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High Glucose Increases Extracellular Matrix Production in Pancreatic Stellate Cells by Activating the Renin–Angiotensin System

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Abstract Pancreatic stellate cells (PSCs) are involved in pancreatic inflammation and fibrosis. Recent studies have shown that blocking the renin–angiotensin system (RAS) attenuates pancreatic inflammation and fibrosis. However, there are few data about the direct effects of high glucose on extracellular matrix (ECM) protein synthesis and angiotensin II (Ang II) induction in PSCs. PSCs were isolated from male Sprague–Dawley rats and cultured in medium containing 5.5 mM (LG group) or 27 mM D-glucose (HG group). Levels of Ang II and transforming growth factor-β (TGF-β) in culture media were measured and Ang II-positive cells were counted. We used real-time polymerase chain reaction (PCR) to detect Ang II receptor expression and Western blot analysis for the expression of ECM proteins such as connective-tissue growth factor (CTGF) and collagen type IV. Cells were also treated with an Ang II-receptor antagonist (candesartan, 10 μM) or angiotensin-converting enzyme (ACE) inhibitor (ramiprilat, 100 nM). Thymidine uptake by PSCs increased fourfold with high glucose treatment. Ang II levels and the proportion of Ang II-positive PSCs were significantly increased after 6 h under high-glucose conditions. TGF-β concentrations also increased significantly with high glucose. After 72 h, the expression of CTGF and collagen type IV proteins in high-glucose cultures increased significantly and this increase was effectively attenuated by the candesartan or the ramiprilat. All together, high glucose induced PSCs proliferation and ECM protein synthesis, and these effects were attenuated by an Ang II-receptor antagonist. The data suggest that pancreatic inflammation and fibrosis aggravated by hyperglycemia, and Ang II play an important role in this pathogenesis. J. Cell. Biochem. 98: 343–355, 2006.

Key words: pancreatic stellate cells; angiotensin II; high glucose; extracellular matrix protein

Since their discovery in 1998, pancreatic stellate cells (PSCs) have been identified as the major source of the extracellular matrix (ECM) proteins found in chronic pancreatitis or pancreatic fibrosis in both experimental animals and humans [Apte et al., 1999]. In the quiescent state, PSCs contain numerous vitamin A-storing lipid droplets in their cytoplasm [Apte et al., 1998; Bachem et al., 1998]. When activated by inflammatory cytokines or oxidative stress, PSCs transform into myofibroblast cells, and are immunostainable for alpha smooth muscle actin (α-SMA) [Apte et al., 1999]. PSCs show markedly increased ECM protein synthesis in response to various stimuli, such as cytokines and growth factors. Of these, platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF-β) exert potent proliferative effects on PSCs [Kruse et al., 2000; Jaster et al., 2002].

Classically, the systemic renin–angiotensin system (RAS) plays a crucial role in the

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maintenance of blood pressure and electrolyte balance through its action on vascular smooth muscle cells and aldosterone secretion. In particular, angiotensin II (Ang II) is a vasoactive agent that participates in hemodynamic regulation. However, Ang II also plays an important role in tissue inflammation, beyond its hemodynamic effects [Ruiz-Ortega et al., 2000; Suzuki et al., 2003]. The local RAS mainly contributes to this phenomenon. In addition to the circulating RAS, a local RAS exists in the kidney, adrenal gland, pituitary gland, brain, adipose tissue, and pancreas [Crandall et al., 1994; Vinson et al., 1998; McKinley et al., 2003]. Ang II receptor subtypes are present in the rodent pancreas, predominantly in the epithelia of pancreatic ducts and vessels, and in pancreatic acinar cells [Leung et al., 1999; Leung and Carlsson, 2001].

Locally produced Ang II promotes the recruitment of inflammatory cells, induces the expression and secretion of ECM proteins, and inhibits collagen degradation [Wolf and Neilson, 1993; Ruiz-Ortega and Egido, 1997; Suzuki et al., 2003]. Ang II also modulates cell growth by inducing hyperplasia/hypertrophy, depending on the cell type [Ruiz-Ortega et al., 2000]. RAS is believed to play a key role in tissue remodeling and fibrogenesis in the kidney, heart, and liver, suggesting that Ang II plays some part in pancreatic inflammation. More recent studies have shown that blocking the RAS attenuates pancreatic inflammation and fibrosis [Kuno et al., 2003] and liver fibrosis [Yoshiji et al., 2001]. However, the relationship between PSCs and Ang II has not been clarified.

In our previous report, the angiotensin-converting enzyme (ACE) inhibitor, ramipril, significantly attenuated the islet fibrosis in Otsuka Long Evans Tokushima fatty (OLETF) rats, an animal model of type 2 diabetes mellitus [Ko et al., 2004]. Interestingly, the proliferation of α-SMA-positive PSCs, fibrosis of the pancreatic islets, and ECM production in the pancreas were significantly increased in OLETF rats, and these traits are reduced with ramipril treatment. In general, the pathological manifestation of pancreatic islets in type 2 diabetic patients includes a reduced beta cell mass, amyloid deposition, and eventually islet fibrosis similar to the islet fibrosis observed in OLETF rats. OLETF rats with diabetic progression display severe islet destruction from fibrosis, accompanied by increased pancreatic expression of α-SMA, a specific marker of PSCs, especially surrounding the destroyed islets [Yoshikawa et al., 2002; Ko et al., 2004]. These data suggest that the ACE inhibitor attenuated islet destruction by fibrosis and had some beneficial effects on glucose tolerance through the suppression of PSC activation and proliferation in an animal model of type 2 diabetes mellitus. However, we could not determine whether the ACE inhibitor directly affected PSCs or did so via some other systemic effects. Furthermore, there have been few data about hyperglycemia and its effects on the RAS in PSC.

Herein, we investigated the effects of high glucose on PSC proliferation and Ang II production by PSCs. We also tested the direct effects on cultured PSCs of blocking the RAS.

MATERIALS AND METHODS

Isolation and Culture of PSCs

Rat PSCs were isolated from normal male Sprague–Dawley rats weighing 200–250 g, as described by Apte et al. [1998] with minor modifications. Cells were cultured under standard conditions in Dulbecco's minimal essential medium (DMEM) containing 10% fetal calf serum (FCS). Only the third and fourth cell passages were used in this experiment. Briefly, after the pancreas was excised, it was digested in Gey's balanced salt solution (GBSS) containing collagenase P (1 mg/ml solution; Boehringer-Manheim, Indianapolis, IN), 0.02% protease (Sigma Chemical Co., St Louis, MO), and 0.001% DNase (Boehringer-Manheim) at 37°C for 20 min. The cell suspension was filtered through 70 μm nylon mesh and washed three times in GBSS buffer. The cells were then centrifuged through an 8.2% Nycodenz gradient (Nycomed Pharma AS, Oslo, Norway) at 1,400*g* for 20 min. PSCs were collected at the interface between the Nycodenz and medium, and washed. The cells were plated on DMEM supplemented with 10% FCS, 100 IU/ml penicillin, and 100 mIU/ml streptomycin.

The viability of PSCs was over 95%, as assessed by Trypan-blue staining. The cells were plated at a density of 0.5×10^4 cells/ml on 60-mm plastic dishes. PSCs were cultured at 37°C in a humidified incubator under 95% air and 5% CO_2.

PSC Proliferation Induced by High Glucose

After growing to confluence, the PSCs were washed with phosphate-buffered saline (PBS),

and the medium was then changed to DMEM containing 10% FCS. These PSCs were incubated at 37°C for 24 h. After the cells were confluent, the medium was changed every 72 h.

To evaluate the effects of glucose on PSC proliferation, cells were serum-starved for 24 h and then incubated with 5.5 or 27 mmol/L D-glucose for 48 h in the presence of 0.5% FCS. DNA synthesis was assessed by [^{3}H]thymidine uptake. At the end of incubation, 10% trichloric acid was added. After the cells were washed, they were solubilized in 500 μl of cell dissolution solution (0.25 mM NaOH, 0.2% sodium dodecyl sulfate) and added to each well, to which was added 50 μl of 5 N HCl. The samples were transferred to scintillation vials with 4 ml of scintillant. The radioactivity of the samples was measured using a Packard β-counter (Packard Instruments Co., Downers Grove, IL). Each experiment was repeated at least three times. To exclude bias from the effects of hyperosmolarity, PSCs were incubated in a medium containing 5 mM glucose supplemented with 25 mM mannitol. PSCs were pretreated with the Ang II-receptor antagonist, candesartan (10 μM; Astrazeneca Pharmaceutical, UK), or the active form of the ACE inhibitor, ramiprilat (100 nM; Aventis Pharmaceutical, Germany).

Ang II and TGF-β Assays

To observe the effects of high glucose on Ang II production in PSCs, all culture media were collected for Ang II assays at 0, 2, 6, and 24 h. Ang II secretion from PSCs was measured by radioimmunoassay (Buhlmann Ang II RIA Kit, Buhlmann Laboratories AG, Schonenbuch, Switzerland) in the supernatants of culture media, according to the manufacturer's instructions. We investigated the changes in Ang II levels induced by glucose (5.5, 16.7, or 27 mmol/L). The immunoassay showed cross-reactivity of only 0.14% with angiotensinogen I.

TGF-β concentrations were also measured using an ELISA (TGF-β Immunoassay, R&D Systems, Inc., Minneapolis, MN). For the TGF-β assay, PSCs were cultured for 24, 48, or 72 h under high-glucose conditions. All culture media were supplemented with angiotensinogen (10^{-8} mol/L) during the incubation period. Experiments were performed in quadruplicate.

Immunohistochemistry and Quantification of Ang II-Positive PSCs

PSCs were cultured in low and high concentrations of glucose for 6 h and then fixed for immunostaining. After overnight incubation at 4°C with goat anti-human Ang II antibody (1:50; Santa Cruz Biotechnology, Santa Cruz, CA), sections were washed with PBS. The secondary antibody, fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse (1:100; Jackson ImmunoResearch Laboratories, PA), was applied for 1 h at room temperature, and the samples were then washed. The sections were mounted on slides with anti-fade medium containing 4′,6′-diamidino-2-phenylindole (DAPI, 1.5 μg/ml; Vector Laboratories, Inc., Burlingame, CA). Digital images at two fluorescent emission wavelengths were acquired using a confocal microscope (Bio-Rad Laboratories, Hercules, CA). FITC and Texas Red were excited at 488 and 568 nm, respectively, using an argon/helium laser. FITC and Texas Red images were scanned separately at different laser wavelengths and captured as overlapping images.

To calculate the relative percentage of Ang II-positive cells in total PSCs, the numbers of total PSCs and Ang II-stained PSCs were determined. The rate of Ang II positivity was calculated as a percentage of total PSCs, as described by the following equation: (number of Ang II-positive cells/number of total PSCs)×100. On average, 140 PSCs were counted for each slide of experimental conditions.

Reverse Transcription and Quantitative Polymerase Chain Reaction (RT-PCR)

Total RNA from PSCs cultured under each set of conditions was extracted with Trizol reagent (Gibco BRL, Grand Island, NY). After extraction, the RNA was precipitated with isopropanol, and the pellet was then resuspended in water. The integrity of the RNA was confirmed by gel electrophoresis and total RNA was quantified by spectrophotometry. Total RNA (4 μg) was used to synthesize first-strand cDNA according to the protocol of the SuperScript Preamplification System (Gibco BRL). Total RNA was incubated with 0.5 μg oligo(dT)$_{12-18}$ primer at 85°C for 3 min, and the reaction was then carried out in a mixture of 5× first-strand buffer from the kit containing 250 mM Tris-HCl, 375 mM KCl, and 15 mM $MgCl_2$, and 10 mM dithiothreitol, and 0.5 mM deoxynucleotide

phosphates in a final volume of 25 μl. The contents of the tubes were incubated at 42°C for 2 min and superscript II transcriptase was added and incubated at 42°C for 50 min. After the reaction, the enzyme was denatured at 70°C for 15 min. The final solution was used directly for PCR amplification. The relative expression levels of genes were determined with reference to the expression of the cyclophilin gene. Gene-specific primers were designed on the basis of published sequences. The PCR primers were: AT1a, sense 5′-GCACACTGGCAATGTAATGC-3′, anti-sense 5′-GTTGAACAGAACAAGTGACC-3′; AT1b, sense 5′-GCCTGCAAGTGAAGTGATTT-3′, anti-sense 5′-TCTGGCTGTGGCTGACTT-3′; ACE, sense 5′-CAGCTTCATCATCCAGTTCC-3′, anti-sense 5′-CTAGGAAGAGCAGCACCCAC-3′; AT2, sense 5′-CA-AGACTTGGTCACGGGT-3′, anti-sense 5′-T-CTGGCTGTGGCTGACTT-3′; angiotensinogen, sense 5′-ACACCCCTGCTACAGTCCAC-3′,anti-sense 5′-ACCCCCTCTAGTGGCAAGTT-3′; and cyclophilin, sense 5′-ACCACAGTCCATGCCATCAC-3′, anti-sense 5′-TCCACCACCCTGTTGCTGTA-3′. The PCR conditions were as follows. After the samples were boiled at 95°C for 10 min, amplification was repeated for 40 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min. The samples were then incubated at 72°C for 10 min to complete the elongation step and then stored at 4°C. The PCR products were visualized by 1.5% agarose gel electrophoresis, and the DNA bands were detected by ethidium bromide staining. The density of each band was measured using a VDS densitometer (Pharmacia Biotech).

Real-Time PCR

Total RNA was obtained using a PicoPure RNA isolation kit and a RiboAmp HS RNA amplification kit. cDNA was synthesized using superscript II RT (Invitrogen, Carlsbad, CA) and 1 mg of total RNA according to the manufacturer's instructions. Primers were as follows: AT1a (70 bp) 5′-TCGCTACCTGGCCATTGT-3′ (forward) and 5′-AGGTGACTTTGGCTACCAGCAT-3′ (reverse); AT1b (204 bp) 5′-GCCTGCAAGTGAAGTGATTT-3′ (forward) and 5′-TCTGGCTGTGGCTGACTT-3′ (reverse); AT2 (161 bp) 5′-CCCTGGCAAGCATCTTATGT-3 (forward) and 5′-CCAGCAGACCACTGAGCATA-3′ (reverse); ACE (97 bp) 5′-CACCGGCAAGGTCTGCTT-3′ (forward) and 5′-CTTGGCATAGTTTCGTGAGGAA-3′ (reverse); Angiotesinogen (212 bp) 5′-ACACCCCTGCTACAGTCCAC-3′ (forward) and 5′-ACCCCCTCTAGTGGCAAGTT-3′ (reverse); TBP 5′-ACCCTTCACCAATGACTCCTATG-3 (forward) and 5′-ATGATGACTGCAGCAAATCGC-3′ (reverse). PCR reactions were carried out in a volume of 20 ml containing 2 ml of cDNA template, 10 pM of each primer, and 1× SYBR Green dye (Invitrogen). The products were detected with the MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad) and the cycle values (C_t) were determined as a measure of the cycle number at which a statistically significant increase in fluorescence intensity was first detected and normalized to the value for the control gene TBP to yield the relative abundance. The values of gene/TBP were normalized to that of control.

Western Blot Analysis

Proteins were isolated from PSCs cultured for 72 h under various conditions. For analysis of AT II type 1 receptor expression in response to high glucose, we additionally isolated proteins from PSCs in 24- and 72-h culture.

All culture media contained angiotensinogen (10^{-8} mol/L), and some cells were also treated with ramiprilat or candesartan. PSCs were resuspended in lysis buffer (150 mM NaCl, 50 mM Tris-HCl [pH 7.4], 2 mM EDTA, 1% NP-40, 10 mM NaF, 1 mM Na_3VO_4, 10 mM sodium pyrophosphate, 1 mM PMSF, 10 mg/ml aprotinin, 10 mg/ml leupeptin, and 0.1 mg/ml soybean inhibitor). The lysate was centrifuged at 15,000 rpm for 5 min at 4°C. Protein concentrations were measured by the Bradford method using BSA as the standard.

Proteins (40 μg) were separated by SDS–PAGE on 10% polyacrylamide and transferred to polyvinylidene difluoride membrane (Hybond-P, Amersham Pharmacia Co., Buckinghamshire, England). The membrane was blocked with 5% fat-free dry milk for 1 h in tris-buffered saline (TBS; pH 7.6) and then incubated overnight at 4°C with the primary antibodies at dilution of 1:500 in TBS: anti-Ang II type 1 receptor antibody (Abcam Ltd., Cambridgeshire, UK), anti-connective-tissue growth factor (CTGF) antibody (Abcam Ltd.), fibronectin (DAKO, Denmark), and anti-collagen type IV antibody (Abcam Ltd.). After the membranes were washed, they were incubated for 1 h at room temperature with secondary peroxidase-conjugated anti-rabbit

or anti-mouse antibodies (Amersham Pharmacia Co.) diluted 1:1,000 in TBS-0.01% Tween 20. An antibody detection system (ECL, Amersham Pharmacia Biotech) was used and the membranes were exposed to X-ray film. Protein band intensities were quantified using a VSD densitometer (Amersham Pharmacia Biotech).

Statistical Analysis

Data are expressed as means ± SD. Differences between groups were evaluated using the SPSS 10.0 program (Chicago). The independent *t*-test and one-way ANOVA were used to analyze the quantitative variables between groups. A P value <0.05 was deemed significant.

RESULTS

Expression of RAS Components in PSCs

Cultured PSCs expressed mRNAs of the local RAS components, including angiotensinogen, ACE, AT1a, AT1b, and AT2, as shown in Figure 1. When cells were cultured at high glucose concentrations for 24 h, AT1a and ACE mRNAs were significantly upregulated ($P < 0.05$). At 72 h, transcripts of AT1a, ACE, and angiotensinogen were upregulated ($P < 0.05$). However, the expression of the genes for AT1b and AT2 did not change, even after 72 h under high-glucose conditions. To exclude the effects of osmolarity, we also compared the expression of the same genes in the cells cultured with the mannitol or

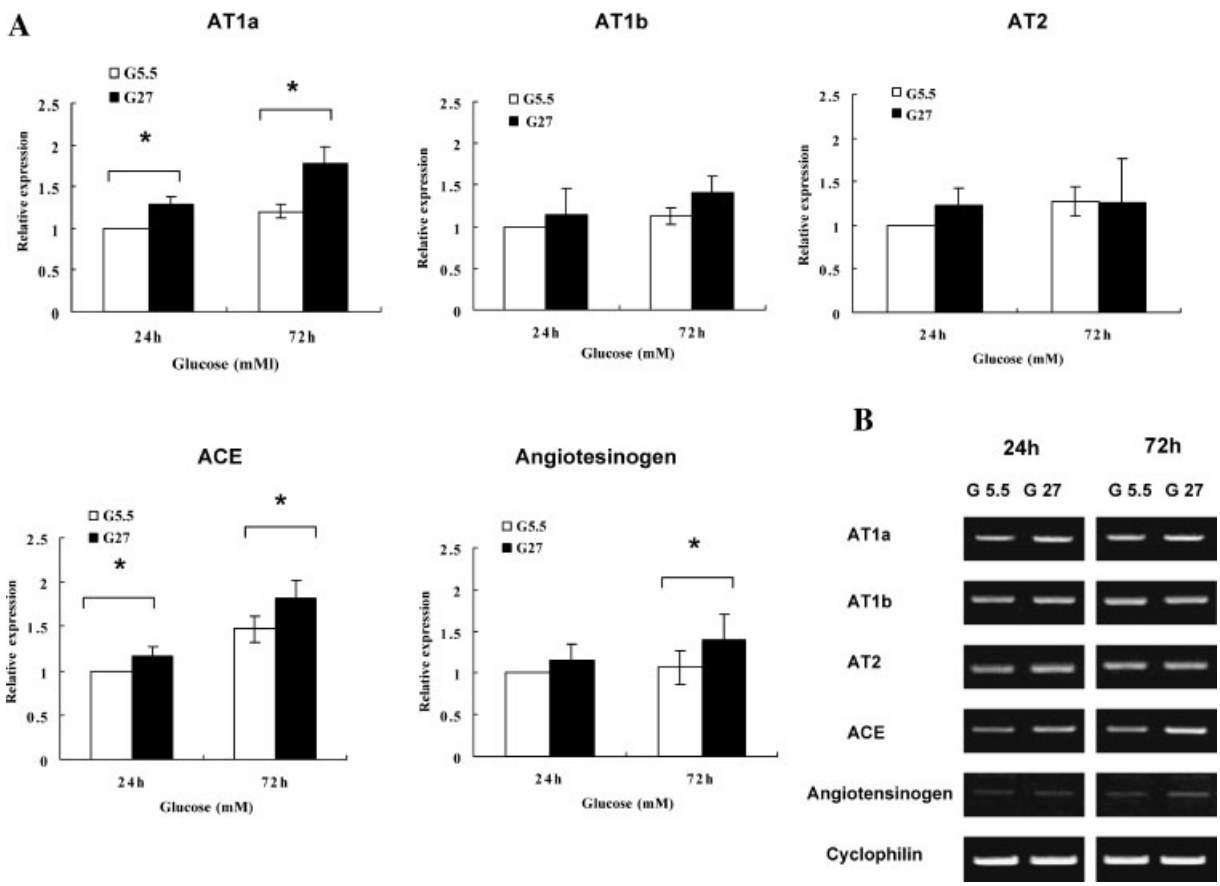

Fig. 1. Real-time PCR (**A**) and RT-PCR analysis (**B**) of local renin–angiotensin system (RAS) components in cultured pancreatic stellate cell (PSCs). When cells were cultured at a high glucose (27.7 mM) concentration for 24 h, AT1a and angiotensin-converting enzyme (ACE) mRNAs were significantly upregulated (*$P < 0.05$). At 72 h, AT1a, ACE, and angiotensinogen mRNAs were upregulated (*$P < 0.05$). However, expression of the genes for AT1b and AT2 was unchanged, even after 72 h under high-glucose conditions. Relative gene expression was normalized to cyclophilin expression. **C**: AT1 receptor expression in Western blot analysis. AT1 receptor expression was significantly increased after 24 and 72 h in 27 mM glucose compared to their expression in medium containing 5.5 mM glucose (*$P < 0.05$).

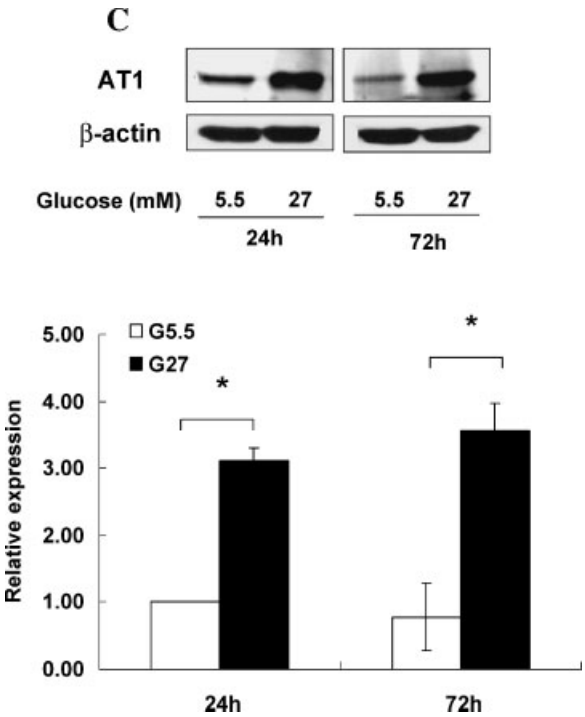

Fig. 1. (*Continued*)

L-glucose-containing medium. Neither mannitol nor L-glucose at 25 mM concentrations had a significant effect on the expression of these genes (data not shown).

Localization and Expression Changes of AT1 Receptor in PSCs

AT1 receptor expression was evaluated by Western blot analysis (Fig. 1C). AT1 receptor expression was significantly increased after 24 and 72 h in 27 mM glucose compared to their expression in medium containing 5.5 mM glucose.

High Glucose Induces PSC Proliferation, Which Is Attenuated by Blocking RAS

PSCs were cultured in high-glucose-containing medium (27 mM) with no angiotensinogen or serum and cell proliferation was measured by [^{3}H]thymidine uptake. Cell proliferation was significantly increased at high glucose concentrations in a dose-dependent manner ($P < 0.05$, Fig. 2). After 48 h of culture under high-glucose conditions, PSC proliferation was markedly increased, up to fourfold that in low-glucose cultures. This increase was dramatically attenuated by treatment with candesartan or ramiprilat. PSC proliferation was more

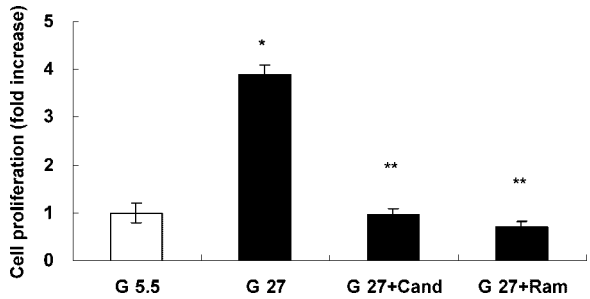

Fig. 2. PSC proliferation was measured by [^{3}H]thymidine uptake. Compared with that of low-glucose (5.5 mM) cultures, PSC proliferation was markedly increased in high-glucose (27.7 mM) cultures at 48 h. The increase was dramatically attenuated by treatment with candesartan (10 μM) or ramiprilat (100 nM) (*$P < 0.05$ compared with low-glucose culture; **$P < 0.05$ compared with high-glucose culture; G 5.5, 5.5 mM glucose; G 27, 27 mM glucose; Cand, candesartan; Ram, ramiprilat).

strongly suppressed in the ramiprilat-treated group than in the candesartan-treated group ($P < 0.05$).

Increased Ang II Production Induced by High Glucose Is Attenuated by Blocking RAS in PSCs

The Ang II concentration in the culture medium was measured in PSC cultures pretreated with angiotensinogen. The Ang II concentration was significantly increased after 6 h under high-glucose conditions (5.5 mM, 17.0 ± 4.9 pg/ml vs. 27 mM, 36.4 ± 8.2 pg/ml, $P < 0.05$). After 24 h, the Ang II level was reduced below that at 6 h (Fig. 3A). When PSCs were treated with ramiprilat or candesartan, the increase in Ang II concentration induced under high-glucose conditions was significantly attenuated at 6 h (Fig. 3B). Thus, high-glucose-induced Ang II production was virtually abolished by preincubation of the PSCs with an ACE inhibitor. This decrease was greater in the ramiprilat-treated PSCs than in the candesartan-treated PSCs. In contrast, the Ang II concentration was not altered by these drug treatments in the low-glucose (5.5 mM) cultures (Fig. 3B).

The results of Ang II immunostaining are similar to the Ang II assay findings. Although the relative percentage of Ang II-positive cells did not change for 2 h, Ang II-positive cells increased significantly thereafter (Fig. 4A,B). Compared with the low-glucose cultures, Ang II-positive cells were markedly increased in the high-glucose cultures at 6 h (5.5 mM glucose,

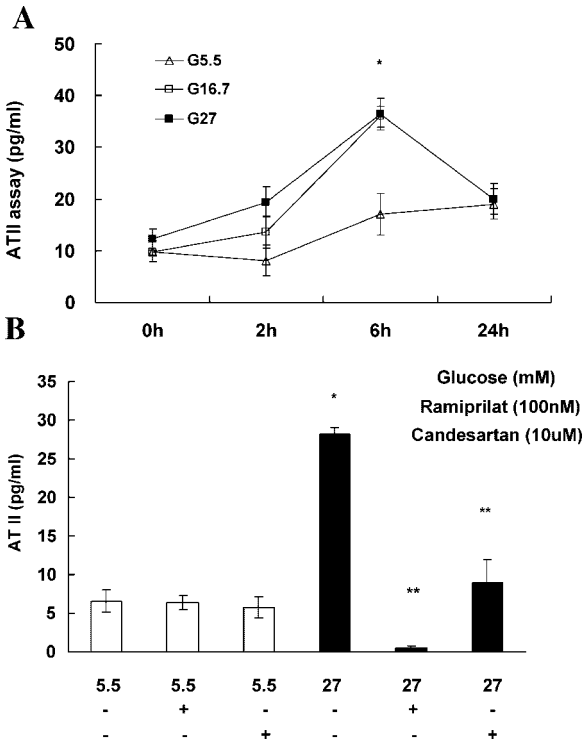

Fig. 3. **A**: Ang II concentrations in culture media of PSCs pretreated with angiotensinogen. Ang II concentration was significantly increased after 6 h under high-glucose conditions (16.7 and 27 mM, $*P<0.05$ compared with low-glucose culture). After 24 h, Ang II levels had decreased relative to those at 6 h. **B**: When PSCs were treated with ramiprilat or candesartan, the increased Ang II concentration under high-glucose conditions was significantly attenuated at 6 h. Ang II production induced by high glucose was virtually abolished by preincubation of PSCs with ACE inhibitor (ramiprilat, 100 nM) or AT-receptor antagonist (candesartan, 10 μM). Ang II concentrations did not change with drug treatments in low-glucose (5.5 mM) cultures, and the decrease was greater in ramiprilat-treated PSCs ($*P<0.05$ compared with low-glucose culture; $**P<0.05$ compared with high-glucose culture).

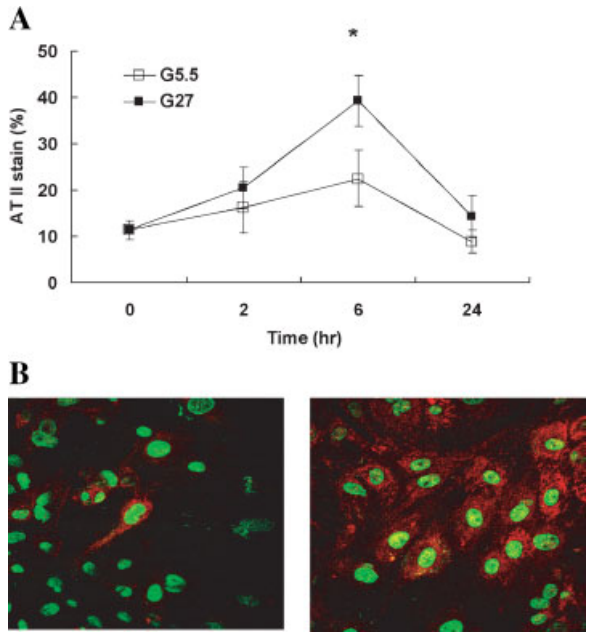

Fig. 4. **A**: Ang II immunostaining. The relative percentage of Ang II-positive PSCs did not differ for 2 h. However, Ang II-positive cells in high-glucose culture (27.7 mM) increased significantly after 6 h, compared with those in low-glucose cultures. G 5.5, 5.5 mM glucose; G 27, 27 mM glucose ($*P<0.05$ compared with low-glucose culture). **B**: Confocal microscopic results of Ang II immunostaining in PSC cultures. The rate of Ang II positivity was calculated as a percentage of total PSCs, as described by the following equation: (number of Ang II-positive cells/number of total PSCs)×100. Ang II-positive cells increased markedly in high-glucose cultures at 6 h (Lt, 5.5 mM glucose; Rt, 27.7 mM glucose. Red, Ang II-positive cells; green, DAPI-stained nuclei, 400×).

22.5% ± 11.0% vs. 27 mM glucose, 39.3% ± 12.0%, $P < 0.05$).

TGF-β in Culture Medium

We measured TGF-β concentrations in the culture medium to determine whether high glucose stimulates TGF-β production in PSCs. Although there was no difference in high-glucose cultures at 24 h, the TGF-β concentration in the medium was significantly increased after 48 h (Fig. 5). Compared with 5.5 mM glucose, 27 mM glucose markedly stimulated the production of TGF-β, even after 72 h (5.5 mM glucose, 436.3 ± 169.0 pg/ml vs. 27 mM glucose, 1,115.1 ± 434.0 pg/ml, $P < 0.05$).

ECM Protein Expression Induced by High Glucose in PSCs

The expression in response to high glucose of ECM proteins such as CTGF and collagen type IV, was quantified by Western blot analysis. The amounts of ECM proteins were measured in PSCs pretreated with angiotensinogen. The expressions of CTGF and collagen type IV proteins were significantly increased after 48 h in 27mM glucose compared with their expression in medium containing 5.5 mM glucose (Fig. 6A,B). After 72 h, the expression of these proteins in 27 mM glucose had increased more than threefold relative to that in 5.5 mM glucose. Thus, ECM protein expression increased in a dose- and time-dependent manner. Fibronectin expression also showed similar pattern (Fig. 6C). The increased expression of all these proteins was significantly

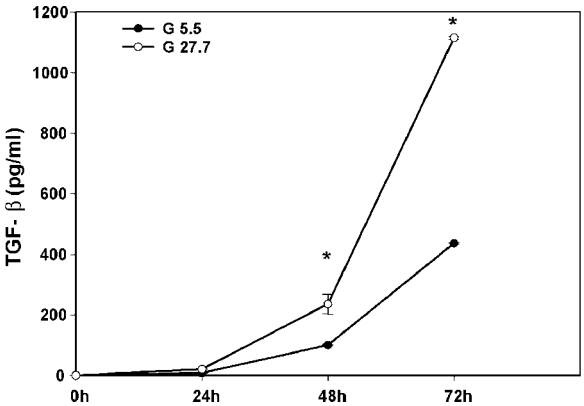

Fig. 5. Effects of glucose on TGF-β production in PSCs. Although there was no increase in TGF-β after 24 h in glucose, the TGF-β concentration in the medium increased significantly after 48 h in high-glucose cultures (*$P < 0.05$ vs. low glucose). G 5.5, 5.5 mM glucose; G 27, 27 mM glucose.

attenuated by ramiprilat or candesartan treatment after 72 h in culture (Fig. 7A–C). The degree of attenuation of protein expression was greater after ramiprilat treatment than after candesartan treatment.

DISCUSSION

In this study, we have shown that high glucose significantly increased PSC proliferation and the PSC proliferation rate well correlated with Ang II production. High glucose also induced ECM protein synthesis, and this was attenuated by an ACE inhibitor or Ang II-receptor antagonist. To our knowledge, these results are the first report that PSC proliferation is enhanced under high-glucose conditions via activation of the RAS.

PSCs were first described in the pancreas in 1982 [Watari et al., 1982]. In the pancreas, stellate-shaped cells comprise about 4% of all pancreatic cells with a periacinar distribution [Apte et al., 1998]. When activated by profibrogenic mediators, such as cytokines or oxidative stress, quiescent PSCs transform into myofibroblast-like cells, which are positive for α-SMA [Wolf and Neilson, 1993; Apte et al., 1999; Kuno et al., 2003]. PDGF and TGF-β are strong stimuli of PSC proliferation. Many recent reports provide evidence that activated PSCs are one of the cell types that play a role in pancreatic fibrogenesis, including chronic pancreatitis and pancreatic fibrosis [Haber et al., 1999; Yokota et al., 2002].

In general, the RAS plays a crucial role in the maintenance of blood pressure. Besides the systemic effects of the RAS, some studies have described the presence of a tissue RAS in

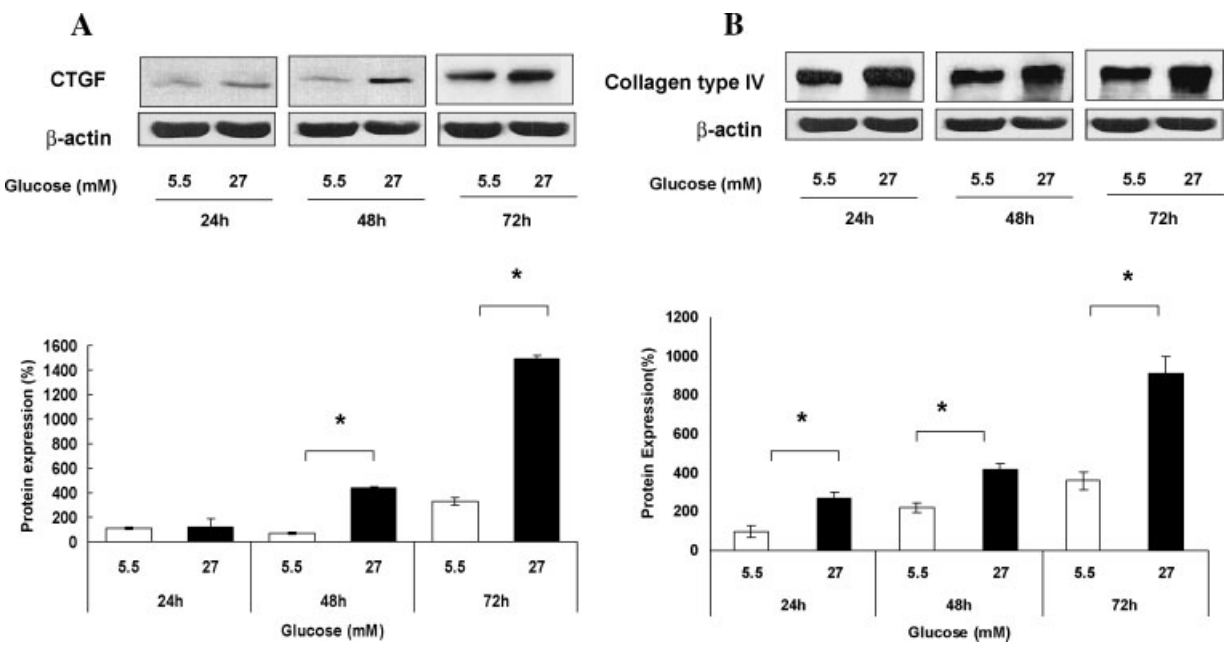

Fig. 6. The expression of extracellular matrix (ECM) proteins in response to high glucose stimulation was determined by Western blot analysis. The expression of CTGF (**A**), collagen type IV (**B**), and fibronectin (**C**) proteins was significantly increased after 48 h in 27 mM glucose, compared with culture in 5.5 mM glucose. G 5.5, 5.5 mM glucose; G 27, 27 mM glucose; CTGF, connective-tissue growth factor (*$P < 0.05$ vs. low glucose).

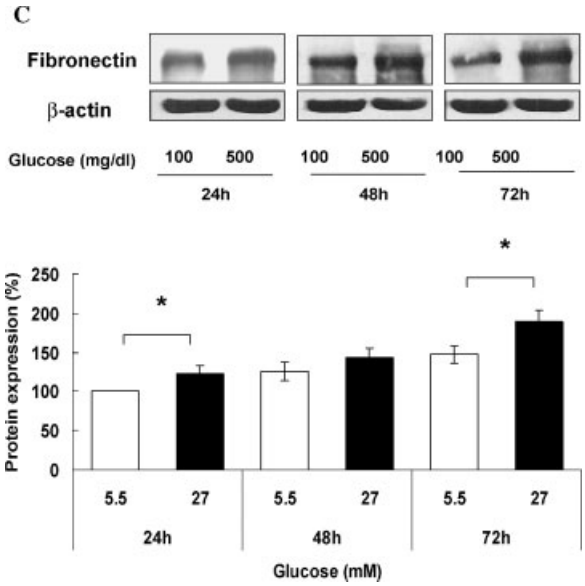

Fig. 6. (*Continued*)

kidney, adrenal gland, adipose tissue, pituitary gland, and brain, in addition to the pancreas [Crandall et al., 1994; Vinson et al., 1998; Leung et al., 1999; Leung and Carlsson, 2001; McKinley et al., 2003]. In rats, the mRNAs of angiotensinogen, renin, and the Ang II receptor subtypes have been observed in the pancreas and localized to the epithelium of pancreatic ducts and the endothelium of blood vessels [Leung et al., 1999; Leung and Carlsson, 2001]. These findings suggest that Ang II and ACE inhibitors might have a direct influence on pancreatic islets. The pancreatic tissue RAS has some regulatory effects in the pancreas, for example, in pancreatic microcirculation, ductal secretion, and islet hormone secretion [Leung and Chappell, 2003; Leung and Carlsson, 2005]. Moreover, recent studies reported that local pancreatic islet RAS with its major components were identified in mouse islets, having a role in the physiological regulation of glucose-induced insulin secretion and improving islet graft blood flow [Fliser et al., 1997; Lau et al., 2004; Kampf et al., 2005]. These findings showed that blockade of local islet RAS might be a good candidate of treatment target to increase islet graft survival and function of transplanted islet grafts, in addition to control of glucose-stimulated insulin secretion.

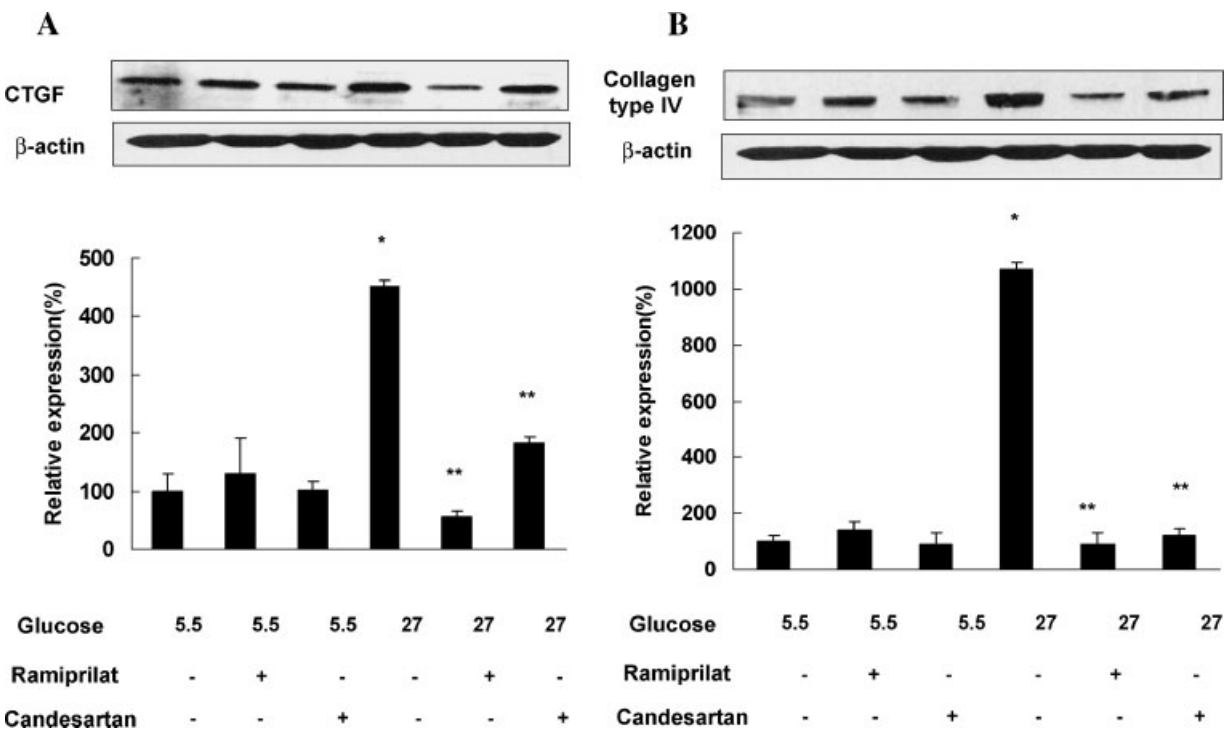

Fig. 7. Effects of ramiprilat and candesartan on ECM protein expression in PSCs. The expression of CTGF (**A**), collagen type IV (**B**), and fibronectin (**C**) proteins was significantly attenuated after 72 h of culture by ramiprilat or candesartan pretreatment. G 5.5, 5.5 mM glucose; G 27, 27 mM glucose; CTGF, connective-tissue growth factor (*$P < 0.05$ compared with low-glucose culture; **$P < 0.05$ compared with high-glucose culture).

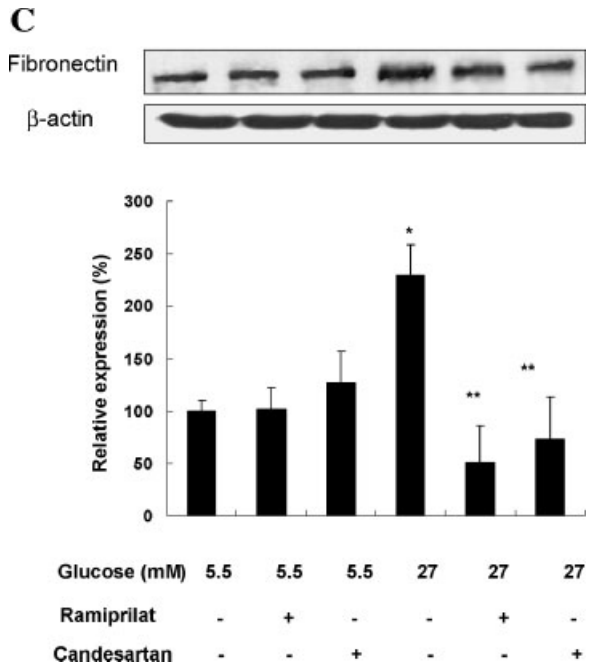

Fig. 7. (*Continued*)

Expression of the mRNAs for components of the tissue RAS, such as AT1a, ATb, angiotensinogen, and AT2, was also observed in PSC cultures in this study, suggesting that the RAS operates in PSC proliferation or activation. Ang II acts through two specific receptor subtypes. Most-known actions of Ang II are mediated by AT1, including vasoconstriction and the deposition of matrix proteins [Leehey et al., 2000; Bataller et al., 2003]. In our result, mRNA expression of AT1a receptor was significantly upregulated in response to high glucose stimuli, whereas AT1b and AT2 showed no significant changes of their mRNA expressions according to high glucose. In Western blot analysis, AT1 protein expression was significantly upregulated by high glucose concentration, suggesting that the effect of Ang II on PSCs be also mediated by AT1.

In renal fibrosis, Ang II activates mesangial cells, tubular cells, and interstitial fibroblasts, increasing the expression and synthesis of ECM proteins mediated by the release of growth factors such as TGF-β and PDGF [Wolf and Neilson, 1990; Wolf et al., 1993]. In addition to its hemodynamic effects, Ang II participates in inflammatory cell recruitment, mesangial cell activation, and the synthesis of ECM proteins during renal injury [Wolf et al., 1993]. Therefore, Ang II has a possible role in fibrinogenesis in the pancreas. Previously, we observed that PSCs are stimulated around the pancreatic islets in OLETF rats [Ko et al., 2004]. This increased PSC proliferation is compatible with the expression of α-SMA mRNA and protein, which is related to ECM proteins. This suggests that PSCs contribute to the pathogenesis of islet fibrosis in this animal model of type 2 diabetes. We could not determine previously whether Ang II directly influences PSC proliferation. However, in this in vitro study, the presence of AT receptor subtypes and the upregulated expression of AT1a, angiotensinogen, and ACE in response to increased glucose concentrations suggest that the RAS is directly involved in PSC proliferation. This finding contributes to our understanding of the mechanism by which sustained hyperglycemia induced progressive islet destruction by proliferation of PSCs in the islets.

In our results, there is a difference between the time course of RNA induction (Fig. 1) versus protein (Fig. 3). It is generally expected that RNA is elevated prior to the protein levels and not the reverse. Ang II, in contrast to Ang I, is largely cell-associated, either due to AT1 receptor-dependent internalization or intracellular generation. Ang II degradation is most likely due to rapid metabolism in the vascular wall by angiotensinases such as aminopeptidases A and M, prolyl endopeptidase, and carboxypeptidases [Iyer et al., 1998]. The in vivo half-life of intact Ang II in heart, kidney, and adrenal was approximately 15 min, compared with 0.5 min in the circulation [van Kats et al., 1997]. But there is little-known data about the angiotensinases in vitro system, especially in PSCs. We could not explain exactly this phenomenon; however, it is possible that Ang II degradation showed some discrepant pattern to in vivo condition.

The mechanism underlying the Ang II increase in response to glucose has not been clarified. In the proximal tubule cells of the kidney, a glucose-response element has been identified in the angiotensinogen gene promoter, and high glucose stimulates angiotensinogen synthesis in a concentration-dependent manner [Zhang et al., 1999; Hsieh et al., 2002; Giacchetti et al., 2005]. In mesangial cells, high glucose induces an increase in Ang II generation, resulting from an increase in intracellular renin activity mediated by at least three factors: the time-dependent stimulation of (pro)renin

gene transcription, a reduction in prorenin enzyme secretion, and an increased rate of conversion of prorenin to active renin, probably mediated by cathepsin B [Vidotti et al., 2004]. Further studies are required to clarify the exact mechanism of high-glucose-induced Ang II production in PSCs.

We have shown that PSCs are activated by high glucose concentrations without PDGF or TGF-β stimulation. We found that PSC proliferation following high glucose stimulation was accompanied by Ang II production. Moreover, TGF-β expression increased after 48 h in culture medium containing high glucose, after the increase in Ang II. Therefore, Ang II could stimulate TGF-β synthesis, which subsequently leads to ECM protein synthesis. Although it is possible that high glucose itself stimulates TGF-β production directly, independently of Ang II, the effects of the ACE inhibitor and AT-receptor antagonist on ECM production are additional evidence of the role of Ang II in ECM production. As described previously, Ang II is closely associated with the inflammatory response, leading to tissue fibrinogenesis [Powell et al., 1999; Jaster, 2004; Reinehr et al., 2004].

PSCs are involved in pancreatic fibrosis, which causes extensive fibrotic changes in the whole pancreas, especially in acute or chronic pancreatitis models. Why pancreatic fibrosis was localized to the pancreatic islets of the animal model of type 2 diabetes in our previous study was not elucidated. The ACE inhibitor and AT-receptor antagonist clearly downregulated CTGF and collagen type IV expression in PSCs. Expression of these proteins was also increased in high-glucose cultures, compared with the non-significant changes observed in low-glucose cultures. These findings are compatible with our experiments using an animal model. The ACE inhibitor had a greater suppressive effect on ECM production than did the AT-receptor antagonist. We anticipated that the ACE inhibitor would have a more powerful protective action against tissue fibrogenesis than the AT-receptor antagonist.

The beneficial effect of ACE inhibitor on pancreatic inflammation has some important clinical meaning. According to UKPDS results, ramipril showed some preventive effect for diabetes mellitus in high-risk patients group [Yusuf et al., 2000]. The mechanism of this preventive effect is not known, however, our findings of in vivo [Ko et al., 2004] and this in vitro studies could add on more evidence of the beneficial effect of the ACE inhibitor in the pancreatic inflammatory process.

In summary, our study shows that the exposure of PSCs to high glucose concentrations stimulated Ang II production, which was blocked by an ACE inhibitor or an AT-receptor antagonist, mediated by the AT1 receptor. These findings suggest that ACE inhibitors or AT-receptor antagonists might be useful agents for the prevention and/or attenuation of the islet fibrosis induced by sustained hyperglycemia. However, the clinical role of PSCs in type 2 diabetes mellitus should be confirmed in future studies. Because the pathogenetic mechanism of pancreatic fibrosis in chronic pancreatitis is known to be related to PSCs, normalization of blood glucose should be helpful in improving the patient's progress to pancreatic inflammation. Future studies of stellate cells should clarify other diseases related to tissue fibrosis, such as renal fibrosis, liver cirrhosis, and pulmonary fibrosis.

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REFERENCES

Apte MV, Haber PS, Applegate TL, Norton ID, McCaughan GW, Korsten MA, Pirola RC, Wilson JS. 1998. Periacinar stellate shaped cells in rat pancreas: Identification, isolation, and culture. Gut 43:128–133.

Apte MV, Haber PS, Darby SJ, Rodgers SC, McCaughan GW, Korsten MA, Pirola RC, Wilson JS. 1999. Pancreatic stellate cells are activated by proinflammatory cytokines: Implications for pancreatic fibrogenesis. Gut 44:534–541.

Bachem MG, Schneider E, Gross H, Weidenbach H, Schmid RM, Menke A, Siech M, Beger H, Grunert A, Adler G. 1998. Identification, culture, and characterization of pancreatic stellate cells in rats and humans. Gastroenterology 115:421–432.

Bataller R, Sancho-Bru P, Gines P, Lora JM, Al-Garawi A, Sole M, Colmenero J, Nicolas JM, Jimenez W, Weich N, Gutierrez-Ramos JC, Arroyo V, Rodes J. 2003. Activated human hepatic stellate cells express the renin-angiotensin system and synthesize angiotensin II. Gastroenterology 125:117–125.

Crandall DL, Herzlinger HE, Saunders BD, Armellino DC, Kral JG. 1994. Distribution of angiotensin II receptors in rat and human adipocytes. J Lipid Res 35:1378–1385.

Fliser D, Schaefer F, Schmid D, Veldhuis JD, Ritz E. 1997. Angiotensin II affects basal, pulsatile, and glucose-stimulated insulin secretion in humans. Hypertension 30:1156–1161.

Giacchetti G, Sechi LA, Rilli S, Carey RM. 2005. The renin-angiotensin-aldosterone system, glucose metabolism and diabetes. Trends Endocrinol Metab 16:120–126.

Haber PS, Keogh GW, Apte MV, Moran CS, Stewart NL, Crawford DH, Pirola RC, McCaughan GW, Ramm GA, Wilson JS. 1999. Activation of pancreatic stellate cells in human and experimental pancreatic fibrosis. Am J Pathol 155:1087–1095.

Hsieh TJ, Zhang SL, Filep JG, Tang SS, Ingelfinger JR, Chan JS. 2002. High glucose stimulates angiotensinogen gene expression via reactive oxygen species generation in rat kidney proximal tubular cells. Endocrinology 143: 2975–2985.

Iyer SN, Chappell MC, Brosnihan KB, Ferrario CM. 1998. Role of AT1 and AT2 receptors in the plasma clearance of angiotensin II. J Cardiovasc Pharmacol 31:464–469.

Jaster R. 2004. Molecular regulation of pancreatic stellate cell function. Mol Cancer 6:26.

Jaster R, Sparmann G, Emmrich J, Liebe S. 2002. Extracellular signal regulated kinases are key mediators of mitogenic signals in rat pancreatic stellate cells. Gut 51:579–584.

Kampf C, Lau T, Olsson R, Leung PS, Carlsson PO. 2005. Angiotensin II type 1 receptor inhibition markedly improves the blood perfusion, oxygen tension and first phase of glucose-stimulated insulin secretion in revascularised syngeneic mouse islet grafts. Diabetologia 48: 1159–1167.

Ko SH, Kwon HS, Kim SR, Moon SD, Ahn YB, Song KH, Son HS, Cha BY, Lee KW, Son HY, Kang SK, Park CG, Lee IK, Yoon KH. 2004. Ramipril treatment suppresses islet fibrosis in Otsuka Long-Evans Tokushima fatty rats. Biochem Biophys Res Commun 26:114–122.

Kruse ML, Hildebrand PB, Timke C, Folsch UR, Schmidt WE. 2000. TGF beta1 autocrine growth control in isolated pancreatic fibroblastoid cells/stellate cells in vitro. Regul Pept 90(1–3):47–52.

Kuno A, Yamada T, Masuda K, Ogawa K, Sogawa M, Nakamura S, Nakazawa T, Ohara H, Nomura T, Joh T, Shirai T, Itoh M. 2003. Angiotensin-converting enzyme inhibitor attenuates pancreas inflammation and fibrosis in male wistar Bonn/Kobori rats. Gastroenterology 124: 1010–1019.

Lau T, Carlsson PO, Leung PS. 2004. Evidence for a local angiotensin-generating system and dose-dependent inhibition of glucose-stimulated insulin release by angiotensin II in isolated pancreatic islets. Diabetologia 47:240–248.

Leehey DJ, Singh AK, Alavi N, Singh R. 2000. Role of angiotensin II in diabetic nephropathy. Kidney Int Suppl 77:S93–S98.

Leung PS, Carlsson PO. 2001. Tissue renin-angiotensin system: Its expression, localization, regulation and potential role in the pancreas. J Mol Endocrinol 26: 155–164.

Leung PS, Carlsson PO. 2005. Pancreatic islet renin angiotensin system: Its novel roles in islet function and in diabetes mellitus. Pancreas 30:293–298.

Leung PS, Chappell MC. 2003. A local pancreatic renin-angiotensin system: Endocrine and exocrine roles. Int J Biochem Cell Biol 35:838–846.

Leung PS, Chan WP, Wong TP, Sernia C. 1999. Expression and localization of the renin-angiotensin system in the rat pancreas. J Endocrinol 160:13–19.

McKinley MJ, Albiston AL, Allen AM, Mathai ML, May CN, McAllen RM, Oldfield BJ, Mendelsohn FA, Chai SYI. 2003. The brain renin-angiotensin system: Location and physiological roles. Int J Biochem Cell Biol 35:901–918.

Powell DW, Mifflin RC, Valentich JD, Crowe SE, Saada JI, West AB. 1999. Myofibroblasts. I. Paracrine cells important in health and disease. Am J Physiol 277(1 Pt 1):C1–C9.

Reinehr R, Zoller S, Klonowski-Stumpe H, Kordes C, Haussinger D. 2004. Effects of angiotensin II on rat pancreatic stellate cells. Pancreas 28:129–137.

Ruiz-Ortega M, Egido J. 1997. Angiotensin II modulates cell growth-related events and synthesis of matrix proteins in renal interstitial fibroblasts. Kidney Int 52:1497–1510.

Ruiz-Ortega M, Lorenzo O, Ruperez M, Egido J. 2000. ACE inhibitors and AT(1) receptor antagonists-beyond the haemodynamic effect. Nephrol Dial Transplant 15:561–565.

Suzuki Y, Ruiz-Ortega M, Lorenzo O, Ruperez M, Esteban V, Egido J. 2003. Inflammation and angiotensin II. Int J Biochem Cell Biol 35:881–900.

van Kats JP, de Lannoy LM, Jan Danser AH, van Meegen JR, Verdouw PD, Schalekamp MA. 1997. Angiotensin II type 1 (AT1) receptor-mediated accumulation of angiotensin II in tissues and its intracellular half-life in vivo. Hypertension 30(1 Pt 1):42–49.

Vidotti DB, Casarini DE, Cristovam PC, Leite CA, Schor N, Boim MA. 2004. High glucose concentration stimulates intracellular renin activity and angiotensin II generation in rat mesangial cells. Am J Physiol Renal Physiol 286:F1039–F1045.

Vinson GP, Teja R, Ho MM, Hinson JP, Puddefoot JR. 1998. The role of the tissue renin-angiotensin system in the response of the rat adrenal to exogenous angiotensin II. J Endocrinol 158:153–159.

Watari N, Hotta Y, Mabuchi Y. 1982. Morphological studies on a vitamin A-storing cell and its complex with macrophage observed in mouse pancreatic tissues following excess vitamin A administration. Okajimas Folia Anat Jpn 58:837–858.

Wolf G, Neilson EG. 1990. Angiotensin II induces cellular hypertrophy in cultured murine proximal tubular cells. Am J Physiol 259(5 Pt 2):F768–777.

Wolf G, Neilson EG. 1993. Angiotensin II as a renal growth factor. J Am Soc Nephrol 3:1531–1540.

Wolf G, Mueller E, Stahl RA, Ziyadeh FN. 1993. Angiotensin II-induced hypertrophy of cultured murine proximal tubular cells is mediated by endogenous transforming growth factor-beta. J Clin Invest 92: 1366–1372.

Yokota T, Denham W, Murayama K, Pelham C, Joehl R, Bell RH, Jr. 2002. Pancreatic stellate cell activation and MMP production in experimental pancreatic fibrosis. J Surg Res 104:106–111.

Yoshiji H, Kuriyama S, Yoshii J, Ikenaka Y, Noguchi R, Nakatani T, Tsujinoue H, Fukui H. 2001. Angiotensin-II type 1 receptor interaction is a major regulator for liver fibrosis development in rats. Hepatology 34(4 Pt 1):745–750.

Yoshikawa H, Kihara Y, Taguchi M, Yamaguchi T, Nakamura H, Otsuki M. 2002. Role of TGF-beta1 in the development of pancreatic fibrosis in Otsuka Long-Evans Tokushima Fatty rats. Am J Physiol Gastrointest Liver Physiol 282:G549–G558.

Yusuf S, Sleight P, Pogue J, Bosch J, Davies R, Dagenais G. 2000. Effects of an angiotensin-converting-enzyme inhibitor, ramipril, on cardiovascular events in high-risk patients. The Heart Outcomes Prevention Evaluation Study Investigators. N Eng J Med 34:145–153.

Zhang SL, Filep JG, Hohman TC, Tang SS, Ingelfinger JR, Chan JS. 1999. Molecular mechanisms of glucose action on angiotensinogen gene expression in rat proximal tubular cells. Kidney Int 55:454–464.