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Hyperglycemia and Hyperinsulinemia Have Additive Effects on Activation and Proliferation of Pancreatic Stellate Cells: Possible Explanation of Islet-specific Fibrosis in Type 2 Diabetes Mellitus

Oak-Kee Hong,[1] Seung-Hwan Lee,[2] Marie Rhee,[1] Seung-Hyun Ko,[2] Jae-Hyoung Cho,[2] Yoon-Hee Choi,[2] Ki-Ho Song,[2] Ho-Young Son,[2] and Kun-Ho Yoon[1,2]*

[1]Immunology & Cell Biology Core Laboratory, Catholic Research Institutes of Medical Science, The Catholic University of Korea, Seoul, Korea
[2]Division of Endocrinology and Metabolism, Department of Internal Medicine, The Catholic University of Korea, Seoul, Korea

Abstract Pancreatic islet fibrosis observed in Type 2 diabetes is one of the major factors leading to progressive β-cell loss and dysfunction. Despite its importance, the mechanism of islet-restricted fibrogenesis associated with pancreatic stellate cell (PSC) activation and proliferation remains to be defined. Therefore, we studied whether the islet-specific environment represented by hyperglycemia and hyperinsulinemia had additive effects on the activation and proliferation of cultured rat PSCs. Cells were stimulated to activate and proliferate with glucose and insulin, either individually or concomitantly. Both stimuli promoted PSC proliferation and extracellular signal-regulated kinase (ERK) 1/2 phosphorylation independently, but an additive effect was also demonstrated. Blockade of ERK signaling by the mitogen-activated protein kinase kinase (MEK) inhibitor, U0126, suppressed both glucose- and insulin-induced ERK 1/2 phosphorylation and PSC proliferation. Glucose and insulin-induced ERK 1/2 phosphorylation also stimulated connective tissue growth factor gene expression. Thus, hyperglycemia and hyperinsulinemia are two crucial mitogenic factors that activate and proliferate PSCs, and the presence of both states will amplify this response. J. Cell. Biochem. 101: 665–675, 2007.

Key words: islet fibrosis; pancreatic stellate cell; hyperinsulinemia; hyperglycemia; extracellular signal-regulated kinase 1 and 2

It is well known that β-cell dysfunction and insulin resistance are the two central, interrelated defects in patients with Type 2 diabetes mellitus [Welsh et al., 1993]. Progressive fibrosis and the subsequent loss of functional tissue that is replaced by extracellular matrix (ECM)-rich connective tissue containing amylin is the main characteristic finding in the pancreatic islets of such patients and in those with chronic pancreatitis and pancreatic cancer [Gepts and Lecompte, 1981; Longnecker, 1982]. In contrast to chronic pancreatitis, in which fibrosis mainly involves the exocrine pancreatic tissue, pancreatic fibrosis in patients with Type 2 diabetes mellitus is mainly restricted to the endocrine pancreatic tissue [Yoshikawa et al., 2002; Ko et al., 2004]. It might play an important role for progressive β-cell dysfunction and loss as well.

Treatment of Otsuka Long-Evans Tokushima Fatty (OLETF) rats with the long-term angiotensin-converting enzyme (ACE) inhibitor, ramipril, improved glucose tolerance by a significant reduction of islet destruction by fibrosis [Ko et al., 2004]. Therefore, protection against islet fibrosis could be a new target for prevention of the development and progression

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*Correspondence to: Kun-Ho Yoon, MD, PhD, 505, Banpo-Dong, Seocho-Gu, Department of Internal Medicine, Kangnam St. Mary's Hospital, Seoul, Korea 137-701.
E-mail: yoonk@catholic.ac.kr

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of Type 2 diabetes mellitus. Despite its importance, the mechanism of fibrogenesis in the islets remains unclear.

Pancreatic stellate cells (PSCs) resemble hepatic stellate cells (HSCs) in their morphology and biochemical nature. The role of HSCs in the development of hepatic fibrosis is well defined [Blomhoff and Wake, 1991]. Profibrogenic inflammatory cytokines, such as transforming growth factor (TGF)-β, tumor necrosis factor (TNF)-α, and oxidative stress, serve to activate HSCs to proliferate and transdifferentiate into myofibroblast-like cells. These secrete ECM proteins, leading eventually to tissue fibrosis [Bachem et al., 1993]. Development of methodology for the isolation and culture of stellate cells from the pancreas has provided many opportunities for in vitro studies on the biology of these cells. An important aspect of such studies is the identification and characterization of factors that may be responsible for activating these cells in pathological states. Stellate cell activation by inflammatory mediators released during acute pancreatic injury may be one mechanism relevant to hyperglycemia in patients with diabetes. A functional relationship between hyperglycemia and fibrosis has been shown in a model of diabetic nephropathy and hepatic fibrosis [Ziyadeh et al., 1994; Han et al., 1999; Paradis et al., 2001]. We found that high glucose treatment produced a fourfold increase of ECM protein synthesis in PSCs, suggesting that hyperglycemia also has a central role in the pathogenesis of pancreatic fibrosis [Ko et al., 2006].

However, it remains to be shown why this fibrosis is restricted only to the islets of patients with diabetes and in animal models of the disease, even though the whole pancreatic tissue is exposed to hyperglycemia. One possible explanation is that PSCs in the islets may be exposed not only to hyperglycemia but also to local hyperinsulinemia. Insulin and insulin-like growth factor (IGF)-1 are mitogenic for fibroblasts and smooth muscle cells, and there is evidence that HSCs are highly sensitive to these molecules, resulting in mitogenesis and collagen synthesis [Svegliati-Baroni et al., 1999]. Because insulin is a well-known growth factor for various cells in the body, PSCs in the islets might be predisposed to activate and proliferate by hyperinsulinemia.

Therefore, the aim of this study was to test whether the additive effects of hyperglycemia and hyperinsulinemia on PSC activation and proliferation might explain islet-specific fibrosis in the pathogenesis of Type 2 diabetes mellitus.

MATERIALS AND METHODS

PSC Isolation and Culture

PSCs were prepared from 12-week-old male Sprague–Dawley rats, as described by Apte et al. [1998] with minor modifications. Briefly, rat pancreas was digested in Gey's balanced salt solution (GBSS) containing 1 mg/ml collagenase P (Boehringer-Mannheim, Indianapolis, IN), 0.02% protease (Sigma Aldrich, St. Louis, MO), and 0.001% DNase I (Boehringer-Mannheim). After filtering the digested tissue through 70 μm nylon mesh, the cells were centrifuged into a 28.7% (w/v) solution of Nycodenz (Nycomed Pharma AS, Oslo, Norway) at 1,400*g* for 20 min. The cells of interest were separated into a fuzzy band just above the interface of the Nycodenz cushion and the GBSS with bovine serum albumin (BSA). This band was harvested and the cells were washed and resuspended in a mix of Dulbecco's Modified Eagle's Medium (DMEM) and F-12 medium (1:1, v/v) containing 20% calf serum, 4 mM glutamine and antibiotics (1,000 units/ml penicillin; 1,000 μg/ml streptomycin, and 25 μg/ml amphotericin B). Viability of the isolated cells was assessed by trypan blue exclusion and the cells were counted using a hemocytometer. An aliquot of freshly isolated cells were cytospun directly onto a slide for immunostaining and fluorescence microscopy. Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2/95% air.

Immunostaining for Alpha Smooth Muscle Actin (SMA) in PSCs

Cultured rat PSCs (Passage 2) were seeded at a density of 0.5×10^4 cells per well onto glass coverslips in DMEM containing 10% calf serum and 100 mg/dl glucose. After serum starvation for 24 h, the cells were treated with D-glucose (100 and 500 mg/dl) and insulin (10 IU/ml) for 12 h. The cultured cells were fixed with 4% paraformaldehyde at room temperature for 30 min. After washing with phosphate-buffered saline (PBS), fixed cells were stored at 4°C in 2 ml of Sorensen's buffer until staining. Normal donkey serum, as the blocking solution, was added for 15 min. Cells were incubated overnight at 4°C after treatment with primary

antibodies against α-SMA (1:400, Sigma Aldrich). After washing with PBS, cells were incubated in the dark for 1 h at room temperature with fluorescein isothiocyanate (FITC)-conjugated donkey antimouse antibody (1:100 dilution, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). After washing, cells were mounted in 1 mg/ml propidium iodide with an antifading medium. Digital images at two fluorescent emission wavelengths were acquired using a confocal microscope (Bio-Rad Laboratories, Hercules, CA).

Determination of Cell Proliferation

The rate of cell proliferation was assessed by measuring the incorporation of [^{3}H]-thymidine into DNA. Cultured rat PSCs (at Passages 2–7) were seeded at a density of 5×10^4 cells per well in 48-well plates in DMEM containing 100 mg/dl D-glucose and 10% fetal calf serum. After 24 h, the medium was replaced with serum-free medium. The cells were incubated in medium containing D-glucose (100–500 mg/dl), insulin (0–100 IU/ml) for 48 h, with 1 μCi/well [^{3}H]-thymidine present for the last 24 h. To control the effects of hyperosmolality, PSCs were also cultured in medium containing 100 mg/dl D-glucose supplemented with 25 mM mannitol. At the end of the incubation, 10% trichloroacetic acid was added and the cells were held on ice for 20 min. Cells were washed twice with 80% ethanol and solubilized in 500 μl of cell dissolution solution (0.25 mM NaOH and 0.2% sodium dodecyl sulfate). These were added into each well followed by 50 μl of 5 N HCl after 15 min. The mixture was transferred to the scintillation vials with 4 ml of scintillant. Radioactivity was measured using a Packard β-counter (Packard Instruments Co., Downers Grove, IL). Each experiment was performed in quadruplicate and repeated at least three times.

Western Blotting

PSCs were lysed with ice-cold lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 2 mM EDTA, 1% Nonidet P-40, 10 mM NaF, 1 mM Na_3VO_4, 10 mM sodium pyrophosphate, 1 mM phenylmethylsulfonylfluoride, 10 mg/ml aprotinin, 10 mg/ml leupeptin, and 0.1 mg/ml soybean inhibitor). Cell lysates were centrifuged at 15,000 rpm for 5 min at 4°C. Protein concentration was measured by the Bradford method using BSA as a standard. Proteins (40 μg) were separated by 10% SDS–PAGE and transferred onto nitrocellulose transfer paper. The membranes were blocked with 5% fat-free dry milk or 5% BSA for 1 h in Tris-buffered saline (TBS; 25 mM Tris-HCl, pH 7.6, and 150 mM NaCl) containing 0.1% Tween 20 (TBS-T), and then incubated with the following primary polyclonal antibodies: anti-extracellular signal-regulated kinase (ERK) 1/2 and antiphsopho-ERK 1/2 (Cell Signaling Technology Inc., Beverly, MA); and anti-SMA, anticonnective tissue growth factor (CTGF), and anti-β-actin (Sigma-Aldrich). These were diluted 1:1,000–1:5,000 in 1% skim milk or BSA in TBS-T and incubated at 4°C overnight. After washing, the membranes were incubated with secondary peroxidase-conjugated antimouse and antirabbit antibodies (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) diluted 1:1,000 in 1% fat-free dry milk in TBS-T at room temperature for 1 h. Detection was achieved using an enhanced chemiluminescence kit (GE Healthcare Bio-Sciences Corp.). Quantitation of Western blots by densitometry was performed with ImageMaster VSD software (GE Healthcare Bio-Sciences Corp.).

Statistical Analysis

The results are presented as the mean ± SD of at least three independent experiments. Analysis of variance was used to compare different groups. Statistical significance was determined using Student's t tests and $P < 0.05$ was assumed to be significant.

RESULTS

Effects of High Glucose and Insulin Treatment on Activation of PSCs

As activated stellate cells exhibit positive staining for the cytoskeletal protein α-smooth muscle actin (SMA), a marker of PSC proliferation and ECM synthesis, we examined the degree of α-SMA expression by immunostaining (Fig. 1A) and Western blotting (Fig. 1B). The α-SMA stained cells in the culture dishes of the control group at 12 h after culture with 100 mg/dl glucose-containing medium were 1.3 ± 0.5% of the total cells (n = 8 replicates). When PSCs were treated with 10 IU/ml insulin or high glucose (500 mg/dl), the percentages of α-SMA positive cells increased significantly (2.1 ± 0.2% and 2.9 ± 0.2%, respectively, n = 8). Those were significantly higher than that of control group ($P < 0.05$) and high-glucose stimulation was

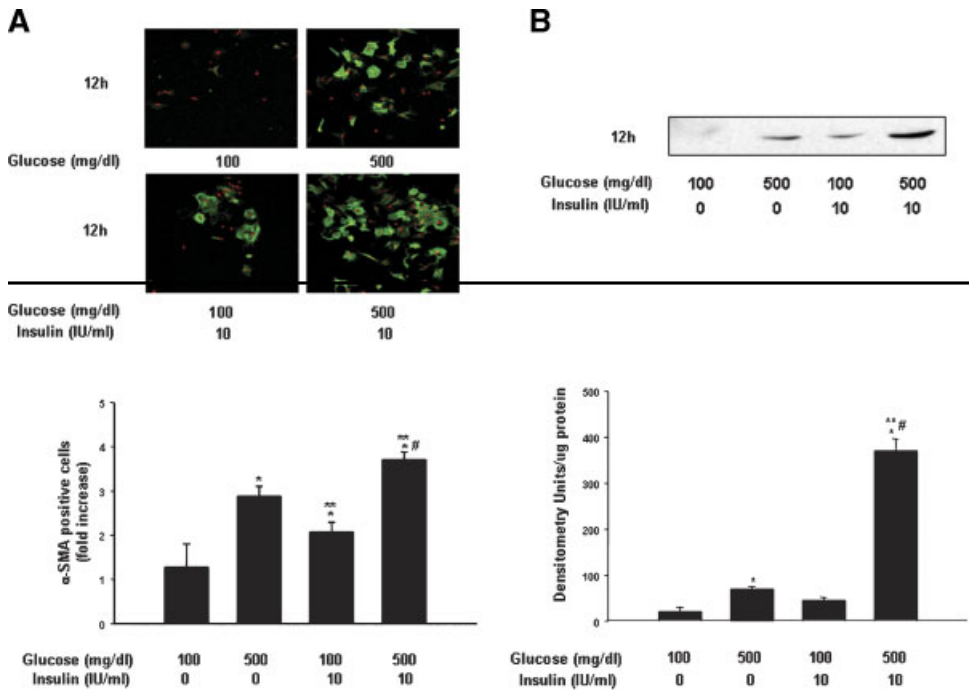

Fig. 1. Effects of high glucose and insulin treatments on the activation of quiescent PSCs. Cells were cultured with 100 or 500 mg/dl glucose in medium supplemented with or without 10 IU/ml insulin for 12 h. Cells were harvested and α-SMA expression was determined by immunostaining (**A**) and Western blotting (**B**) with the anti-α-SMA antibody. After 12 h, the combined stimulation of high glucose (500 mg/dl) and/or insulin (10 IU/ml) produced strong α-SMA immunofluorescence (A). A single band was detected in each lane corresponding to the known molecular weight (42 kDa) of α-SMA. Compared with the control (100 mg/dl glucose), there was a marked increase in band density in cells incubated with the combined stimulation of high glucose and insulin (B). Means ± SD of three independent experiments; $^{*}P < 0.05$ vs. 100 mg/dl glucose; $^{**}P < 0.05$ vs. 500 mg/dl glucose; $^{\#}P < 0.05$ vs. 100 mg/dl glucose +10 IU/ml insulin. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

more potent for the activation of PSCs than insulin ($P < 0.05$). Furthermore, the combined treatment of 500 mg/dl glucose and 10 IU/ml insulin significantly increased the percentage of α-SMA positive cells in the dishes ($3.7 \pm 0.2\%$, $n = 8$) over the other three groups ($P < 0.05$, Fig. 1A). Similar results were observed in Western blot analysis (Fig. 1B). These results suggest that treatment with high glucose combined with insulin accelerated rat PSC activation more than high glucose or insulin treatments by themselves.

The Additive Effects of Glucose and Insulin on PSC Proliferation

PSC proliferation was significantly enhanced by increased glucose concentrations from 100 to 500 mg/dl with various concentrations of insulin in a dose-dependent manner ($P < 0.05$). The additive effect of insulin on PSC proliferation was clearly demonstrated in all groups using various glucose concentrations (Fig. 2, $P < 0.05$). The highest rate of cell proliferation was observed when PSCs were stimulated with the combination of 500 mg/dl and 1 IU/ml insulin concentrations at 48 h: it was almost sixfold higher than that of the basal condition ($P < 0.05$). The addition of a supraphysiological concentration of insulin (100 IU/ml) in the medium had an inhibitory effect on the rate of cell proliferation.

Role of Mitogen-Activated Protein Kinase (MAPK) Signaling Pathway in PSC Proliferation Stimulated by Glucose and Insulin

High glucose and insulin treatment induced the activation of ERK 1/2. Both glucose and insulin independently enhanced the phosphorylation of ERK 1/2 (Fig. 3A,B). ERK 1/2 phosphorylation by glucose was increased significantly only at a high concentration of glucose (Fig. 3A, $P < 0.05$). In contrast, ERK 1/2 phosphorylation was gradually increased by insulin in a dose-dependent manner (Fig. 3B). Interestingly, ERK 1/2 phosphorylation at 500 mg/dl glucose peaked even with

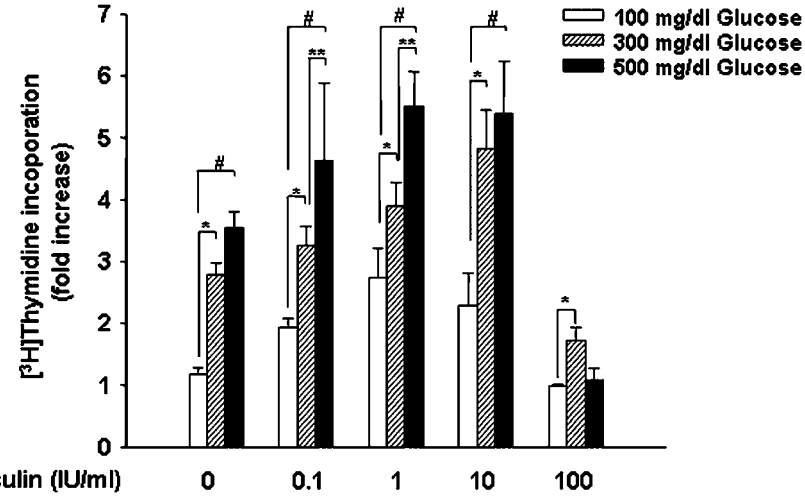

Fig. 2. Additive effect of glucose and insulin on the proliferation of PSCs. Cells were cultured with 100, 300, or 500 mg/dl glucose in medium supplemented with 0, 0.1, 1, 10, or 100 IU/ml insulin for 48 h. Cultures were stopped and cell proliferation was measured quantitatively as described in the methodology. The highest rate of cell proliferation was observed when PSCs were stimulated with the combination of 500 mg/dl and 1 IU/ml insulin at 48 h: it was almost sixfold higher than the basal level. Results are expressed as fold increases in [^{3}H] thymidine incorporation of controls. Means ± SD of three independent experiments (n = 8 each); *$P < 0.05$, 100 mg/dl vs. 300 mg/dl glucose; **$P < 0.05$, 300 mg/dl vs. 500 mg/dl glucose; $^{\#}P < 0.05$, 100 mg/dl vs. 500 mg/dl glucose.

the addition of a very low concentration of insulin in the medium, with a mild decrease at higher concentrations of insulin (Fig. 3C).

Inhibition of PSC proliferation with U0126 (a MEK inhibitor) pretreatment. The pretreatment of PSCs with U0126 inhibited the ERK 1/2 activation level induced by 500 mg/dl glucose and 10 IU/ml insulin in a dose-dependent manner (Fig. 4A,B). The ERK 1/2 phosphorylation level induced by 500 mg/dl glucose was reduced by more than 50% with U0126 ($P < 0.05$), whereas that induced by insulin was only suppressed by a high concentration of U0126 ($P < 0.05$). Furthermore, ERK 1/2 phosphorylation by the combined stimulation of high glucose and insulin was completely maintained in the range of 1–10 μM of U0126 (Fig. 4C). At 50 μM U0126, ERK 1/2 phosphorylation induced by the combined addition of high glucose and insulin was reduced to 50% of the peak: this level was still significantly higher than that of the control ($P < 0.05$).

PSC proliferation was also tested under the same conditions. Pretreatment with U0126 reduced the PSC proliferation stimulated by various concentrations of glucose and insulin (Fig. 5). The inhibitory effect of U0126 was more prominent for glucose-stimulated PSC proliferation. When PSCs were incubated with 500 mg/dl glucose, there was a 4.5-fold increase in cell proliferation, which was completely abolished by 1 μM U0126. However, the effect of U0126 on cell proliferation by insulin was ineffective at 1 μM U0126. Levels of proliferation of both insulin-stimulated and glucose plus insulin-stimulated PSCs were reduced only at 10 μM U0126.

Effects of High Glucose, Insulin, and U0126 on CTGF

CTGF expression was significantly increased by treatment with high glucose (500 mg/dl), insulin (10 IU/ml), or both ($P < 0.05$). Pretreatment with 10 μM U0126 abolished both glucose- and insulin-induced CTGF expression (Fig. 6A,B).

DISCUSSION

The discovery of PSCs, which are morphologically and functionally similar to HSCs, has provided a foundation to determine the mechanism of pancreatic fibrosis in various diseases [Watari et al., 1982]. Stable PSCs contain vitamin A in lipid droplets, and this is observed in normal tissues expressing musculoskeletal markers such as desmin or glial fibrillary acidic protein (GFAP) [Apte et al., 1998; Bachem et al., 1998]. When pancreatic injury leads to the increased production of profibrogenic factors,

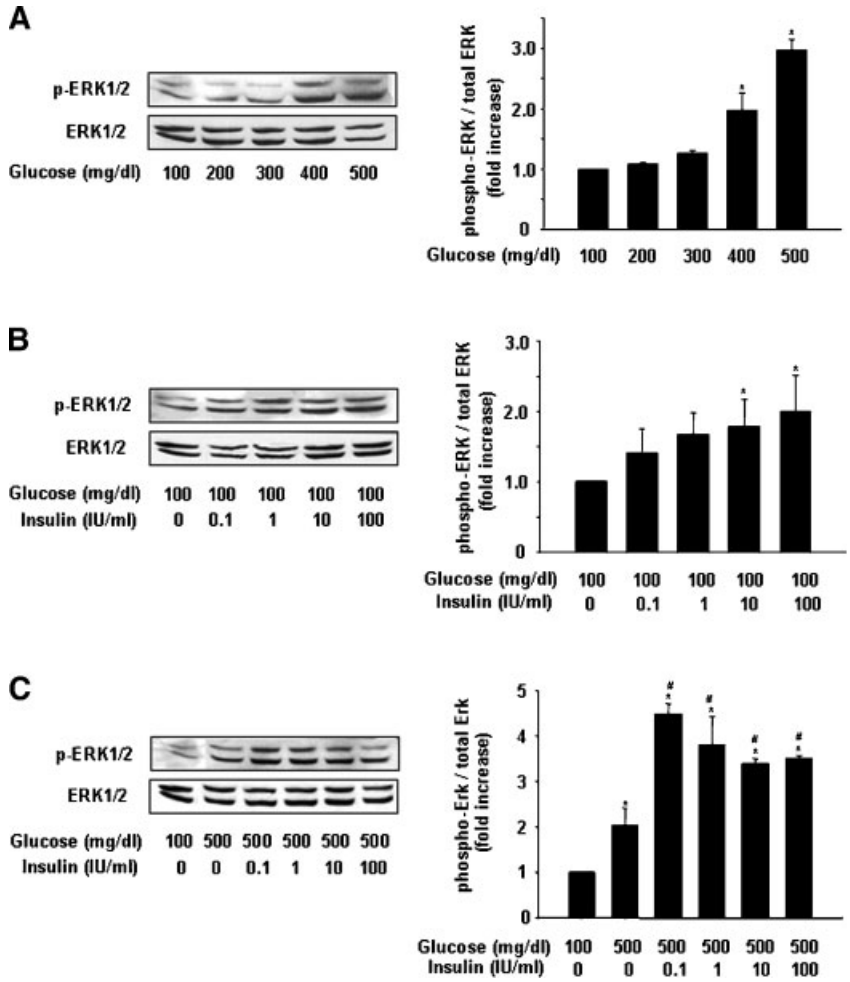

Fig. 3. Glucose and insulin induced activation of ERK 1/2 in rat PSCs in dose-dependent manners. Rat PSCs were plated at a density of 5×10^5 cells per 60 mm dish. After 24 h, the medium was replaced with serum-free medium. **A**: Cells were cultured with various concentrations of glucose-containing medium. After incubation for 10 min, cells were harvested and ERK 1/2 activity was determined by Western blotting with antiphosphorylated ERK antibody. Western blotting with anti-ERK antibody was carried out as an internal control. **B**: Cells were cultured with 100 mg/dl glucose medium supplemented with 0, 0.1, 1, 10, and 100 IU/ml insulin for 10 min. **C**: Cells were cultured with 500 mg/dl glucose medium supplemented with 0, 0.1, 1, 10, and 100 IU/ml insulin for 10 min. ERK1/2 activation was induced by glucose and insulin in a dose-dependent manner. However, ERK activation in 500 mg/dl glucose reached a peak level even with the addition of a very low concentration of insulin. Means ± SD of three independent experiments; $^{*}P < 0.05$ vs. 100 mg/dl glucose; $^{\#}P < 0.05$ vs. 500 mg/dl glucose.

PSCs proliferate and transform into myofibroblast-like cells, resulting in the increased synthesis and secretion of ECM with increased expression of α-SMA [Apte et al., 1999]. That the activation of PSC and fibrosis are related has already been proven in animal models and human pancreatic tissue [Bachem et al., 1998]. The increased expression of α-SMA in the fibrotic areas of pancreatic tissue has also been observed [Yoshikawa et al., 2002]. Cytokines that stimulate PSC activation include platelet-derived growth factor (PDGF), the TGF-β family members TGF-β1 and activin A, TGF-α, basic fibroblast growth factor, TNF-α, and interleukin (IL)-1 and IL-6 [Apte et al., 1999; Schneider et al., 2001; Mews et al., 2002]. Among these, TGF-β1, an isoform of TGF-β, is a most potent fibrogenic mediator that regulates the synthesis and secretion of ECM molecules from stellate cells [Menke and Adler, 2002]. Angiotensin II (ATII), which is induced by high levels of glucose, also stimulates PSC proliferation and induces cell contraction in vitro [Reinehr et al., 2004; Ko et al., 2006]. Although the exact mechanism of high glucose-induced ATII production needs further

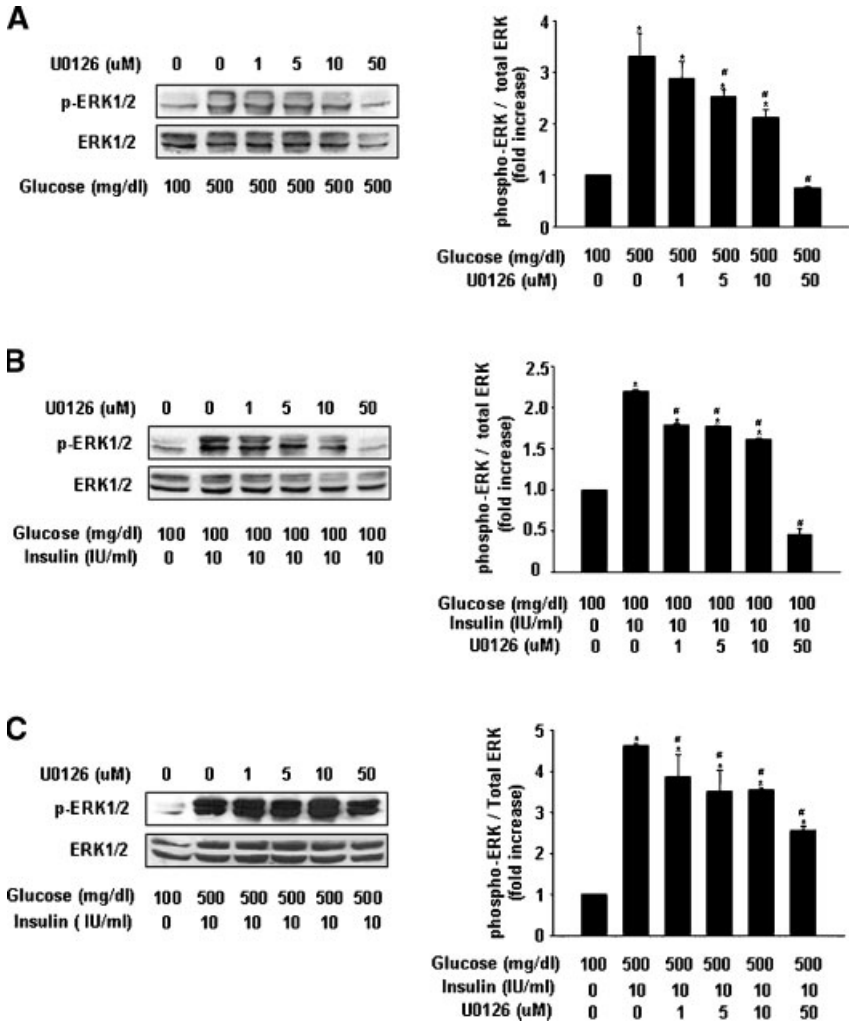

Fig. 4. Suppression of glucose- and insulin-induced ERK1/2 phosphorylation by U0126. Rat PSCs were preincubated with U0126 (0–50 μM) for 30 min before stimulation with glucose and/or insulin. Cells were cultured for 10 min with 500 mg/dl glucose (**A**), with 100 mg/dl glucose medium supplemented with 10 IU/ml insulin (**B**), or with 500 mg/dl glucose medium supplemented with 10 IU/ml insulin (**C**). Cells were harvested and ERK 1/2 activity was determined by Western blotting using an antiphosphorylated ERK antibody. Pretreatment of PSCs with U0126 inhibited the ERK1/2 activation induced by glucose and insulin in a dose-dependent manner. ERK1/2 activation induced by the combined stimulation of glucose and insulin was reduced to only 50% of the peak even with 50 μM U0126. Means ± SD of three independent experiments; $^{*}P < 0.05$ vs. 100 mg/dl glucose; $^{\#}P < 0.05$ vs. U0126 untreated controls.

clarification, activation of the local renin-angiotensin system (RAS) in the pancreas seems to be closely associated with the inflammatory response, leading to tissue fibrogenesis. We have reported that application of the ACE inhibitor ramipril suppressed pancreatic inflammation and fibrosis in an animal model of type 2 diabetes mellitus, the OLETF rat [Ko et al., 2004]. By an in vitro study [Ko et al., 2006], we demonstrated AT receptor subtypes and upregulated expression of the ATII receptor 1a (AT1a), angiotensinogen, and ACE in response to increased glucose concentrations, suggesting a direct effect of the RAS on PSC proliferation. TGF-β expression was increased later in culture medium, after the increase in ATII. Those results suggested that hyperglycemia could stimulate the activation and proliferation of PSC through activation of ATII-TGF-β1 signals [Ko et al., 2006]. Thus, ACE inhibitors or AT-receptor antagonists could be effective in preventing or attenuating islet fibrosis in hyperglycemic patients.

Although a hyperglycemic environment should have influenced the whole pancreas, PSC activation and fibrosis were limited mainly

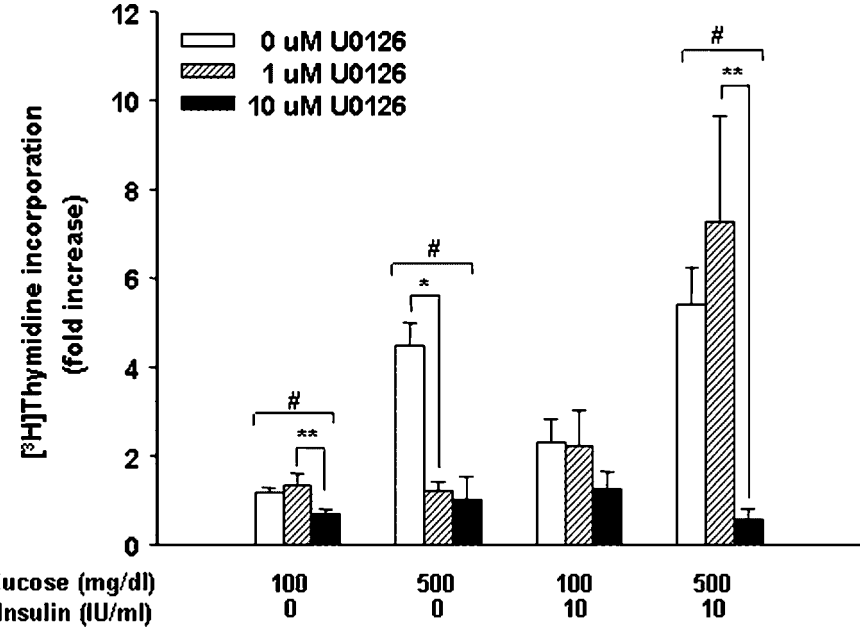

Fig. 5. U0126 inhibited glucose- and insulin-induced proliferation of PSC. Rat PSCs were pretreated with U0126 (0, 1, and 10 μM) for 3 h and then stimulated with high glucose (500 mg/dl), 10 IU/ml insulin, or combination of high glucose and insulin. After 48 h incubation in serum-free medium, cell proliferation was measured quantitatively as described in the methodology. The inhibitory effect of U0126 was more prominent in glucose-stimulated PSC proliferation, and the PSC proliferation induced by the combined stimulation of glucose and insulin was suppressed only at 10 μM U0126. Means ± SD of three independent experiments; $^{*}P < 0.05$, 0 μM vs. 1 μM U0126; $^{**}P < 0.05$, 1 μM vs. 10 μM U0126; $^{\#}P < 0.05$, 0 μM vs. 10 μM U0126.

to the islets of OLETF rats. It seems that hyperglycemia alone may not be enough to activate PSCs in the whole pancreas, so other factors might induce the activation of PSCs in the islets of these diabetic rats. Insulin is a well-known cell growth factor and is continuously secreted into the capillary of the islets at a relatively high concentration, although patients with Type 2 diabetes mellitus shown relative insulin deficiency in the whole body. Therefore, we hypothesized that local hyperinsulinemia in the islets may predispose towards PSC activation and proliferation in a hyperglycemic environment.

In this study, we demonstrated that hyperglycemia and hyperinsulinemia both promote PSC proliferation in vitro, although the pattern was a little different between the two treatments. Glucose was more potent and enhanced PSC proliferation gradually in a dose-dependent manner. Although not as effective as glucose, insulin also significantly influenced PSC proliferation within a limited range of concentrations. PSC proliferation in response to insulin and glucose peaked at 500 mg/dl and 1 IU/ml, respectively. When treated concomitantly, the peak activation was at the aforementioned concentration with almost sixfold that of the basal level, confirming the additive effect. Although PSC proliferation increased in a dose-dependent manner with glucose, the addition of a high level of insulin (100 IU/ml) seemed to play a negative role in this. The additive effect of high glucose and insulin was verified once again by the increase in α-SMA expression noted by immunostaining and Western blotting.

The signaling pathways activating stellate cells are not yet fully understood, although the ERK [Jaster et al., 2002] and phosphatidylinositol 3-kinase (PI3-k) [Svegliati-Baroni et al., 1999] pathways are known to be involved. In our study, both glucose and insulin induced ERK 1/2 phosphorylation in dose-dependent manners. A prominent effect on the proliferation of PSCs was observed at low concentrations of insulin, whereas the effect of glucose was limited to high concentrations (400–500 mg/dl). Concomitant treatment of insulin with high glucose also showed a similar tendency.

U0126 is a selective inhibitor of mitogen-activated protein kinase kinase (MEK) [Duncia

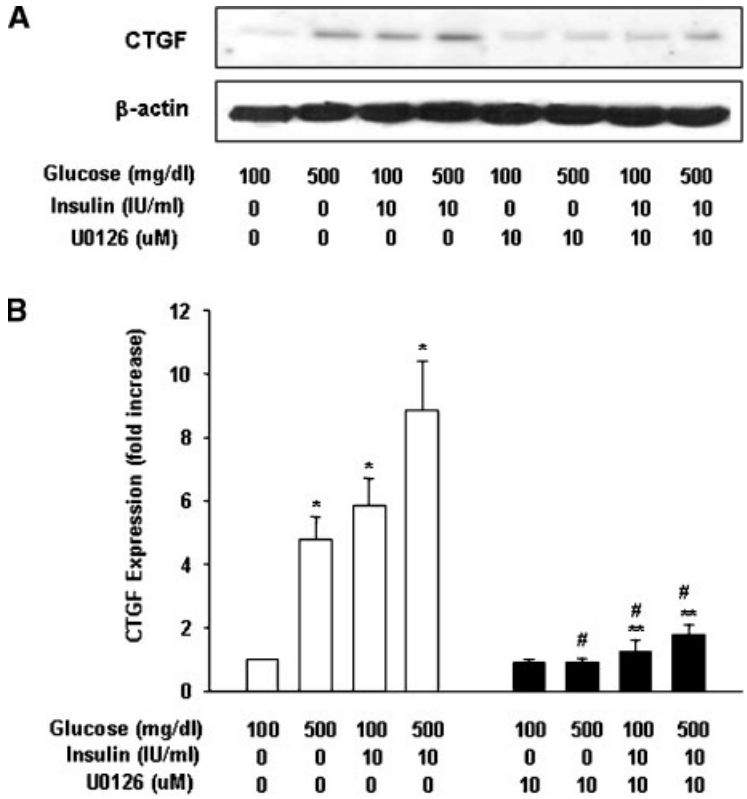

Fig. 6. Glucose- and insulin-induced CTGF expression in the PSCs were suppressed by U0126. Rat PSCs were treated with 10 μM U0126 for 3 h and then stimulated with high glucose (500 mg/dl) and/or 10 IU/ml insulin. After 48 h incubation in serum-free medium, Western blot analysis (**A**) and scanning densitometry (**B**) showed increased CTGF expression in PSCs treated with high glucose and/or insulin. With the 10 μM U0126 treatment, CTGF expression was reduced significantly in all groups. Means ± SD of three independent experiments; $^{*}P < 0.05$ vs. 100 mg/dl glucose; $^{**}P < 0.05$ vs. 500 mg/dl glucose; $^{\#}P < 0.05$ untreated controls vs. 10 μM U0126.

et al., 1998]. When ERK 1/2 activity was observed with U0126 pretreatment, the cells treated with glucose showed blocking of activity in a dose-dependent manner. However, the increased ERK 1/2 activity produced by insulin or insulin plus high glucose was suppressed only at a high concentration (50 μM) of U0126. PSC proliferation was measured under the same conditions, which showed that U0126 was more effective in the glucose-stimulated group; 1 μM of U0126 was enough to suppress the PSC proliferation induced by the high glucose treatment, whereas 10 μM was needed to suppress the cells treated with high glucose plus insulin. From these concordant results, we may presume that insulin is an important stimulus in augmenting the ERK pathway-related PSC proliferation. However, another pathway might play a role in insulin-induced PSC proliferation.

CTGF, an important downstream mediator of TGF-β activity [Igarashi et al., 1993; Hong et al., 1999], was significantly upregulated by high glucose and insulin treatments, but almost totally suppressed by U0126. Several studies have demonstrated the importance of the ERK pathway in CTGF production [Chen et al., 2002; Stratton et al., 2002; Leask et al., 2003], and our findings are in agreement with them.

The results of the present study are in line with previous studies showing the stimulatory and inhibitory effects of various mediators (i.e., PDGF, ATII, insulin, IGF-1 and trapidil, PD98059, AG1478, respectively) on stellate cell proliferation in a similar manner [Marra et al., 1999; Svegliati-Baroni et al., 1999; Jaster et al., 2002; Hama et al., 2004]. Although there have been several studies evaluating the effect of glucose and insulin on cell proliferation, this study was the first to demonstrate the response of PSC. Recently, studies on various agents such as the HMG CoA reductase inhibitor [Jaster et al., 2003], antioxidants [Kim et al., 2001], and thiazolidinedione [Shimizu et al., 2004] have

been shown to inhibit stellate cell activity. These results suggest that the MAPK pathway might be an important target for inhibiting pancreatic fibrosis, as demonstrated here.

In conclusion, it appears that hyperglycemia and hyperinsulinemia are two crucial mitogenic factors leading PSCs to proliferate, and that they act synergistically to amplify the response. Therefore, rigorous control of the glucose level and improvement in insulin resistance in diabetes to regulate such conditions may suppress pancreatic fibrosis. Also, products such as U0126 which inhibit the fibrosis pathway might be a promising agent in treating diseases that involve this pathology, including diabetes.

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