IGFBP5 mediates high glucose-induced cardiac fibroblast activation

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Abstract

This study examined whether IGF-binding protein 5 (IGFBP5) is involved in the high glucose-induced deteriorating effects in cardiac cells. Cardiac fibroblasts and cardiomyocytes were isolated from the hearts of 1- to 3-day-old Sprague Dawley rats. Treatment of fibroblasts with 25 mM glucose increased the number of cells and the mRNA levels of collagen III, matrix metalloproteinase 2 (MMP2), and MMP9. High glucose increased ERK1/2 activity, and the ERK1/2 inhibitor PD98059 suppressed high glucose-mediated fibroblast proliferation and increased collagen III mRNA levels. Whereas high glucose increased both mRNA and protein levels of IGFBP5 in fibroblasts, high glucose did not affect IGFBP5 protein levels in cardiomyocytes. The high glucose-induced increase in IGFBP5 protein levels was inhibited by PD98059 in fibroblasts. While recombinant IGFBP5 increased ERK phosphorylation, cell proliferation, and the mRNA levels of collagen III, MMP2, and MMP9 in fibroblasts, IGFBP5 increased c-Jun N-terminal kinase phosphorylation and induced apoptosis in cardiomyocytes. The knockdown of IGFBP5 inhibited high glucose-induced cell proliferation and collagen III mRNA levels in fibroblasts. Although high glucose increased IGF1 levels, IGF1 did not increase IGFBP5 levels in fibroblasts. The hearts of Otsuka Long-Evans Tokushima Fatty rats and the cardiac fibroblasts of streptozotocin-induced diabetic rats showed increased IGFBP5 expression. These results suggest that IGFBP5 mediates high glucose-induced profibrotic effects in cardiac fibroblasts.

Introduction

The increased incidence of cardiovascular diseases is an important factor contributing to a higher mortality rate in diabetic individuals (Zoungas & Patel 2010). Although vascular diseases, such as coronary artery disease, atherosclerosis, and hypertension, play an important role in diabetes-related cardiac dysfunction, diabetes also directly affects the heart at the level of the myocardium, leading to structural and functional changes in the heart (Boudina & Abel 2010). Cardiac fibroblasts are one of the main cellular components of the heart (Banerjee et al. 2007) and play a key role in extracellular matrix (ECM) turnover in the normal heart (Souders et al. 2009). However, the activity of the cardiac fibroblast is greatly increased in a variety of acute and chronic heart diseases, which leads to the accumulation of ECM and the stiffening of the myocardium (Boudina & Abel 2010, Wei 2011). The hearts of diabetic individuals are characterized by increased
ECM deposition, myocyte apoptosis, left ventricular hypertrophy, and systolic and diastolic dysfunction (Boudina & Abel 2010). Furthermore, type III collagen levels are increased in the biopsied myocardial tissues of type 2 diabetic patients who do not have predisposing vascular diseases (Shimizu et al. 1993).

The mechanism underlying diabetes-related heart disease has recently been studied, with hyperglycemia emerging as a leading candidate among the suggested factors. High glucose accelerates the proliferation of human cardiac fibroblasts in vitro (Neumann et al. 2002) and promotes the synthesis of DNA and collagen in rat cardiac fibroblasts (Tokudome et al. 2004). Activation of the MAPK pathway is also a critical factor to induce high glucose-mediated vascular and nephritic complications (Liu et al. 2000).

Insulin-like growth factor (IGF)-binding proteins (IGFBPs) constitute a family of proteins that bind to and modulate the mitogenic actions of IGFs by regulating the ability of IGFs to interact with their signaling receptor (Hwa et al. 1999). IGFBPs can also exert IGF-independent effects by binding to putative receptors on the surface of cells or directly regulating gene expression in the nucleus (Schneider et al. 2002). IGFBP5 is overexpressed in the lung tissue of patients with idiopathic pulmonary fibrosis (IPF) and in cultured fibroblasts derived from IPF patients (Pilewski et al. 2005). Furthermore, IGFBP5 expression is increased in fibroblasts cultured from keloid nodules (Russell et al. 2010), and injection of mice with adenovirus expressing human IGFBP-5 induces skin fibrosis (Yasuoka et al. 2006a).

In this context, we hypothesized that IGFBP5 plays a role in high glucose-induced cardiac fibrosis because high glucose increases the expression of IGFBP5 in mesangial cells (Park et al. 1998). In this study, we examined whether IGFBP5 plays a role in high glucose-induced cardiac fibroblast activation in primary cultured rat fibroblasts.

Materials and methods

Animals

Female and male Sprague Dawley rats (Koatech, Pyeongtaek, Korea) were housed in a room with a 12 h light:12 h darkness cycle, with lights-on at 0700 h and lights-off at 1900 h. The rats were fed a standard chow diet with free access to water. Pups from these rats were used for this experiment. Male Sprague Dawley rats (250–280 g) were administered a single i.p. injection of streptozotocin (STZ; Sigma) at a dose of 65 mg/kg body weight. The STZ was prepared in 0.1 M sodium citrate buffer (pH 4.5) immediately before use. One day after the STZ injection, the rats were checked twice for the development of diabetes by measuring urine glucose levels with Diastix strips (Bayer). Age-matched non-diabetic Sprague Dawley rats were injected with sodium citrate buffer and were used as controls. One week after the confirmation of diabetes, the rats were anesthetized in a CO2 chamber, and blood samples were collected from the abdominal aorta for the determination of plasma glucose concentrations. Heart samples were quickly removed under sterile conditions, washed twice with ice-cold PBS (Gibco), and immediately used for cardiac fibroblast isolation. Male Otsuka Long-Evans Tokushima Fatty (OLETF) and Long-Evans Tokushima Otsuka (LETO) rats were kindly supplied by the Tokushima Research Institute of Otsuka Pharmaceuticals (Tokushima, Japan) and were maintained in the same manner as the Sprague Dawley rats. After overnight fasting, the OLETF rats were anesthetized by an i.p. injection of tiletamine and zolazepam (25 mg/kg) and xylazine (10 mg/kg). Blood samples were collected from the abdominal aorta and the plasma was stored at –80 °C for the measurement of plasma glucose concentrations. The hearts were removed and stored at –80 °C until analysis. The study was conducted in accordance with the guidelines for the care and use of laboratory animals provided by Yeungnam University, and all the experimental protocols were approved by the Ethics Committee of Yeungnam University.

Cell isolation and culture

Cardiac fibroblasts were isolated from the hearts of 1- to 3-day-old Sprague Dawley rats by proteolytic digestion. Each heart was cut into small segments and digested by shaking in 100 units/ml collagenase type II ( Worthington, Lakewood, NJ, USA) at 37 °C for 15 min four times. The dissociated cell suspension was filtered through a stainless steel mesh. The cells were collected by centrifugation at 1500 g for 5 min and suspended in DMEM supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% antibiotics (Gibco). The cells were plated onto culture plates and incubated at 37 °C in a humidified environment containing 5% CO2 and 95% air state for 2 h. The non-adherent cells (mostly cardiomyocytes) were collected, resuspended in DMEM, plated in a culture dish, and used for the experiments. The adherent cells (mostly fibroblasts) were cultured until they reached 80% confluence, and the cells from passage 2–3 were used. Cardiac fibroblasts from the heart of STZ diabetic and control
rats were isolated, cultured in DMEM, and used for the experiments when they reached 80% confluence.

**Cell counting**

In the cell proliferation experiments, 2×10⁴ cells were seeded in each well of 24-well culture plates and grown in culture medium. The rate of cell proliferation in the presence of varying doses of glucose or recombinant mouse IGFBP5 (R&D Systems, Minneapolis, MN, USA) was determined by direct cell counting. The cells were detached by the addition of 0.05% trypsin and suspended in PBS, and the viability of the cells was determined by trypan blue exclusion.

**RNA isolation and real-time PCR**

Total RNA was extracted from cells or tissues and reverse transcribed with High-Capacity cDNA RT Kits (Applied Biosystems). Quantitative real-time PCR (RT-PCR) was performed using the Real-Time PCR 7500 Software system under the following thermocycling conditions: 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 s, annealing at 55°C for 20 s, and hybridization elongation at 72°C for 35 s. The sequences of the β-actin, IGFBP5, collagen-I, collagen III, matrix metalloproteinase 2 (MMP2), and MMP9 primers were based on the sequences available in NCBI’s Nucleotide database and were designed using the Primer Express program (Applied Biosystems): β-actin (71 bp), forward 5'-TGG ACA GTG AGG CAA GGA TAG-3' and reverse 5'-TAC TGC CCT GGC TCC TAG CA-3'; IGFBP5 (71 bp), forward 5'-ATG AAG CTG CCG GGC GCC-3' and reverse 5'-TCA ACG TTA CTG CGG TCG TCG AAG-3'; collagen-I (71 bp), forward 5'-AGA CAG GCC GCC TGA ATA-3' and reverse 5'-GTT GCC CTC AGC AAC AAG T-3'; collagen III (71 bp), forward 5'-TAC TAG AGG ATG GCT GCA GTA-3' and reverse 5'-TTG CAT GTA-3'.

**Western blot analysis**

The antibodies for the phosphorylated ERK, ERK, phosphorylated c-Jun N-terminal kinase (pJNK), JNK, and α-tubulin were obtained from Cell Signaling Technology (Danvers, MA, USA). The antibody for IGFBP5 was from Upstate Biotechnology (Lake Placid, NY, USA), glyceraldehyde 3-phosphate dehydrogenase was from Santa Cruz Biotechnology, and TATA-binding factor (TFIIB) was from BD Biosciences (San Jose, CA, USA). Total protein extraction and western blotting were performed as described previously (Kim et al. 2010). Briefly, the cells were harvested by trypsinization and resuspended in lysis buffer, and protein concentrations were determined by a Bradford assay (Sigma-Aldrich). The proteins were separated by 12% SDS–PAGE and transferred to a PVDF membrane (Millipore, Bedford, MA, USA). After blocking the membrane with TBST solution (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, and 0.1% Tween 20) containing 5% nonfat dry milk, the membrane was incubated with the primary antibodies overnight at 4°C. The membrane was washed in TBST, incubated with a goat-anti-rabbit IgG secondary antibody (Bio-Rad) for 1 h at room temperature and washed again in TBST. The bands were visualized with a chemiluminescent reagent (Millipore).

**Isolation of nuclear and cytosol extract**

The cells were lysed in lysis buffer (20 mM HEPES buffer, pH 7.9), 10 mM KCl, 1 mM phenylmethylsulfonyl fluoride, 2 mM MgCl₂, 50 mM NaF, 10 mM β-glycerophosphate, 1 mM dithiothreitol, 1 mM EDTA, 1 mM EGTA, 1% Nonidet P-40, 10% glycerol, 1 mM Na₃VO₄, and 10 μg/ml each aprotinin, pepstatin, and leupeptin). The lysates were centrifuged at 12 000 × g for 1 min at 4°C, and the supernatants were used as the cytoplasmic fractions. The pellets containing the crude nuclear fractions were resuspended in lysis buffer containing 150 mM NaCl at 4°C with vigorous shaking and centrifuged at 20 000 × g for 30 min. The supernatants were used as nuclear fractions.

**Immunoprecipitation**

The conditioned mediums from cells treated with 25 mM glucose were incubated with antibodies against IGFBP5 overnight at 4°C on a rotating device, followed by incubation with protein A-G PLUS Agarose (Genscript Biology, Piscataway, NJ, USA) for 2 h. After centrifugation, the supernatants were removed, and the immunoprecipitated complexes were washed three times with PBS. The pellets were then resuspended in 20 μl of 2× sample buffer. After boiling for 10 min, the samples were centrifuged and analyzed by western blotting with the corresponding antibodies as described earlier.
Transfection of cells with siRNA targeting IGFBP5

The IGFBP5 siRNA and control siRNA were purchased from Invitrogen and used according to the manufacturer’s instructions. Briefly, 3×10^5 cells were seeded in each well of a six-well plate 1 day before transfection with siRNA. On the following day, the media were replaced with the Opti-MEM (Gibco), and the cells were supplemented with 100 pmol IGFBP5 siRNA or 100 pmol non-targeting siRNA complexed with Lipofectamine 2000 transfection reagent (5 μl/well; Invitrogen). The Opti-MEM was removed after 4 h, at which time normal growth medium was added. The specific silencing of the IGFBP5 gene was confirmed by RT-PCR and western blots.

Flow cytometry

Apoptosis was determined by flow cytometry using annexin V conjugated with FITC, as described previously (Kim et al. 2010). In brief, 1×10^5 cells/100 μl binding buffer were transferred to a tube and incubated with 5 μl annexin V-FITC (BD Biosciences) containing 0.01 M HEPES, pH 7.4, 0.14 M NaCl, and 2.5 mM CaCl for 15 min at room temperature. Four hundred microliters of binding buffer were then added to the tubes, and the level of annexin V-FITC conjugation was detected using the FL1 setting on a FACSCalibur flow cytometer (BD Biosciences).

Statistical analyses

The results are expressed as the mean ± S.E.M. The differences among the groups were analyzed with a one-way ANOVA followed by Tukey’s post hoc test. The differences between two groups were analyzed with Student’s t-test. A P value < 0.05 was considered significant.

Results

The effect of high glucose on fibroblast activation

Glucose increased the number of cardiac fibroblasts in a dose-dependent manner compared with the osmotic control (5.6 mM glucose and 19.4 mM mannitol) at 48 h (Fig. 1A). Mannitol did not affect the proliferation of cardiac fibroblasts (data not shown). The mRNA levels of collagen III and MMP2 were increased by 25 mM glucose in a time-dependent manner (Fig. 1C and D). The MMP9 mRNA levels tended to be increased by 25 mM glucose at 24 h (Fig. 1E). The signaling pathway involved in high glucose-induced fibroblast activation was examined. Treatment of cells with 25 mM glucose increased ERK phosphorylation in a time-dependent manner (Fig. 1F), and 10 μM PD98059, ERK inhibitors, inhibited high glucose-induced ERK phosphorylation at 30 min (Fig. 1G). High glucose-induced increases in cell proliferation and collagen III mRNA levels in cardiac fibroblasts were also suppressed by 10 μM PD98059 (Fig. 1H and I).

The effect of high glucose on IGFBP5 expression in fibroblasts

Treatment with 25 mM glucose increased the IGFBP5 mRNA levels compared with the osmotic control at 1, 6, and 24 h. The mRNA levels of IGFBP5 at 24 h tended to be lower than that of IGFBP5 at 6 h. Mannitol did not affect IGFBP5 mRNA expression in cardiac fibroblasts (Fig. 2A). The mRNA levels of IGFBP5 were significantly enhanced with increasing concentrations of glucose compared with the osmotic control at 6 h (Fig. 2B). Treatment with 25 mM glucose significantly increased the protein levels of IGFBP5 at 24 and 48 h. Mannitol did not affect IGFBP5 protein levels in cardiac fibroblasts (Fig. 2C). The protein levels of IGFBP5 were also increased in the cell culture medium, cytoplasm, and nuclei of cells treated with 25 mM glucose for 48 h (Fig. 2D). The high glucose-induced increase in IGFBP5 mRNA and protein levels at 6 and 48 h was significantly inhibited by the ERK inhibitor PD98059 (Fig. 2E and F).

The effect of IGFBP5 on fibroblast activation

Fibroblast proliferation and the expression of ECM genes were measured after treatment with recombinant IGFBP5 to measure the effect of IGFBP5 on cardiac fibroblast activation. The number of cardiac fibroblasts was significantly increased by 50, 100, and 200 nM IGFBP5 at 48 h (Fig. 3A). However, the number of fibroblasts treated with 200 nM IGFBP5 was lower than the number of fibroblast treated with 100 nM IGFBP5. Therefore, we used 100 nM IGFBP5 in this experiment. The mRNA levels of collagen III, MMP2, and MMP9 were significantly increased by IGFBP5 (Fig. 3C, D and E). Treatment with IGFBP5 increased ERK phosphorylation (Fig. 3F).

Next, the effect of IGFBP5 on high glucose-induced activation of cardiac fibroblast was examined in cells transfected with IGFBP5 siRNA. The mRNA and protein levels of IGFBP5 decreased significantly after transfection.
Treatment with 25 mM glucose increased the number of cells transfected with control siRNA at 24 and 48 h. By contrast, treatment with 25 mM glucose did not affect the number of cells transfected with \textit{IGFBP5} siRNA at 24 or 48 h (Fig. 3I). While 25 mM glucose increased the mRNA levels of collagen III in cells transfected with control siRNA, the mRNA levels of collagen III was not affected in the cells treated with \textit{IGFBP5} siRNA (Fig. 3J).

\textbf{The effect of IGF1 on \textit{IGFBP5} expression}

Although treatment of cardiac fibroblasts with 25 mM glucose for 48 h increased IGF1 expression (Fig. 4A), treating cells with 10 or 100 nM IGF1 for 24 or 48 h did not affect \textit{IGFBP5} protein levels (Fig. 4B). The number of cells treated with \textit{IGFBP5} in the absence of FBS, which contains IGF1, also increased in a dose-dependent manner at 24 and 48 h (Fig. 4C).
The effect of IGFBP5 on cardiomyocytes

The IGFBP5 protein levels in cardiomyocytes and in the cell culture medium were not affected by 25 mM glucose (Fig. 5A and B). Treatment with IGFBP5 significantly decreased the number of cardiomyocytes in a dose-dependent manner at 48 h (Fig. 5C). The cells treated with 100 nM IGFBP5 for 24 or 48 h exhibited increased apoptosis, as measured by annexin V staining (Fig. 5D). Treatment with 100 nM IGFBP5 increased JNK phosphorylation, whereas ERK phosphorylation was not affected by IGFBP5 (Fig. 5E and F).

Figure 2

The effects of high glucose on IGFBP5 mRNA and protein levels in cardiac fibroblasts. IGFBP5 gene expression was measured in cells treated with 25 mM glucose for the indicated times (A). IGFBP5 mRNA levels were measured in cells treated with varying doses of glucose for 6 h (B). IGFBP5 protein levels were measured in cells treated with glucose for 24 or 48 h (C). Cells were treated with 25 mM glucose (HG) for 48 h, and IGFBP5 protein levels in the culture medium were determined by immunoprecipitation (IP) followed by western blotting. IGFBP5 protein levels in the nuclear fraction and the cytoplasmic fraction were measured (D). Cells were treated with 10 mM PD98059 for 30 min before being treated with 25 mM glucose for 6 h (E) or 48 h (F). Gene expression was assessed by real-time PCR, and protein levels were measured by western blotting throughout the experiment. The results are reported as the mean ± S.E.M. of five separate experiments, each performed in triplicate, except for (D). Mannitol (5.6 mM glucose + 19.4 mM mannitol) was used as an osmotic control (OC). NG indicates normal glucose (5.6 mM). *P < 0.05 vs OC. §P < 0.05 vs 10 and 17 mM glucose. #P < 0.05 vs 25 mM glucose.
IGFBP5 expression in the heart of diabetic animals

OLETF rats, an animal model of type 2 diabetes, showed increased plasma glucose levels compared with LETO rats. The mRNA levels of collagen III were higher in the hearts of OLETF rats than the heart of LETO rats. The IGFBP5 protein levels were also higher in the heart of OLETF rats than the heart of LETO rats (Fig. 6A, B and C). Injecting rats with STZ increased plasma glucose levels and collagen III mRNA levels in the cardiac fibroblast. The mRNA levels of IGFBP5 were also increased in the cardiac fibroblast of diabetic rats treated with STZ (Fig. 6D, E and F).

Discussion

In this study, we first demonstrated that high glucose increases IGFBP5 expression and IGFBP5 accelerates cell proliferation and collagen synthesis in cardiac fibroblasts. Furthermore, IGFBP5 knockdown attenuates high glucose-induced cell proliferation and collagen synthesis. While high glucose has no affect on IGFBP5 expression, IGFBP5 induces apoptosis in cardiomyocytes. These findings indicate that IGFBP5 may play important pathological roles in the diabetic heart.

High glucose-induced increases in the cell proliferation and collagen synthesis of cardiac fibroblasts,
mesangial cells, and vascular smooth muscle cells contribute to the cardiovascular and nephritic complications of diabetes (Neumann et al. 2002, Tokudome et al. 2004, Maile et al. 2010, Tang et al. 2010), and ERK1/2 activation has been linked to high glucose-induced fibrosis (Tang et al. 2007). Consistent with these previous reports, high glucose presently increased cell proliferation and affected the expression of ECM-related genes in cardiac fibroblasts by modulating ERK activities.

Although the activation of the intracellular renin-angiotensin system is a major mediator of the high glucose-induced fibrotic effect in cardiac fibroblasts (Singh et al. 2008a), a new mediator, thrombospondin 1, has recently been reported (Tang et al. 2011). The present results support the possibility that IGFBP5 mediates high glucose-induced cardiac fibrosis. IGFBP5 is constitutively expressed in the heart of experimental animals (Kluge et al. 1995). Despite several reports investigating the modulation of IGFBP5 by ischemic reperfusion or cardiac hypertrophy stimuli in the heart, the role of IGFBP5 in the heart has remained unclear (Kluge et al. 1995, Cook et al. 2002, Baurand et al. 2007). Consistent with our suggestion, IGFBP5 has been previously postulated to contribute to diabetic nephropathy through increased matrix accumulation, although no direct evidence in support of this hypothesis has been presented. High glucose increases IGFBP5 levels in glomerular mesangial cells (Berfield et al. 2006, Schaeffer et al. 2010), and IGFBP5 mRNA levels are increased in the glomeruli, cortical, and inner medullary peritubular interstitial cells in the kidneys of diabetic rats (Park et al. 1998).

IGFBP5 is involved in many biological functions, including proliferation, differentiation, senescence, and apoptosis. IGFBP5 increases the cell proliferation of osteoblasts (Amaar et al. 2005), human intestinal smooth muscle cells (Kuemmerle & Zhou 2002), and neuroblastoma cells (Cesi et al. 2004). IGFBP5 is also involved in the cell differentiation of gingival epithelial cells (Hung et al. 2008) and muscle cells (Ren et al. 2008), the senescence of human umbilical endothelial cells (Kim et al. 2007), and the apoptosis of hepatic stellate cells (Sokolovic et al. 2010). Presently, IGFBP5 increased the proliferation of cardiac fibroblasts but induced apoptosis in cardiac myocytes. These cell type-dependent effects of IGFBP5 in heart tissue are similar to the apoptotic and profibrotic effects of angiotensin II on the heart (Singh et al. 2008b).

**Figure 4**
The role of IGF1 in IGFBP5 expression in cardiac fibroblasts. Cells were treated with 25 mM glucose for 24 or 48 h and then IGF1 protein levels were measured by western blotting (A). Cells were treated with IGF1 for 24 or 48 h, and IGFBP5 protein levels were measured by western blotting (B). Cells were treated with recombinant (rm) IGFBP5 for 24 h (white bar) or 48 h (black bar) in the absence of FBS and cell number was measured by cell counting (C). Mannitol (5.6 mM glucose + 19.4 mM mannitol) was used as an osmotic control (OC). The results are reported as the mean ± S.E.M. of five separate experiments, each performed in triplicate. *P < 0.05 vs OC or control.
IGFBP5 not only increased fibroblast proliferation in this study but also increased collagen synthesis. These profibrotic effects of IGFBP5 have been well documented in the lung (Pilewski et al. 2005, Yasuoka et al. 2006b, 2009) and skin (Feghali & Wright 1999, Yasuoka et al. 2006a,b). IGFBP5 is overexpressed in the lungs of IPF patients, and IGFBP5 induces the production of collagen and fibronectin by fibroblasts and fibroblast/myofibroblast transdifferentiation in vitro and in vivo (Pilewski et al. 2005, Yasuoka et al. 2006a,b). IGFBP5 expression is increased in dermal fibroblasts of patients with systemic sclerosis (Feghali & Wright 1999), and overexpression of IGFBP5 induces fibrosis of the skin, including increased dermal thickness and collagen bundle diameter (Yasuoka et al. 2006a,b). Our results, together with previous reports, lead us to postulate that IGFBP5 may be involved in cardiac fibrosis. Furthermore, the profibrotic effect of high glucose was presently suppressed by inhibiting IGFBP5, suggesting that IGFBP5 may function as a mediator of high glucose-induced cardiac fibrosis. Additionally, the increased IGFBP5 expression in the diabetic heart and cardiac fibroblasts of diabetic rats also supports our hypothesis. Like the profibrotic effect of high glucose on the heart, high glucose appeared to increase IGFBP5 expression through the ERK1/2 pathway in this study, indirectly suggesting a role of IGFBP5 as a mediator of the profibrotic effect of high glucose. Moreover, the IGFBP5-induced increase in ERK phosphorylation observed in the current study suggests that IGFBP5 is a positive regulator of high glucose-induced ERK phosphorylation. It seems that IGFBP5 is not only a mediator but also an exacerbating factor of high glucose-induced cardiac fibrosis. The mechanism regulating the high glucose-induced ERK1/2 activation remains unclear and requires further elucidation in future studies.

The high glucose-induced increase in IGFBP5 expression in cardiac fibroblasts could be indirect, as some of the pathological responses to high glucose are...
mediated indirectly (Busik et al. 2008). It is possible to hypothesize the involvement of IGF1 in the high glucose-mediated increase in IGFBP5 expression, as high glucose affects IGF1 expression in a variety of cells, such as hepatocytes, cardiomyocytes, mesangial cells, and osteoblasts (Han et al. 2006, Zhen et al. 2010). Moreover, IGFBP5 expression is also increased by IGF1 in chondrocytes and mammary fibroblasts (Kiepe et al. 2005). As IGF1 activity is modulated by IGFBPs, the regulatory effect of IGF1 on IGFBP5 expression seems to be a physiological response for homeostasis. Additionally, the ERK1/2 pathway is also responsible for the IGF1-mediated regulation of IGFBP5 in rat intestinal smooth muscle cells (Xin et al. 2004). As IGF1 activity is modulated by IGFBPs, the regulatory effect of IGF1 on IGFBP5 expression seems to be a physiological response for homeostasis. Additionally, the ERK1/2 pathway is also responsible for the IGF1-mediated regulation of IGFBP5 in rat intestinal smooth muscle cells (Xin et al. 2004). However, it appears that IGF1 is not involved in high glucose-induced IGFBP5 expression. Our hypothesis is supported by the facts that IGFBP5 protein levels remained unchanged after treatment with IGF1 and the proliferative effect of IGFBP5 was maintained in culture medium lacking serum.

Putative IGFBP5 receptors have been suggested to exist in human intestinal smooth muscle cells, mouse osteoblasts, and rat kidney mesangial cells (Mohan et al. 1995, Kuemmerle & Zhou 2002, Mohan & Baylink 2002). It is possible that the binding of IGFBP5 to its putative receptors in fibroblasts may stimulate a signaling pathway, such as the p38 MAPK kinase and ERK1/2 pathway (Kuemmerle & Zhou 2002). Another mechanism by which IGFBP5 could exert effects is through the transcriptional regulation of genes (Mohan & Baylink 2002).

Figure 6
IGFBP5 expression in the heart of diabetic rats. Plasma glucose levels of LETO and OLETF rats were measured after overnight fasting (A). The mRNA levels of collagen III (B) and protein levels of IGFBP5 (C) were measured in the hearts of LETO and OLETF rats. Fasting plasma glucose levels were measured in STZ-induced diabetic rats (D). The mRNA levels of collagen III (E) and IGFBP5 (F) in cardiac fibroblasts isolated from STZ diabetic hearts were measured. Gene expression was assessed by RT-PCR and protein levels were measured by western blotting throughout the experiment. The results are reported as the mean ± S.E.M. for seven experiment cases. *P<0.05 vs LETO or control.

Figure 7
IGFBP5 may play a role in the hyperglycemia-induced activation of cardiac fibroblasts. High glucose activates ERK1/2, increasing cytoplasmic IGFBP5 levels. IGFBP5 is then secreted into the extracellular space, where the protein affects the cell itself or neighboring cells by binding to putative receptors in the cell membrane. IGFBP5 also translocates to the nucleus and may regulate gene expression.
The presence of a nuclear localization signal in the C-terminal regions of IGFBP5 (Schedlich et al. 1998) and the observation of nuclear localization (Amaar et al. 2002) and a nuclear binding partner of IGFBP5 (Amaar et al. 2002) support this suggestion. We also confirmed that high glucose increased IGFBP5 secretion into the culture medium and increased the IGFBP5 level in the nuclear fraction of cardiac fibroblasts. Therefore, it is possible that the high glucose-induced increase in IGFBP5 mediates its profibrotic effects by interacting with receptors in the cell membrane and/or regulating gene transcription after nuclear translocation. The identification of these putative receptors, nuclear transport proteins, and binding partners or receptors is necessary.

As mentioned earlier, IGFBP5 affects cell survival and apoptosis in a variety of cells, and the response to IGFBP5 depends on cell types. IGFBP5 induces apoptosis in mammary epithelial cells (Tonner et al. 2002), breast cancer cells (Butt et al. 2005), and osteosarcoma cells (Su et al. 2011) but prevents apoptosis in neuroblastoma cells (Tanno et al. 2006), C2 myoblasts (Cobb et al. 2004), and human stellate cells (Sokolovic et al. 2010). Although high glucose presently did not increase IGFBP5 protein levels in cardiomyocytes, the IGFBP5 released from cardiac fibroblasts may promote the apoptosis of cardiac myocytes. The IGFBP5-mediated increase in JNK phosphorylation observed in our study also supports a proapoptotic effect of IGFBP5, as JNK is involved in high glucose-induced apoptosis in cardiomyocytes (Kuo et al. 2012, Singh et al. 2012). JNK may mediate IGFBP5-induced apoptosis in cardiomyocytes.

Overall, hyperglycemia activates the ERK1/2 pathway, leading to increased IGFBP5 levels in cardiac fibroblasts. IGFBP5 translocates into the nucleus, regulating gene expression, and/or is released and binds to cell membrane receptors, regulating signaling pathways (Fig. 7). The released IGFBP5 may induce apoptosis in cardiomyocytes. The role of IGFBP5 as a mediator of high glucose-induced cardiac fibrosis requires confirmation in vivo.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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