48 hours (4760 ± 288 cpm [HG]; 1.42-fold over NG, 3298 ± 242 cpm; $P < .02$) and then dropped down to the basal level at 72 hours. Fig. 1B shows the dose-dependent effects of glucose on leucine incorporation. The increase in leucine incorporation became significant when glucose concentration was 25 (1.67 ± 0.2-fold of NG, n = 4, $P < .01$) or 30 mmol/L (1.82 ± 0.2-fold of NG, n = 4, $P < .01$). To inspect if osmotic pressure played a role in HG-induced leucine incorporation, 3 groups of myocytes were treated with NG (5.5 mmol/L D-glucose), 5.5 mmol/L D-glucose + 25 mmol/L mannitol, or HG (30 mmol/L D-glucose), respectively. Leucine incorporation in the cells treated with 5.5 mmol/L D-glucose + 25 mmol/L mannitol was similar to cells treated with 5.5 mmol/L D-glucose, suggesting that osmotic pressure did not play a role in HG-induced leucine incorporation.

### 3.2. High glucose reduces EF-2 protein phosphorylation in cardiac myocytes

To further evaluate the effect of HG on protein synthesis, the effect of HG on EF-2 phosphorylation was studied. The top panel of Fig. 2 shows phosphorylated EF-2 protein bands and nonphosphorylated EF-2 protein bands from a representative gel. The lower panel of Fig. 2 shows a densitometric quantification of phosphorylation levels, which were expressed as the ratio of the density of phosphorylated band vs nonphosphorylated band. High glucose significantly reduced EF-2 protein phosphorylation at 5 minutes by 64% ($P < .01$, n = 3). Angiotensin II (0.1 μmol/L) was used as a positive control. Angiotensin II + HG did not further reduce the levels of EF-2 phosphorylation.

### 3.3. High glucose activates PKB/Akt in cardiac myocytes

The PI3K-PKB/Akt pathway plays an important role in cell protein synthesis. We therefore first tested whether HG activates PKB/Akt in cardiac myocytes. The cellular proteins were extracted after 0 to 48 hours of treatment with HG. High glucose activation of PKB/Akt occurred at 12 hours (1.65 ± 0.17-fold over that of NG at the same time point, n = 3, $P < .05$) but reached a maximum at 18 hours (2.42 ± 0.23-fold over that of NG at the same time point, n = 3, $P < .01$), and the effect was sustained to 24 hours (1.94 ± 0.34-fold over that of NG at the same time point, n = 3, $P < .01$) and then the level decreased at 48 hours. Fig. 3 shows a representative Western blot of phosphory-

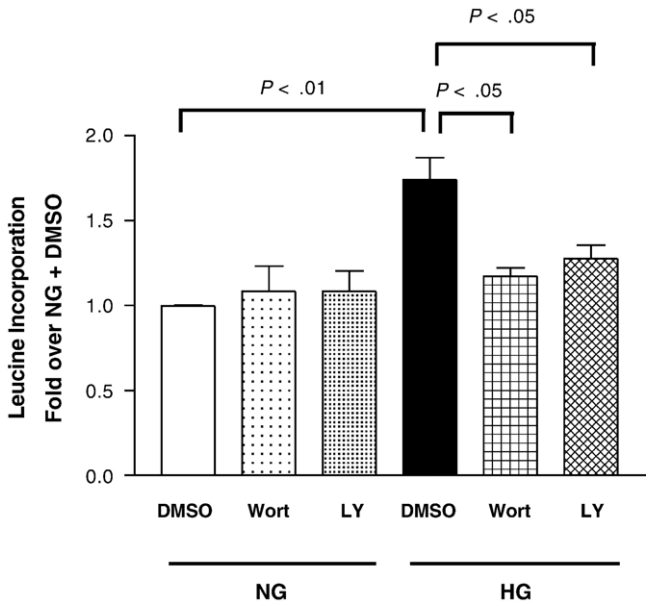


Fig. 4. The inhibitory effects of wortmannin and LY294002 on HG-induced leucine incorporation. Equal number of myocytes were seeded and grown, depleted in the same condition as described in Fig. 1. Myocytes were pretreated with wortmannin (100 nmol/L) or LY294002 (2 μmol/L) for 30 minutes and cells were then treated with NG or HG medium containing the same concentration of wortmannin or LY294002 and 2 μCi/mL $^{3}$H-leucine for 24 hours and the $^{3}$H-leucine incorporation was determined as described in Methods. Four wells of cells were for each treatment condition. The incorporation was calculated as fold of incorporation in NG + DMSO condition. The results are expressed as mean ± SEM from 3 separate experiments using different batches of cells. $P < .01$ between HG + DMSO and NG + DMSO. $P < .05$ between HG + DMSO and HG + wortmannin or LY294002.

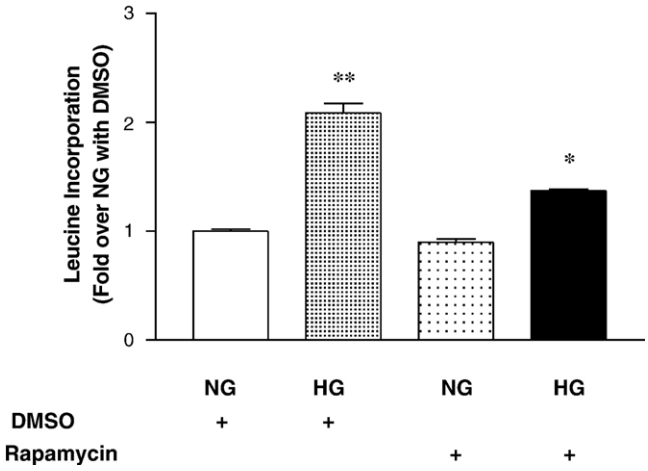


Fig. 5. The inhibitory effect of rapamycin on HG-induced leucine incorporation. Equal number of myocytes were seeded and grown, depleted in the same condition as described in Fig. 1. Myocytes were pretreated with rapamycin (100 nmol/L) for 30 minutes and cells were then treated with NG or HG medium containing the same concentration of rapamycin and 2 $\mu$Ci/mL $^3$H-leucine for 24 hours and the $^3$H-leucine incorporation was determined as described in Methods. Four wells of cells were for each treatment condition. The incorporation was expressed as fold over that in NG + DMSO condition. Finally, the results are expressed as mean ± SEM from 3 separate experiments using different batches of cells. $^{**}P < .01$ vs NG with DMSO vehicle; $^{*}P < .05$ vs HG.

lated PKB/Akt and the corresponding total PKB/Akt as a control under NG and HG conditions (upper panel). The densitometric quantification of kinase activities were shown in lower panel at 18 hours.

PKB/Akt is a downstream target of PI3K. To further address whether the PI3K pathway mediates HG-induced leucine incorporation, two specific PI3K inhibitors were used. Cardiac myocytes were pretreated with wortmannin (100 nmol/L) or LY294002 (2 $\mu$mol/L) or with a vehicle (dimethyl sulfone [DMSO]) for 30 minutes and then treated with NG or HG in the presence of inhibitors and $^3$H-leucine for 24 hours. Fig. 4 again shows that HG increased leucine incorporation by 1.74 ± 0.2-fold vs NG. Interestingly, 100 nmol/L wortmannin and 2 $\mu$mol/L LY294002 inhibited HG-induced leucine incorporation by 72% and 64%, respectively. Neither inhibitor affected basal leucine incorporation.

### 3.4. Rapamycin inhibits HG-induced leucine incorporation

Rapamycin, a specific inhibitor of mTOR, blocks the effects of insulin or phorbol ester on the phosphorylation of 4E-BP1. Rapamycin was used for evaluating whether mTOR mediates HG-induced leucine incorporation. Cardiac myocytes were preincubated with rapamycin (100 nmol/L) or the vehicle DMSO for 30 minutes and then treated with NG or HG in the presence of rapamycin or DMSO. Fig. 5 shows that 30 mmol/L glucose increases leucine incorporation in the presence of DMSO. However, HG only partially increased leucine incorporation in the presence of rapamycin. To elucidate the possible action of p70$^{s6k}$, a protein kinase that phosphorylates ribosomal protein S6 in mammalian cells, we measured p70$^{s6k}$ activity in cardiac myocytes treated with HG or NG using Western blot with a phospho-specific anti-p70$^{s6k}$ antibody and with an anti-p70$^{s6k}$ antibody to detect the corresponding total p70$^{s6k}$ protein. No difference in p70$^{s6k}$ activity was seen in HG or NG condition, suggesting that p70$^{s6k}$ does not play a role in HG-induced protein synthesis (data not shown).

### 3.5. High glucose activates ERK1/2 activity in cardiac myocytes

Extracellular signal–regulated kinase 1/2 is a key growth related signal. Extracellular signal–regulated kinase 1/2

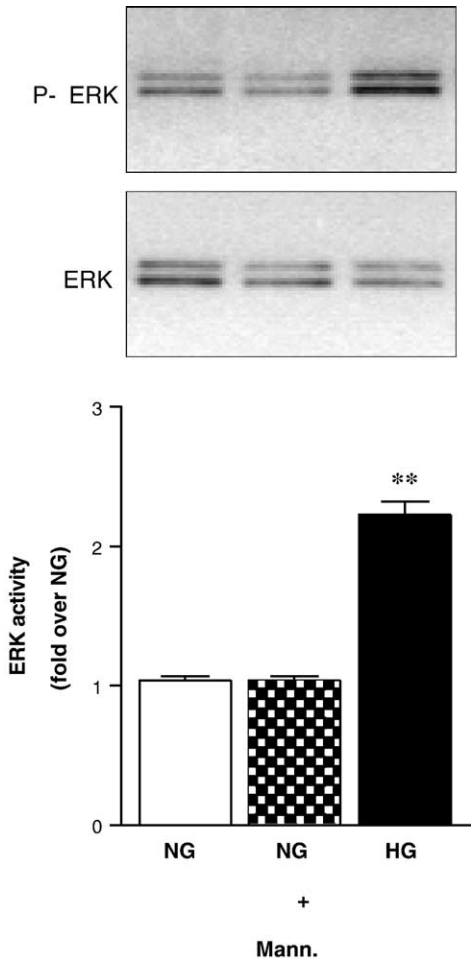


Fig. 6. High glucose activates ERK1/2 activity and mannitol does not change ERK1/2 activity. Myocytes were seeded in 60-mm plates with 3 million cells each and grown, depleted in the same condition as described in Fig. 1. Myocytes were then treated with NG or HG or NG plus 20 mmol/L mannitol for 5 minutes. The cells were then lysed, and equal amounts of protein extractions were examined for ERK activity by using Western blot with phospho-specific ERK antibody. The same membrane was stripped and reprobed with ERK antibody as described in Methods. The upper panel of Fig. 7 shows pairs of phosphorylated or nonphosphorylated ERK1/2 protein bands under 5.5 mmol/L glucose or 5.5 mmol/L glucose + 20 mmol/L mannitol or 25 mmol/L glucose conditions in a representative experiment. Protein band density was quantitated with scanning densitometry as shown in lower panel. The value for NG was taken as 1. The results are expressed as mean ± SEM from 3 separate experiments using different batches of cells.

activity was therefore measured in cell extracts after culture in NG or HG. High glucose induced an increase in ERK1/2 activity as early as 5 minutes and this was sustained to 10 minutes compared with the NG condition. High glucose did not produce an increase in ERK1/2 activity at 30 minutes or 2 hours (data not shown). The upper panel of Fig. 6 shows phosphorylated and nonphosphorylated ERK1/2 protein bands under 5.5 mmol/L glucose or 5.5 mmol/L glucose + 20 mmol/L mannitol or 25 mmol/L glucose conditions in a representative experiment. The lower panel of Fig. 6 illustrates the quantification of the phosphorylated ERK protein bands by scanning densitometry. The data show a 2.3-fold increase of ERK1/2 activity by HG at 5 minutes ($P = .01$ vs NG, n = 3) without a change in total ERK1/2 protein expression in NG- or HG-treated samples. Mannitol was also used to evaluate the osmotic pressure effect and the result is also shown in the lower panel of Fig. 6. Extracellular signal–regulated kinase 1/2 activity with 5.5 mmol/L glucose + 20 mmol/L mannitol was similar to the basal activity in NG condition, suggesting that osmotic pressure was not the mechanism whereby HG increased the ERK1/2 activity. To elucidate the role of ERK1/2 activation in HG-induced protein synthesis in cardiac myocytes, PD98059, an inhibitor of MEK (the upstream activator of ERK1/2), was used. Fig. 7 shows that 25 $\mu$mol/L PD98059 completely inhibited HG-induced increases of leucine incorporation ($P < .01$ vs DMSO vehicle under HG condition). PD98059 had no effect on leucine incorporation in cells incubated in NG.

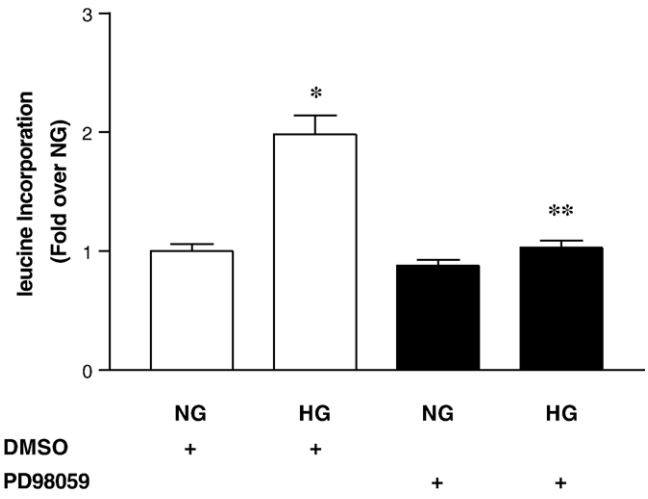


Fig. 7. PD98059 inhibits HG-induced leucine incorporation. Equal number of myocytes were seeded and grown, depleted in the same condition as described in Fig. 1. Myocytes were pretreated with PD98059 (25 $\mu$mol/L) for 30 minutes and cells were then treated with NG or HG medium containing the same concentration of PD98059 and 2 $\mu$Ci/mL $^3$H-leucine for 24 hours and the $^3$H-leucine incorporation was determined as described in Methods. Four wells of cells were for each treatment condition. The incorporation was first calculated as fold over that in NG + DMSO condition, and the results were expressed as an average of 3 separate experiments using different batches of cells. $^{**}P < .01$ vs NG and $^{**}P < .01$ vs HG with DMSO.

## 4. Discussion

In this study, in addition to the use of EF-2 dephosphorylation, leucine incorporation was also used as an index of protein synthesis. The medium we used contained all 18 types of essential amino acids including L-leucine at 105 mg/L or 0.8 mmol/L. The depletion medium contained the same concentrations of the 18 essential amino acids. The medium used for treatment of cells was the same depletion medium with either 5.5 (NG treatment medium) or 25 mmol/L D-glucose (HG treatment medium). Evidence has shown that extracellular leucine is the main pool of specific radioactivity of leucine [23]. There is no report showing that glucose concentration would affect intracellular or extracellular concentration of leucine. It is possible that the precursor leucine pool varied between experiments. However, we feel that this is very unlikely given the sufficient amount of cold leucine in the media used. We assume that the specific radioactivity of the leucine pool was similar in NG and HG conditions. Therefore, the specific radioactivity of leucine incorporation was used as an indication of protein synthesis.

Our results clearly demonstrate for the first time that HG stimulates leucine incorporation in a time- and dose-depended manner in neonatal cardiac myocytes. The difference of osmotic pressure between HG and NG condition is not the mechanism resulting in the protein synthesis increase induced by HG because 25 mmol/L mannitol did not increase leucine incorporation compared with the baseline. Our results on HG-induced leucine incorporation are different from a report [12] showing that HG has no effect on leucine incorporation. It is not entirely clear why the results varied. However, it is noted that there were major methodological differences between the studies. Collagenase II was used for the myocyte isolation in the earlier report [12]. In contrast, in our myocyte isolation procedure, minced heart tissues were digested once with collagenase II in Leibovitz L15 medium and then repeatedly digested by trypsin in calcium- and glucose-free HBSS. In our study, we emphasized that the dissociated cardiac cells were recovered step-by-step with addition of calcium chloride after trypsin digestion under a calcium-free condition. It is known that calcium plays an important role in cardiac cell function. And finally, the cell suspension medium is also different: DMEM medium with 10% fetal calf serum in Reference [12] vs DMEM/F-12 (1:1) medium containing 5% horse serum. It is very likely that the cell isolation technique has a major effect on cell behavior to HG.

We also show that HG has a regulatory effect on protein synthesis in myocytes by demonstrating that HG significantly reduced EF-2 phosphorylation, which is an essential step involved in extension of the polypeptide chain in protein translation. Additional evidence for this pathway is revealed using Ang II as a positive control. Angiotensin II leads to a significant reduction in EF-2 phosphorylation in

primary cultured neonatal rat cardiomyocytes [16]. The mechanism of HG-induced reduction of EF-2 phosphorylation in cardiac myocytes remains to be elucidated. The dephosphorylation of EF-2 and the subsequent activation of protein elongation no doubt demonstrate that HG activates protein synthesis in cardiac myocytes. The time course of HG-induced reduction of EF-2 phosphorylation indicated that the peak time of dephosphorylation was at 5 minutes, which does closely match the peak time (10 minutes) of Ang II–induced EF-2 dephosphorylation in cardiomyocytes from neonatal rat heart. The peak time (5 minutes) of HG-induced EF-2 dephosphorylation is different from that of HG-induced leucine incorporation at 24 hours. Further analysis in future studies will thus be needed to assess how the early changes in EF-2 dephosphorylation lead to later increases in leucine incorporation. It is reported that the Ang II–induced increase in [$^3$H] phenylalanine incorporation occurred at 24 hours in cardiomyocytes from neonatal rat heart [24], which matches the peak time of HG-induced leucine incorporation at 24 hours in this study. Additional studies will be needed to fill in all the signaling steps involved of how glucose leads to increases in protein synthesis given the complex nature of the interactions.

Our results also demonstrate that HG activates PKB/Akt activity. The involvement of the PI3K signaling pathway was demonstrated by the fact that PI3K-specific inhibitors, wortmannin and LY294002, inhibited HG-induced leucine incorporation. It has been shown that insulin stimulates the phosphorylation of 4E-BP through PI3K-PKB signaling pathway [13,25]. The PI3K action on phosphorylation of 4E-BP1 is thought to be dependent on both the phosphorylation and activation of PKB, indicating an involvement of PI3K-PKB/Akt pathway in protein synthesis [14,26]. Based on our results that PI3K inhibitors could inhibit HG-induced leucine incorporation, it is likely that PKB/Akt mediates the PI3K pathway in HG-induced protein synthesis. Additional experiments using adenoviral vectors encoding dominant-negative PKB/AKT will be necessary to get a conclusive result. Additional studies would also be needed to clarify how insulin would affect the glucose-induced changes in myocytes.

In addition, the mTOR inhibitor rapamycin inhibited HG-induced leucine incorporation, suggesting a role of mTOR in HG effects. Because these inhibitors did not significantly change basal protein synthesis, it was unlikely that these reagents had toxic effects in the cells. Our results are consistent with data in pancreatic islets, which demonstrate that rapamycin inhibits glucose-induced protein synthesis, suggesting an involvement of mTOR. Recent evidence indicates a role for p70$^{s6k}$, a protein kinase that phosphorylates ribosomal protein S6 in mammalian cells in the phosphorylation of PHAS-I in smooth muscle cells and in 3T3-L1 adipocytes [19,27]. However, our results indicated that HG did not activate p70$^{s6k}$, suggesting that it is unlikely that p70$^{s6k}$ is involved in HG-induced protein synthesis in cardiac myocytes. Glucose-induced protein synthesis appears to be mediated primarily by activation of PI3K-PKB/Akt and/or PI3K-mTOR pathway(s).

High glucose also activated ERK1/2 activity. Evidence has shown that high osmotic pressure is able to activate ERK1/2 activity. However, in the current study, mannitol did not increase ERK1/2 activity over the basal level. Our result showing the activation of ERK1/2 by HG treatment is consistent with the results showing that HG activates ERK1/2 in adipocytes and skeletal muscle [28,29]. In addition, our result showing that the inhibition of MEK blocks HG-induced leucine incorporation further strengthens the conclusion that ERK1/2 is an important mediator of HG-induced protein synthesis. PD98059, the MEK inhibitor, had no effect on basal leucine incorporation, suggesting that this compound had no toxic effects. 4E-BP1 is also an excellent ERK1/2 substrate. Extracellular signal–regulated kinase 1/2 phosphorylates all 5 sites in response to insulin [26]. Therefore, we propose that ERK1/2 activation and 4E-BP1 phosphorylation mediate HG-induced leucine incorporation.

Based on our experimental results showing that HG regulates leucine incorporation through PI3K-PKB and PI3K-mTOR as well as ERK1/2, we propose that the HG effect on leucine incorporation might be through both PI3K-PKB/mTOR and ERK1/2 pathways. It is possible that there is cross talk between these two pathways because inhibiting any one of these pathways had effects to reduce the HG response.

It is of interest to note that HG-induced PKB/Akt and ERK1/2 activation has a different time course. Compared with the rapid ERK activation at 5 to 10 minutes, PKB/Akt activation is characterized by a delayed onset in response to HG, which may reflect the time required for production of a glucose metabolite or another signaling molecule such as certain protein kinase C (PKC) isoforms. It has shown that an increase in the membrane/cytosolic PKC activity ratio was detected for all isoforms of PKC activity at 12 hours when exposing adult cardiac muscle cells to 25 mmol/L glucose.

These results provide new evidence for a direct effect of elevated glucose on cardiac myocyte protein synthesis. Further studies will be required to determine the importance of these changes to pathological growth of the heart in diabetes.

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