Direct Effects of High Glucose and Insulin on Protein Synthesis in Cultured Cardiac Myocytes and DNA and Collagen Synthesis in Cardiac Fibroblasts

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The present study examined the direct effects of high glucose and insulin on protein synthesis in cardiac myocytes and DNA and collagen synthesis in cardiac fibroblasts. Cultured rat cardiac myocytes and fibroblasts were grown in media containing normal glucose, high glucose, or osmotic control, and incubated with or without insulin. In cardiac myocytes, high glucose had no effect, but insulin increased protein synthesis and atrial natriuretic peptide (ANP) secretion and gene expression. The extracellular signal-regulated protein kinase (ERK)/mitogen-activated protein kinase (MAPK) inhibitor and the protein kinase C (PKC) inhibitor blocked insulin-induced protein synthesis. In cardiac fibroblasts, high glucose and osmotic control media increased DNA synthesis. Collagen synthesis and fibronectin and transforming growth factor-β1 (TGF-β1) mRNA expression were stimulated by high glucose, but not by osmotic control. Insulin increased DNA and collagen synthesis in fibroblasts, and the insulin-induced increase in DNA synthesis was blocked by the phosphatidylinositol 3 kinase (PI3K) inhibitor. Our findings suggest that cardiomyocyte protein synthesis is mainly regulated by insulin rather than high glucose and both high glucose and insulin contribute to fibroblast DNA and collagen synthesis. High glucose accelerates fibroblast DNA synthesis and collagen synthesis, and fibronectin and TGF-β1 mRNA expression, dependent or independent of osmotic stress. Insulin regulates myocyte protein synthesis and fibroblast DNA synthesis through different intracellular mechanisms.

THE PRINCIPAL pathophysiological feature of cardiovascular complications in diabetes mellitus is the morphological and functional alteration of macro- and microvessels. However, the presence of myocardial dysfunction independent of coronary artery disease in diabetes mellitus has also been documented.[1] Previous experimental and epidemiological studies showed an association between hyperglycemia/hyperinsulinemia and myocardial diabetic complications.[2,3] Therefore, it is important to evaluate the influence of hyperglycemia and hyperinsulinemia on cardiac cells. Nevertheless, few studies have focused on the direct effects of high glucose and insulin on cardiac myocytes and fibroblasts and on the intracellular mechanism of their stimulation.[4,5]

Hyperglycemia itself is an important factor in the development of diabetic vascular and nephrotic complications and several biochemical mechanisms have been proposed, including activation of protein kinase C (PKC),[6] extracellular signal-regulated kinase (ERK) pathway,[7] and P38 mitogen-activated protein kinase (MAPK) pathway.[8] On the other hand, insulin induces mitogenic and metabolic effects in most cell types. The ubiquitous insulin receptor substrate 1 protein appears to play a major role in the transduction of the proliferative effects of insulin.[9] Activation of this protein results in immediate activation of the Ras/ERK-MAPK and phosphatidylinositol 3 kinase (PI3K) pathways.[10] In addition, recent studies demonstrated that the PKC family is involved in the insulin signaling pathway.[11,12] However, the intracellular signaling of high glucose and insulin stimulation in cardiac myocytes and fibroblasts remains to be elucidated.

Thus, we conducted the present study to investigate the direct effects of high glucose and insulin on cultured cardiac myocyte protein synthesis and DNA and collagen synthesis by cardiac fibroblasts, and to examine intracellular signaling of high glucose and insulin stimulation in cardiac myocytes and fibroblasts.

MATERIALS AND METHODS

Cell Culture

Primary cultures of neonatal rat cardiac myocytes and fibroblasts were prepared as previously described.[13] Briefly, apical halves of cardiac ventricles from 1-to 2-day-old Wistar rats were separated, minced, and dispersed with 0.1% collagenase type II. To segregate myocytes from nonmyocytes, a discontinuous gradient of Percoll was prepared. The purified myocytes were plated on gelatin-coated culture plates or culture dishes and then cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Grand Island, NY) containing 5.6 mmol/L glucose supplemented with 10% fetal calf serum (FCS; Life Technologies) for 24 hours. This purification procedure has well been established,[14] and in fact more than 95% of the cells we obtained by this method were cardiomyocytes.

Nonmyocytes were plated on uncoated culture dishes and then cultured in DMEM with 10% FCS. The cells were allowed to grow to confluence, trypsinized, and passaged. This procedure yielded cultures of cells that almost exclusively fibroblasts by the first passage.[15] Fibroblasts at the second or third passage were cultured in DMEM containing 5.6 mmol/ L glucose with 10% FCS for 24 hours.

Experimental Protocol

After incubation in DMEM containing 5.6 mmol/L glucose with 10% FCS, myocytes and fibroblasts were replaced with 1 of 3 types of

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Submitted June 17, 2003; accepted January 9, 2004.

Supported by the Promotion of Fundamental Studies in Health Science of the Organization for Pharmaceutical Safety and Research (OPSR) of Japan, and Grants-in-aid for Scientific Research (14571044) from Japan Society for the Promotion of the Science.

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0026-0495/04/5306-0019$30.00/0

doi:10.1016/j.metabol.2004.01.006

media: DMEM containing 5.6 mmol/L glucose (normal glucose), 5.6 mmol/L glucose plus 16.6 mmol/L mannose (osmotic control), or 22.2 mmol/L glucose (high glucose). These cells were maintained for 72 hours with 10% FCS, and then serum-starved for 24 hours before the experiments. After the preconditioning period, porcine insulin (Sigma Chemical Co, St Louis, MO), PD98059, H7, wortmannin, and/or SB203580 (Wako Pure Chemical Industries, Osaka, Japan) were added. The doses of these inhibitors used in the present study have been confirmed to be sufficient to block ERK, PKC, PI3K, and P38 MAPK activities, respectively, as described previously.[16-19] For protein synthesis in cardiac myocytes, 0.5 μCi of [^{3}H]leucine was added. For DNA or collagen synthesis in cardiac fibroblasts, 0.5 μCi of [^{3}H]thymidine or 0.5 μCi of [^{3}H]proline was added, respectively. After incubation in 1 of 3 types of media with or without insulin and/or inhibitors for 24 hours, the medium was collected and stored at −80°C for the radioimmunoassay, and the cells were submitted for the incorporation study or RNA extraction.

Protein, DNA, and Collagen Synthesis

The effects of various agents on protein synthesis in cardiac myocytes, DNA, and collagen synthesis in cardiac fibroblasts were evaluated by the incorporation of [^{3}H]leucine, [^{3}H]thymidine, and [^{3}H]proline into cells, respectively, as described previously.[20] After cultured cells were treated as described in the experimental protocol, the radioactivity of aliquots of the trichloroacetic acid–insoluble material was determined using a liquid scintillation counter.

Measurement of Immunoreactive Atrial Natriuretic Peptide

The culture medium (0.5 mL) was acidified with acetic acid, boiled for 5 minutes to inactivate intrinsic proteases, and lyophilized. The radioimmunoassay for rat atrial natriuretic peptide (ANP) was performed, as previously reported.[13]

Northern Blot Analysis

Total RNA was extracted from cultured cells with TRIzol LS Reagent (Life Technologies). Northern hybridization was performed with cDNA probes for rat ANP, TGF-β1, endothelin-1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA and oligonucleotide probes for rat fibronectin mRNA (GeneDetect Co, Auckland, New Zealand) and 18S ribosomal RNA, according to the method previously reported.[21]

Statistical Analysis

All values are shown as the mean ± SD. Statistical calculations were done using the StatView (version 5.0) software package (Abacus Concepts, Berkeley, CA). Statistical significance between the 2 groups was determined using unpaired *t* test. For multiple comparisons, data were subjected to 1-way analysis of variance (ANOVA) followed by Fisher's multiple comparison test. The interactions between the 2 factors of glucose, osmotic stress, insulin, and several inhibitors were evaluated using 2-way factorial ANOVA. $P < .05$ was considered statistically significant.

RESULTS

Effects of High Glucose and Insulin on Protein Synthesis and ANP Production in Cardiac Myocytes

There was no significant difference in [^{3}H]leucine incorporation among treatment with 3 types of media (normal glucose, high glucose, and osmotic control), while insulin (10^{-10} to 10^{-6} mol/L) increased the protein synthesis in cardiac myocytes in a dose-dependent manner (Fig 1A). Treatment with high glucose or osmotic control media had no additional effect on the insulin-mediated increase in protein synthesis of myocytes. To examine which intracellular signaling is involved in the insulin-mediated increase in protein synthesis of cardiac myocytes, the effects of the ERK-MAPK inhibitor PD98059, the PKC inhibitor H7, and the PI3K inhibitor wortmannin were tested. PD98059 and H7 individually blocked insulin-induced protein synthesis of myocytes, but wortmannin had no suppressive effect (Fig 1B).

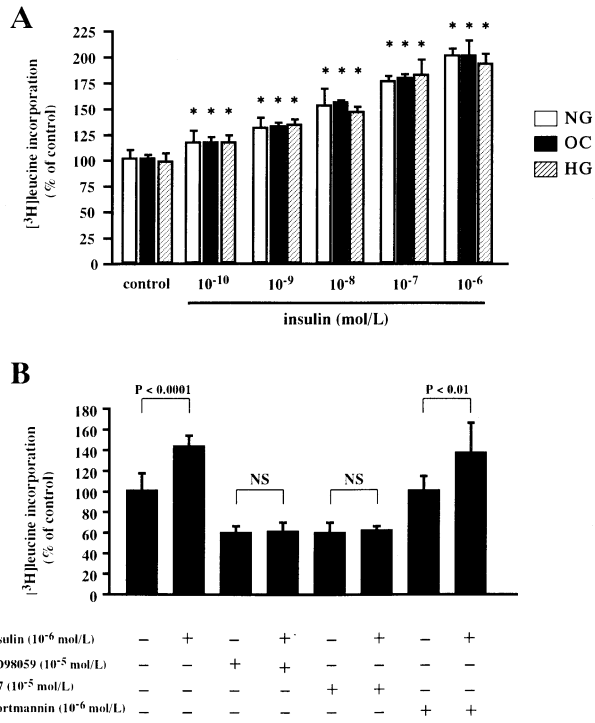

Fig 1. (A) Effects of high glucose and insulin on protein synthesis in cultured cardiac myocytes. Cardiac myocytes were treated in DMEM with 5.6 mmol/L glucose (NG), 5.6 mmol/L glucose plus 16.6 mmol/L mannose (OC), or 22.2 mmol/L glucose (HG) in the absence or presence of various doses of insulin. Values are given as the mean ± SD of 12 measurements. *$P < .05$ *v* control in each medium. (B) Effects of the ERK-MAPK inhibitor PD98059, the PKC inhibitor H7, and the PI3K inhibitor wortmannin on protein synthesis in cultured cardiac myocytes under insulin-stimulated conditions. Cardiac myocytes were incubated in DMEM containing 5.6 mmol/L glucose in the absence or presence of insulin and/or inhibitors. Values are given as the mean ± SD of 6 measurements.

The secretion of immunoreactive ANP from cardiac myocytes was not increased by exposure to high glucose or osmotic control media (Fig 2A). In contrast, insulin dose-dependently stimulated the ANP secretion (Fig 2B). PD98059 and H7 inhibited insulin-induced secretion of ANP, but wortmannin did not (Fig 2C). Insulin also stimulated the expression of ANP mRNA in cardiac myocytes and the increase in ANP gene expression was suppressed by PD98059 and H7, but not by wortmannin (Fig 3).

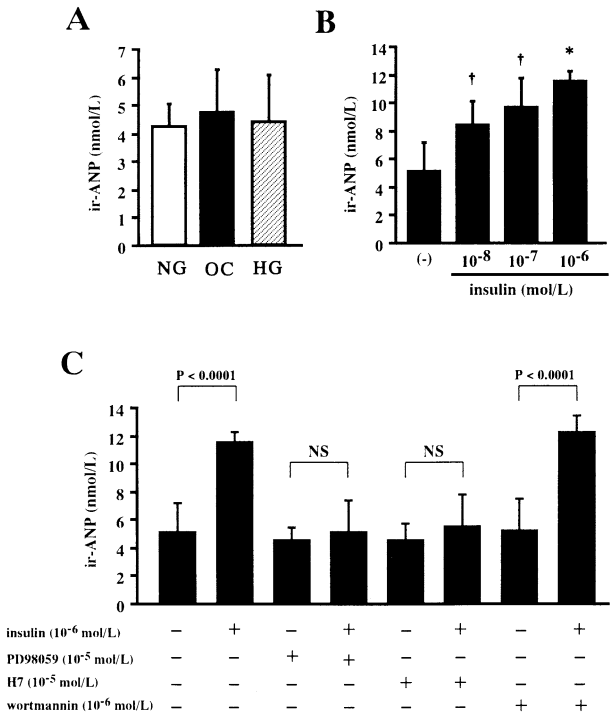

Fig 2. (A) Effects of high glucose on immunoreactive (ir-) ANP secretion in cultured cardiac myocytes. Cardiac myocytes were treated in DMEM with 5.6 mmol/L glucose (NG), 5.6 mmol/L glucose plus 16.6 mmol/L mannose (OC), or 22.2 mmol/L glucose (HG). Values are given as the mean ± SD of 6 measurements. (B) Effects of insulin on ANP secretion in cultured cardiac myocytes. Cardiac myocytes were treated with various doses of insulin in DMEM containing 5.6 mmol/L glucose. Values are given as the mean ± SD of 6 measurements. †*P* < .01 and **P* < .0001 *v* control. (C) Effects of the ERK-MAPK inhibitor PD98059, the PKC inhibitor H7, and the PI3K inhibitor wortmannin on ANP secretion in cultured cardiac myocytes under insulin-stimulated conditions. Cardiac myocytes were incubated in DMEM containing 5.6 mmol/L glucose in the absence or presence of insulin and/or inhibitors. Values are given as the mean ± SD of 6 measurements.

Effects of High Glucose and Insulin on DNA synthesis in Cardiac Fibroblasts

In cultured cardiac fibroblasts, [^{3}H]thymidine incorporation under treatment with high glucose and osmotic control media was higher than that with normal glucose media (Fig 4A). There was no difference in DNA synthesis of fibroblasts between treatment with high glucose and osmotic control media. Insulin (10^{-9} to 10^{-6} mol/L) significantly increased the DNA synthesis. The extent of the increase in [^{3}H]thymidine incorporation by insulin was similar among the 3 types of media. It was confirmed that high glucose and insulin had no synergistic effect. A high glucose- or high osmotic pressure-mediated increase in DNA synthesis was not blocked by PD98059, H7, wortmannin, or the P38 MAPK inhibitor SB203580, although some of them decreased basal [^{3}H]thymidine incorporation (Fig 4B). It was also confirmed using 2-way factorial analysis that these inhibitors did not significantly suppress high glucose- or osmotic stress-stimulated DNA synthesis. On the other hand, insulin-stimulated DNA synthesis was blocked only by wortmannin (Fig 4C).

Effects of High Glucose and Insulin on Collagen Synthesis and Fibronectin, TGF-β1, and Endothelin-1 Gene Expression in Cardiac Fibroblasts

Exposure of cardiac fibroblasts to high glucose media significantly increased [^{3}H]proline incorporation into cells, but treatment with osmotic control media did not (Fig 5A). Insulin (10^{-9} to 10^{-6} mol/L) significantly increased [^{3}H]proline incorporation in cardiac fibroblasts. However, the effect of high glucose on collagen synthesis was not influenced by the absence or presence of insulin. High glucose media increased the expression of fibronectin mRNA in fibroblasts, and this increase was also independent of high osmotic pressure (Fig 5B). Furthermore, high glucose, independent of high osmotic pressure, increased the expression of TGF-β1 mRNA, but not endothelin-1 mRNA (Fig 5C and 5D).

DISCUSSION

We demonstrated that exposure to high glucose had no effect on cardiac myocytes. In addition, high glucose had no synergistic effect on the effect of insulin. In contrast to the lack of an effect of high glucose, insulin significantly increased protein synthesis and stimulated ANP secretion and gene expression in cardiac myocytes. The physiological concentration of fasting serum insulin in patients with glucose intolerance or diabetic model rats is less than 300 or 800 pmol/L and after a meal its concentration reaches up to 2,000 or 1,400 pmol/L, respectively.[2,22] In the present study, 100 to 1,000 pmol/L insulin

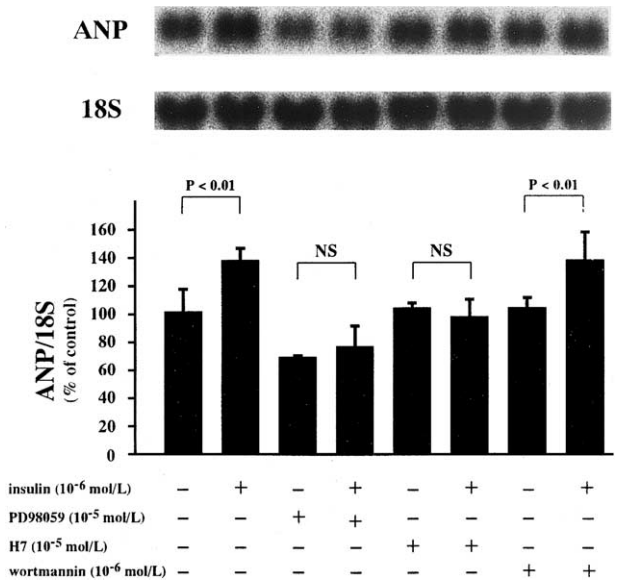

Fig 3. Effects of insulin on the expression of rat ANP mRNA and the effects of the ERK-MAPK inhibitor PD98059, the PKC inhibitor H7, and the PI3K inhibitor wortmannin on insulin-mediated upregulation of ANP transcripts in cultured cardiac myocytes. Cardiac myocytes were incubated in DMEM containing 5.6 mmol/L glucose in the absence or presence of insulin and/or inhibitors. Values are given as the mean ± SD of 4 measurements.

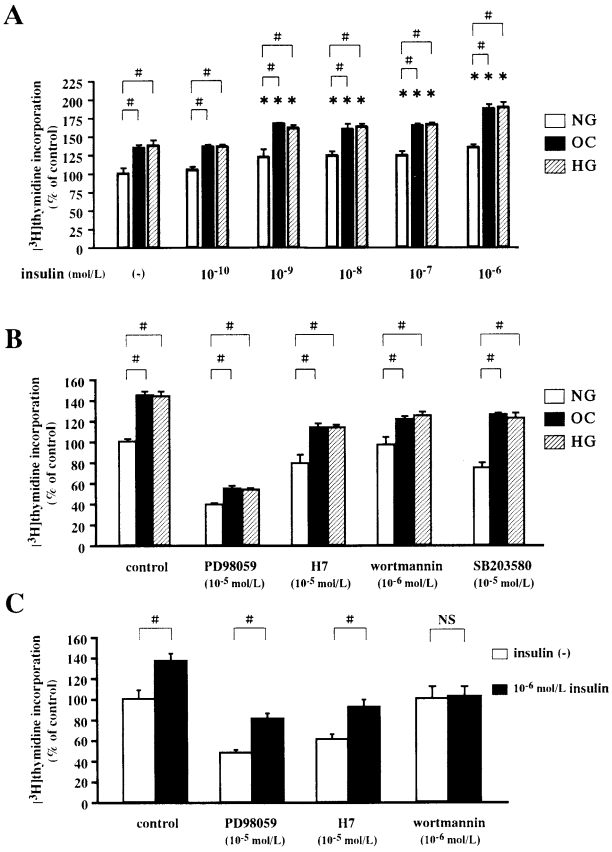

Fig 4. (A) Effects of high glucose and insulin on DNA synthesis in cultured cardiac fibroblasts. Cardiac fibroblasts were treated in DMEM with 5.6 mmol/L glucose (NG), 5.6 mmol/L glucose plus 16.6 mmol/L mannose (OC), or 22.2 mmol/L glucose (HG) in the absence or presence of various doses of insulin. Values are given as the mean ± SD of 12 measurements. *$P < .05$ *v* control in each medium; #$P < .0001$. (B) Effects of the ERK-MAPK inhibitor PD98059, the PKC inhibitor H7, the PI3K inhibitor wortmannin, and the P38 MAPK inhibitor SB203580 on glucose concentration- or osmotic pressure-dependent DNA synthesis in cultured cardiac fibroblasts. Cardiac fibroblasts were treated in DMEM with 5.6 mmol/L glucose (NG), 5.6 mmol/L glucose plus 16.6 mmol/L mannose (OC), or 22.2 mmol/L glucose (HG) in the absence or presence of inhibitors. Values are given as the mean ± SD of 6 measurements. #$P < .0001$. (C) Effects of the ERK-MAPK inhibitor PD98059, the PKC inhibitor H7, and the PI3K inhibitor wortmannin on DNA synthesis in cultured cardiac fibroblasts under insulin-stimulated conditions. Cardiac fibroblasts were incubated in DMEM containing 5.6 mmol/L glucose in the absence or presence of insulin and/or inhibitors. Values are given as the mean ± SD of 6 measurements. #$P < .0001$.

slightly but significantly increased protein synthesis in cardiac myocytes. Therefore, an increase in insulin within the physiological range may stimulate cardiac myocyte protein synthesis.

It has been unknown which intracellular pathway plays a critical role in insulin-mediated cardiomyocyte growth. In the present study, we demonstrated that insulin-mediated protein synthesis and ANP secretion and gene expression were completely blocked by the ERK-MAPK inhibitor PD98059 and the PKC inhibitor H7, but not by the PI3K inhibitor wortmannin. Our results suggest that the ERK and/or PKC pathway, but not PI3K, are chiefly involved in an insulin-mediated myocyte growth effect.

The direct effects of high glucose and insulin on cardiac fibroblasts have not been known. In the present study, we demonstrated that exposure to high glucose clearly induced DNA and collagen synthesis, and gene expression of fibronectin and TGF-β1 in cardiac fibroblasts. Although endothelin-1 has also been shown to be involved in high glucose-induced TGF-β1 gene expression in glomerular cells,[23] high glucose did not stimulate the gene expression of endothelin-1 in our cardiac fibroblasts. In renal cortical fibroblasts, high glucose increases TGF-β1 gene expression and the augmented TGF-β1 promotes DNA and collagen synthesis.[24] Therefore, the stimulatory effect of high glucose on DNA and collagen synthesis in cardiac fibroblasts might be partly mediated by activation of TGF-β1. However, this possibility and more detailed roles of TGF-β1 need elucidation by further investigation.

High osmotic pressure mimicked high glucose-mediated DNA synthesis but not collagen synthesis, fibronectin, and TGF-β1 gene expression in cardiac fibroblasts. Hence, the role of osmotic stress can be concluded to be as a primary inducer of fibroblast DNA synthesis. It has been shown that osmotic stress activates the ERK-MAPK, PKC, and P38 MAPK pathways[25-28] and these pathways are associated with cell growth.

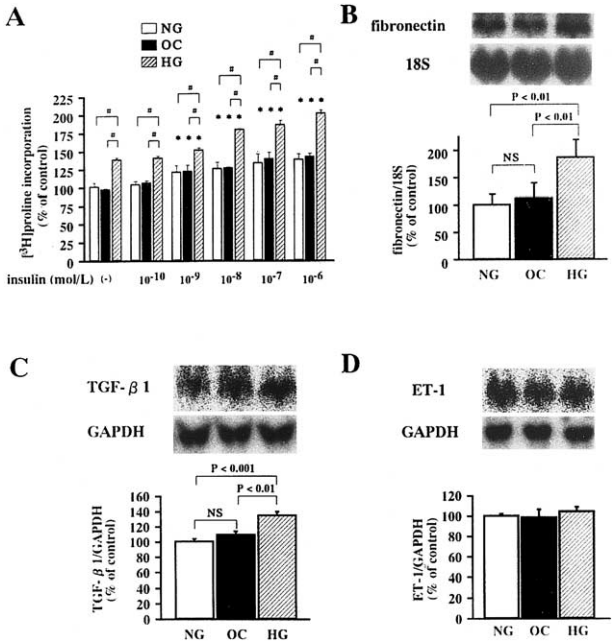

Fig 5. (A) Effects of high glucose and insulin on collagen synthesis in cultured cardiac fibroblasts. Cardiac fibroblasts were treated in DMEM with 5.6 mmol/L glucose (NG), 5.6 mmol/L glucose plus 16.6 mmol/L mannose (OC), or 22.2 mmol/L glucose (HG) in the absence or presence of various doses of insulin. Values are given as the mean ± SD of twelve measurements. *$P < 0.05$ versus control in each medium; #$P < .0001$. (B-D) Effects of high glucose on the expression of rat fibronectin (B), TGF-β1 (C), and endothelin (ET)-1 (D) mRNA in cultured cardiac fibroblasts. Cardiac fibroblasts were treated in DMEM with 5.6 mmol/L glucose (NG), 5.6 mmol/L glucose plus 16.6 mmol/L mannose (OC), or 22.2 mmol/L glucose (HG). Values are given as the mean ± SD of 6 measurements.

In our study, however, the ERK-MAPK inhibitor PD98059, the PKC inhibitor H7, and the P38 MAPK inhibitor SB203580 did not block high osmotic pressure-mediated DNA synthesis. Therefore, it is suggested that another intracellular pathway may be involved in osmotic stress-induced DNA synthesis in cardiac fibroblasts, eg, c-Jun NH2-terminal kinases, because a recent study revealed that this pathway is activated by osmotic stress,[28] and other signaling stress pathways, including cyclic adenosine monophosphate–activated protein kinase, should also be considered.

The present study demonstrated that a physiological concentration of insulin stimulated cardiac fibroblast DNA and collagen synthesis. Surprisingly, inhibition of PI3K, but not of ERK-MAPK, suppressed insulin-mediated fibroblast DNA synthesis. The findings suggest that insulin accelerates growth of cardiac myocytes and DNA synthesis of cardiac fibroblasts through different intracellular mechanisms. Recent studies have shown that not only ERK/MAPK but also the activation of PI3K is related to insulin- and insulin-like growth factor-mediated proliferation of several cell types.[29,30] These findings support an involvement of PI3K in the proliferative response of cardiac fibroblasts to insulin.

It is unclear why myocytes and fibroblasts differently respond to insulin, high glucose, and high osmolarity. As for the response to insulin, cardiac myocytes were more sensitive than fibroblasts. One possible mechanism of the different responses to insulin is the difference in expression levels of insulin receptor between myocytes and fibroblasts. Furthermore, in the present study, insulin stimulated cardiac myocyte protein synthesis and cardiac fibroblast DNA synthesis through different intracellular mechanisms. The different degree of outcome to insulin stimulation may also be due to the difference in downstream pathways of insulin receptors between cells. As for the response to high glucose, cardiac fibroblasts were much more sensitive than myocytes. The activation of polyol pathway-induced increase in fructose formation was previously reported as an important cause of high glucose-induced diabetic complications.[31] One possible mechanism of the varied responses to high glucose is the difference in the activity of this pathway. However, further investigations are needed to clarify the exact mechanisms of the different responses of cardiac myocytes and fibroblasts to insulin and high glucose.

In the present study, PD98059 and H7 decreased basal protein synthesis and ANP gene expression in cardiac myocytes and also decreased DNA synthesis in cardiac fibroblasts. These observations may indicate the substantial role of ERK-MAPK and PKC in preservation of cell homeostasis.

In summary, high glucose showed no effect on protein synthesis in cardiac myocytes but stimulated DNA and collagen synthesis, and fibronectin and TGF-β1 gene expression in cardiac fibroblasts via osmotic stress-dependent or independent mechanisms. Insulin stimulated both cardiac myocytes protein synthesis and fibroblast DNA and collagen synthesis. The intracellular mechanism of insulin-mediated myocyte protein synthesis was different from that of fibroblast DNA synthesis. These findings may be a cellular basis for explaining cardiac remodeling in hyperglycemia and hyperinsulinemia.

Study Limitations

Although the findings of this study were derived from experiments performed in cell cultures of neonatal rat cardiac myocytes and fibroblasts, it is known that the reaction of cultured cells does not necessarily reflect what occurs in vivo. Therefore, it may not be advisable to directly extrapolate the current findings of protein or DNA synthesis to the in vivo situation, ie, cardiac hypertrophy or fibrosis in adult hearts. In addition, the model we used did not account for insulin resistance observed in the diabetic process in humans.

ACKNOWLEDGMENT

The authors thank Dr Hiroyuki Kaiya, Dr Takeshi Soeki, and Yoko Saito for their technical assistance.

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