Testosterone reduces AGTR1 expression to prevent β -cell and islet apoptosis from glucotoxicity

Suwattanee Kooptiwut¹, Wanthanee Hanchang¹, Namoiy Semprasert¹,
Mutita Junking³, Thawornchai Limjindaporn² and Pa-thai Yenchitsomanus³

¹Department of Physiology, ²Department of Anatomy and ³Division of Molecular Medicine, Department of Research and Development, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand

Correspondence
should be addressed
to S Kooptiwut
Email
suwattanee.koo
@mahidol.ac.th

Abstract

Hypogonadism in men is associated with an increased incidence of type 2 diabetes. Supplementation with testosterone has been shown to protect pancreatic β -cell against apoptosis due to toxic substances including streptozotocin and high glucose. One of the pathological mechanisms of glucose-induced pancreatic β -cell apoptosis is the induction of the local rennin–angiotensin–aldosterone system (RAAS). The role of testosterone in regulation of the pancreatic RAAS is still unknown. This study aims to investigate the protective action of testosterone against glucotoxicity-induced pancreatic β -cell apoptosis via alteration of the pancreatic RAAS pathway. Rat insulinoma cell line (INS-1) cells or isolated male mouse islets were cultured in basal and high-glucose media in the presence or absence of testosterone, losartan, and angiotensin II (Ang II), then cell apoptosis, cleaved caspase 3 expression, oxidative stress, and expression of angiotensin II type 1 receptor (AGTR1) and p47^{phox} mRNA and protein were measured. Testosterone and losartan showed similar effects in reducing pancreatic β -cell apoptosis. Testosterone significantly reduced expression of AGTR1 protein in INS-1 cells cultured in high-glucose medium or high-glucose medium with Ang II. Testosterone decreased the expression of AGTR1 and p47^{phox} mRNA and protein in comparison with levels in cells cultured in high-glucose medium alone. Furthermore, testosterone attenuated superoxide production when co-cultured with high-glucose medium. In contrast, when cultured in basal glucose, supplementation of testosterone did not have any effect on cell apoptosis, oxidative stress, and expression of AGTR1 and p47^{phox}. In addition, high-glucose medium did not increase cleaved caspase 3 in AGTR1 knockdown experiments. Thus, our results indicated that testosterone prevents pancreatic β -cell apoptosis due to glucotoxicity through reduction of the expression of AGTR1 and its signaling pathway.

Key Words

- ▶ glucotoxicity
- ▶ testosterone
- ▶ pancreatic β -cell
- ▶ apoptosis
- ▶ angiotensin II type 1 receptor (AGTR1)
- ▶ signaling pathway

Journal of Endocrinology
(2015) 224, 215–224

Introduction

Diabetes mellitus is a chronic metabolic disease that affects the quality of life of millions of people worldwide. Type 2 diabetes (T2D) is characterized by increased levels of glucose due to impairment of insulin action and/or insulin secretion (Lin & Sun 2010). The progressive

failure of pancreatic β -cells to secrete adequate amounts of insulin to compensate for insulin resistance leads to hyperglycemia (Weir *et al.* 2001). Prolonged or chronic hyperglycemia contributes to detrimental effects in various tissues, such as kidneys, eyes, peripheral nerves,

and microvasculature, that lead to diabetic complications (Forbes & Cooper 2013).

Chronic hyperglycemia generates glucotoxicity which causes pancreatic β -cell dysfunction and death, leading to reductions in pancreatic β -cell mass (Federici *et al.* 2001). There are several pathways causing glucotoxicity of pancreatic β -cells, including increased production of reactive oxygen species (ROS; Evans *et al.* 2002), protein glycation (Tajiri *et al.* 1997), and glucose auto-oxidation (Wolff & Dean 1987). Although the accumulation of oxidative stress has been proposed to be a major mechanism of glucotoxicity, antioxidant substances have been shown to confer only partial protection against pancreatic β -cell death due to glucotoxicity (Lonn *et al.* 2005). Therefore, it has been proposed that there are still unidentified pathways involved in glucotoxicity. One of the novel pathological pathways of glucotoxicity is the local rennin–angiotensin–aldosterone system (RAAS). Local RAAS has been reported in several organs including liver and pancreas (Leung & Carlsson 2001). In the pancreas, the local RAAS is divided into the acinar RAAS and islet RAAS. The acinar RAAS regulates exocrine function and the islet RAAS has a role in glucose homeostasis by regulating glucose-induced insulin secretion (Leung 2007). The expression of RAAS components, such as angiotensinogen (AGT), angiotensin-converting enzyme, angiotensin II type 1 receptor (AGTR1) and AGTR2, in human and mouse islets has been demonstrated (Lam & Leung 2002, Lau *et al.* 2004). RAAS blockers decreased blood glucose levels in diabetes patients (Scheen 2004). Also, the expression of *Agtr1* mRNA is upregulated due to hyperglycemia in obesity-induced T2D mice. In these mice, local pancreas angiotensin II (Ang II) can inhibit insulin secretion by decreasing production of (pro)insulin via AGTR1 (Chu *et al.* 2006). In addition, AGTR1 is expressed in rat insulinoma cell line (INS-1) cells and Ang II decreased glucose-stimulated insulin secretion (Leung & Leung 2008). Also, knockout of *Nox2*, a NADPH oxidase components, in mice conferred a protective effect against streptozotocin (STZ)-induced diabetes (Xiang *et al.* 2010). These results indicate that pancreatic RAAS plays an important role in high-glucose-induced pancreatic β -cell death.

The incidence of T2D has been found to increase in males with hypogonadism (Rhoden *et al.* 2005, Grossmann *et al.* 2009). Results from previous studies have indicated that testosterone replacement therapy ameliorated insulin resistance and improved glycemic control in hypogonadal men with T2D (Kapoor *et al.* 2006). Also, testosterone treatment has been shown to improve pancreatic β -cell function by decreasing proinflammatory cytokines such as interleukin-6 (IL6), IL1 β , and tumor necrosis factor- α

in hypogonadal men with T2D (Kapoor *et al.* 2007). In addition, it has been shown that the insulin concentration, insulin receptor mRNA, and glucose oxidation were decreased in orchidectomized rats, and these conditions were reversed by testosterone replacement (Muthusamy *et al.* 2007). In an *in vitro* study, testosterone has been shown to induce uptake of calcium and to stimulate insulin secretion in isolated pancreatic islets (Grillo *et al.* 2005). Moreover, testosterone replacement conferred protection against pancreatic β -cell apoptosis in STZ-diabetic male rats (Palomar-Morales *et al.* 2010). However, it remains unclear whether or not testosterone protects against pancreatic β -cell death via an effect on local RAAS.

Materials and methods

Animals

This work was approved by the Siriraj Animal Care and Use Committee (SI-AUCC). Male imprinting control region (ICR) outbred 8–12-week-old mice were purchased from the National Laboratory Animal Center, Mahidol University, Bangkok, Thailand.

Mouse pancreatic islet isolation

Pancreatic islets were isolated by collagenase digestion according to a modification of the method of Lacy & Kostianovsky (1967) and Gotoh *et al.* (1985). Briefly, pancreases were infused with collagenase-P and digested at 37 °C. The islets were separated by using histopaque gradient and manually picked under a stereomicroscope.

Cell culture

Isolated islets and INS-1 cells were grown in RPMI-1640 supplemented with 10% heat-activated FCS, 100 U/ml penicillin, and 100 μ g/ml streptomycin at 37 °C in 5% CO₂. The culture medium was changed every 2 days. The cells were normally cultured in medium containing 11.1 mM glucose, which was used as the control condition, and they were cultured in approximately four times the concentration (40 mM) of glucose to create the condition of glucotoxicity.

Analysis of cell apoptosis using Annexin V-FITC/propidium iodide staining

INS-1 cells were cultured in 11.1 mM or 40 mM glucose in the presence or absence of 1 μ M Ang II, with or without

0.05 μ g/ml testosterone or 1 μ M losartan or a combination of both. After incubation for 72 h, cells were collected and stained with Annexin V-FITC and propidium iodide (PI) (Becton Dickinson, Franklin Lakes, NJ, USA). The apoptotic cells were then analyzed using a FACSort flow cytometer (Becton Dickinson).

Analysis of cell apoptosis by measurement of cleaved caspase 3 expression

INS-1 cells were cultured in 11.1 mM or 40 mM glucose in the presence or absence 0.05 μ g/ml testosterone or 1 μ M losartan. After incubation for 72 h, cells were lysed in radioimmuno-precipitation assay (RIPA) buffer and western blotting analysis of cleaved caspase 3 expression was performed.

RNA isolation and real-time RT-PCR

Total RNA of isolated male mouse pancreatic islets and INS-1 cells were extracted using a High Pure RNA isolation kit (Roche Diagnostic Corporation) according to the manufacturer's instructions. The RNA concentration was measured using a Nano Drop-1000 spectrophotometer. RT was carried out on 1 μ g of RNA using the SuperScript III RT kit with random hexamer primer (Invitrogen), according to the manufacturer's instructions. Quantitative real-time PCR was then performed using SYBR Green reaction mix and an Mx3005P instrument (Stratagene Agilent, Cedar Creek, TX, USA). The specific primer sequences for rat AGTR1 were forward: 5'-CAGTGTGCGCGTTTCATTATG-3' and reverse: 5'-TGGTAAGGCC-CAGCCCTAT-3'; those for rat p47^{phox} were forward: 5'-GGAGCTTATGAATGACCTCGAT-3' and reverse: 5'-CTACGCAGGTGAACCGTATGTA-3'; those for mouse AGTR1 were forward: 5'-CCATTGTCCACCCGATGAAG-3' and reverse: 5'-TGCAGGTGACTTTGGCCAC-3'; those for mouse p47^{phox} were forward: 5'-AGCCTGAGACA-TACCTGGTG-3' and reverse: 5'-AGACTTCTGCAGATA-CATGG-3' (Leung & Leung 2008); those for mouse β -actin were forward: 5'-ATGAAGGTGACGTTGACATCGTC-3' and reverse: 5'-CCTAGAAGCATTTGCGGTGCACGATG-3'. The comparative threshold cycles (Ct) of tested mRNA and β -actin were measured and their difference (Δ Ct) was calculated. The relative expression was then calculated by $2^{-\Delta\Delta C_t}$ method, compared with the control sample.

Knock down Agtr1

Transfection of siRNA directed against *Agtr1* mRNA (Dharmacon, Lafayette, CO, USA) was performed using

Lipofectamine 2000 (Invitrogen) as detailed by the manufacturer. INS-1 cells were seeded into six-well plates for 24 h before transfection. The double-stranded siRNA were transfected. After 6 h, the medium was changed to a complete culture medium. As a control, cells were treated with siRNA-control (Dharmacon) under identical conditions. At 24 h after siRNA transfection, cells were treated with 11.1 mM or 40 mM glucose for 72 h. Then, the cells were harvested for further examination by western blotting analysis.

Western blotting analysis

The cells were lysed in a RIPA buffer (Pierce, Waltham, MA, USA) and total proteins were quantified using a micro bicinchoninic acid protein assay kit. Total proteins were separated by SDS-PAGE and transferred to polyvinylidene fluoride membrane (Bio-Rad). After that, the membrane was blocked with 5% skimmed milk. The membrane was incubated with one of the following primary antibodies: rabbit polyclonal anti-AGTR1 (Santa Cruz Biotechnology), goat polyclonal anti-p47^{phox} (Santa Cruz Biotechnology), rabbit monoclonal anti-cleaved caspase 3 (Cell Signaling, Boston, MA, USA), or mouse monoclonal anti- β -actin (Santa Cruz Biotechnology), overnight at 4 °C. Then, the membrane was washed and incubated with HRP-conjugated secondary antibody (Santa Cruz Biotechnology). The protein bands were detected by ECL (Pierce). The band intensities of proteins were analyzed using Image J densitometry software version 1.43.

Measurement of intracellular superoxide generation

Superoxide production was detected using the nitroblue tetrazolium (NBT) assay. Briefly, INS-1 cells were cultured in 11.1 mM or 40 mM glucose with or without 0.05 μ g/ml testosterone for 72 h. Next, cells were incubated with NBT for 90 min. The cells were then lysed in potassium hydroxide and were dissolved in DMSO. The amount of superoxide production was determined from optical density at a wavelength of 630 nm measured using a PowerWave microplate scanning spectrophotometer (BIO-TEK, Winooski, VT, USA).

Statistical analyses

All data were generated from triplicated experiments and expressed as mean $\pm$ S.E.M. The differences between the groups of results were determined by one-way ANOVA, followed by Tukey's *post hoc* test. A *P* value of less than 0.05 was considered to be statistically significant.

Results

Testosterone decreased apoptosis of pancreatic β -cells cultured in high-glucose (40 mM) medium

To examine whether testosterone protected against high-glucose-induced pancreatic β -cell apoptosis, apoptotic cells were measured by Annexin V-FITC/PI staining. As shown in Fig. 1A, high-glucose (40 mM) medium significantly increased the percentage of apoptosis in INS-1 cells compared with those cultured in basal-glucose (11.1 mM) medium. The presence of testosterone in INS-1 cells cultured in high-glucose medium significantly reduced the percentage of cell apoptosis. However, testosterone had no effect on the percentage of apoptosis in INS-1 cells cultured in basal-glucose medium.

Testosterone and angiotensin II receptor blocker decreased apoptosis of pancreatic β -cells induced by high-glucose medium and Ang II

Previous studies have reported that Ang II production was increased in mice with T2D, which might be produced by

pancreatic islet RAAS (Lau *et al.* 2004, Leung 2007). To examine whether Ang II or AGTR1 is important for pancreatic β -cell apoptosis, INS-1 were cultured in basal-glucose or high-glucose medium with or without Ang II and in the presence or absence of testosterone, losartan, or both. As shown in Fig. 1B, the percentage of apoptotic cells was significantly increased in INS-1 cells cultured in high-glucose medium or high-glucose medium with Ang II, when compared with those cultured in basal glucose medium. Importantly, testosterone or losartan in INS-1 cells cultured in high-glucose medium with Ang II significantly reduced the percentage of apoptotic cells. However, testosterone and losartan did not have synergic effect on apoptosis of INS-1 cells cultured in high-glucose medium with Ang II. Surprisingly, Ang II did not trigger a significant increase in the percentage of cell apoptosis in basal-glucose medium.

We further confirmed our cell apoptosis results using the levels of cleaved caspase 3. INS-1 cells were cultured in basal- and high-glucose media with or without testosterone or in the presence or absence of losartan. Then, cleaved caspase 3 levels were determined by western blot analysis.

Figure 1

Effect of testosterone, losartan, and Ang II on apoptosis of pancreatic β -cells cultured in basal- and high-glucose media. INS-1 cells were cultured in basal-glucose (11.1 mM) or high-glucose (40 mM) medium in the presence or absence of testosterone (0.05 μ g/ml) for 72 h. Apoptotic INS-1 cells were detected using Annexin V-FITC/PI staining. The bar graph shows the average percentage undergoing early apoptosis (A). INS-1 cells were cultured in basal or high-glucose medium in the presence or absence of Ang II, with or without testosterone or losartan or a combination of both

for 72 h. This bar graph shows the percentage of early apoptosis (B). INS-1 cells were cultured in basal- or high-glucose media in the presence or absence of testosterone or losartan for 72 h. These bar graphs show caspase 3 activity (C and D). All data are expressed as mean $\pm$ s.e.m. of three to four independent experiments. $^{+++}P < 0.001$ compared with the basal glucose-treated group. $^{+++}P < 0.001$, $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the high-glucose-treated group. $^{***}P < 0.001$ compared with the high glucose- and Ang II-treated group.

Figure 2

Effect of basal- and high-glucose media, Ang II, and testosterone on expression of AGTR1 protein in INS-1 cells. INS-1 cells were cultured with basal-glucose medium with or without Ang II for 72 h. The image shows a representative western blot of AGTR1 and β -actin proteins. The bar graph below shows the fold changes in AGTR1 protein normalized to β -actin protein (A). INS-1 cells were cultured in high-glucose medium in the presence or absence of Ang II, with or without testosterone for 72 h. The image shows a western blot of AGTR1 and β -actin proteins. The bar graph below shows fold changes in AGTR1 protein relative to the control group, and normalized to β -actin protein (B). All data are expressed as mean $\pm$ S.E.M. of three independent experiments. $^+P < 0.05$, $^{++}P < 0.01$ compared with the high-glucose-treated group. $^{***}P < 0.001$ compared with the high-glucose- and Ang II-treated group. NS, not significant.

Cleaved caspase 3 was significantly increased in INS-1 cells cultured in high-glucose medium, while treatment with testosterone or losartan of INS-1 cells cultured in high-glucose medium significantly reduced the levels of cleaved caspase 3 compared with those of cells cultured with basal-glucose with or without testosterone or losartan.

Testosterone decreased AGTR1 expression in pancreatic β -cells activated by high-glucose medium and Ang II

To further examine effect of Ang II on expression of AGTR1 protein in basal-glucose medium, INS-1 cells were cultured in basal-glucose medium with or without Ang II and expression of AGTR1 protein was determined by western blotting analysis. Ang II did not affect the expression of AGTR1 protein in INS-1 cells cultured in basal-glucose medium (Fig. 2A). In contrast, the presence of Ang II in INS-1 cells cultured with high-glucose medium significantly increased the expression of AGTR1 compared with that in cells cultured in high-glucose medium alone (Fig. 2B). To verify whether testosterone decreased the expression of AGTR1 in INS-1 cells induced by high-glucose medium or high-glucose medium with Ang II, AGTR1 protein was assessed by western blotting analysis. Testosterone markedly reduced the expression of AGTR1 protein in INS-1 cells co-cultured in high-glucose medium or high-glucose medium with Ang II.

Testosterone attenuated the expression of AGTR1 mRNA and protein in pancreatic β -cells cultured in high-glucose medium

To investigate whether high-glucose-induced pancreatic β -cells apoptosis was associated with upregulation of the AGTR1 pathway and whether testosterone could reduce AGTR1 expression, we assessed the expression of AGTR1 mRNA and protein by real-time RT-PCR and western blotting analysis respectively. As shown in Fig. 3A and B, AGTR1 mRNA and protein expression were markedly increased in INS-1 cells cultured in high-glucose medium compared with those cultured in basal-glucose medium. Testosterone significantly reduced the expression of AGTR1 mRNA and protein in INS-1 cells cultured in high-glucose medium. In contrast, testosterone did not show any effect on expression of AGTR1 mRNA and protein in INS-1 cells cultured with basal-glucose medium. Similarly, expression of AGTR1 mRNA and protein was upregulated in isolated male mouse pancreatic islets cultured in high-glucose medium, whereas addition of testosterone to high-glucose medium downregulated AGTR1 mRNA and protein expression (Fig. 3C and D).

Testosterone decreased p47^{phox} mRNA and protein expression in pancreatic β -cells cultured in high-glucose medium

AGTR1 activation was shown to induce oxidative stress via activation of NADPH oxidase. P47^{phox} is a subunit of

Figure 3

Effect of testosterone on the expression of *Agtr1* mRNA and protein in INS-1 cells (A and B) and isolated male mouse pancreatic islets (C and D) cultured in high-glucose medium. Fold changes in *Agtr1* mRNA normalized to β -actin mRNA at 48 h (A and C). The images show representative western blots of AGTR1 and β -actin from INS-1 cells. The bar graphs below show

AGTR1 protein levels normalized to β -actin protein (B and D). The data are presented as mean $\pm$ S.E.M. of four independent experiments.

+ P < 0.05, ++ P < 0.01 compared with the basal-glucose-treated group.

* P < 0.05, ** P < 0.01 compared with the high-glucose-treated group.

activated NADPH oxidase. To examine whether testosterone decreased NADPH oxidase activation, $p47^{\text{phox}}$ mRNA and protein were measured by real-time RT-PCR and western blot analysis respectively. As shown in Fig. 4A and B, expression of $p47^{\text{phox}}$ mRNA and protein was markedly increased in INS-1 cells cultured in high-glucose medium compared with those cultured in basal-glucose medium. However, testosterone reduced the expression of $p47^{\text{phox}}$ mRNA and protein in INS-1 cells cultured in high-glucose medium but did not affect the expressions of $p47^{\text{phox}}$ mRNA and protein in basal-glucose medium. Moreover, the expression of $p47^{\text{phox}}$ mRNA and protein was significantly increased in isolated male mouse pancreatic islets cultured in high-glucose medium when compared with those cultured in

basal-glucose medium. As expected, testosterone in high-glucose medium significantly decreased the expression of $p47^{\text{phox}}$ mRNA and protein (Fig. 4C and D).

Testosterone reduced superoxide production in pancreatic β -cells cultured in high-glucose medium

To further examine whether the reduction in high-glucose-induced pancreatic β -cell apoptosis due to testosterone was mediated by decreased NADPH oxidase activity that reduced production of ROS, superoxide anion in INS-1 cells was measured by NBT assay. The superoxide anion production in INS-1 cells cultured with high-glucose medium was significantly increased compared with those

Figure 4

Effect of testosterone on the expression of $p47^{\text{phox}}$ mRNA and protein in INS-1 cells (A and B) and isolated male mouse pancreatic islet cells (C and D) cultured in high-glucose medium. Fold change in $p47^{\text{phox}}$ mRNA normalized to β -actin mRNA at 48 h (A and C). The images show representative western blots of AGTR1 and β -actin from INS-1 cells. The bar

graphs below show $p47^{\text{phox}}$ protein levels normalized to β -actin protein (B and D). The data is presented as mean $\pm$ S.E.M. of four independent experiments. ++ P < 0.01, +++ P < 0.001 compared with the basal-glucose-treated group. * P < 0.05, ** P < 0.01 compared with the high-glucose-treated group.

Figure 5

Superoxide production in INS-1 cells cultured in high-glucose medium. Superoxide production was measured using an NBT assay. The amount of superoxide was determined from the optical density (OD). The bar graph shows production of superoxide in INS-1 cells. The data are presented as mean $\pm$ S.E.M. of three independent experiments. $^{++}P < 0.01$ compared with the basal-glucose-treated group (11.1 mM); $^{*}P < 0.05$ compared with the high-glucose-treated group (40 mM).

cultured with basal-glucose medium, whereas testosterone decreased superoxide production in INS-1 cells cultured in high-glucose medium. No significant changes were found in INS-1 cells cultured in basal-glucose medium in the presence or absence of testosterone (Fig. 5).

AGTR1 knockdown rescued pancreatic β -cells apoptosis from high-glucose medium

To confirm the role of testosterone in reducing pancreatic β -cells apoptosis and AGTR1 expression, AGTR1 silencing were performed in INS-1 cells cultured with basal- or high-glucose media (Fig. 6). After AGTR1 knockdown, cellular apoptosis was determined by detection of cleaved caspase 3 using western blotting analysis. Expression of AGTR1 protein was not observed in INS-1 cells cultured in basal- and high-glucose media with si-AGTR1. Cleaved caspase 3 was not detected in INS-1 cells cultured in high-glucose medium with AGTR1 knockdown, which was similar to the results for cells cultured in basal-glucose medium with mock treatment, si-control, and si-AGTR1. Whereas INS-1 cells cultured in high-glucose medium with mock treatment and si-control showed markedly increased cleaved caspase 3 and AGTR1 protein levels when compared with those cultured in basal-glucose medium. These results indicated that AGTR1 silencing protected pancreatic β -cells against glucotoxicity.

Discussion

There have been increasing evidence indicative of a role for the pancreatic RAAS in the pathophysiology of T2D (van der Zijl *et al.* 2012). It has been shown in previous studies that Ang II production was increased in a rodent model of T2D, and that Ang II modulated AGTR1 expression in pancreatic β -cells (Singh *et al.* 2008, Chu & Leung 2009, Hirata *et al.* 2009). Addition of Ang II to β -cells cultured in basal-glucose medium has been shown to reduce glucose-stimulated insulin secretion (Chu *et al.* 2006). Ang II binds to AGTR1, which is a major Ang II receptor in pancreatic β -cells. AGTR1 was also upregulated in animal models of T2D (Nakayama *et al.* 2005, Chu *et al.* 2006) and AGTR1 blocker improved β -cell mass and function in T2D (Chu *et al.* 2006, Hasegawa *et al.* 2009). Therefore, pancreatic RAAS may be another

Figure 6

Effect of AGTR1 knockdown on levels of cleaved caspase 3. INS-1 cells cultured in basal- and high-glucose media with mock treatment, si-control, and si-AGTR1. Cleaved caspase 3 and AGTR1 were detected by western blot analysis. The image shows a representative western blot of cleaved caspase 3, AGTR1, and β -actin from INS-1 cells. The bar graph below shows cleaved caspase 3 protein levels normalized to β -actin protein. The results are presented as mean $\pm$ S.E.M. of four independent experiments. $^{***}P < 0.001$ compared with the high-glucose-treated group.

pathological mechanism of glucotoxicity-induced pancreatic β -cell apoptosis.

In this study, we have shown that testosterone protected pancreatic β -cells against apoptosis when these cells were cultured in high-glucose medium. Also, it has been revealed in this study that the level of pancreatic β -cell apoptosis was approximately the same for INS-1 cells cultured either in high-glucose medium or in high-glucose medium with addition of Ang II, which indicates that a similar pathway might be exploited for either condition. Either testosterone or losartan decreased pancreatic β -cell apoptosis, which was induced by high-glucose medium alone or high-glucose medium with Ang II to approximately equal levels. Interestingly, treatment with both testosterone and losartan reduced pancreatic β -cell apoptosis similarly to testosterone or losartan alone, indicating that testosterone and losartan had no synergistic action in reducing β -cell apoptosis. This result was confirmed by detection of cleaved caspase 3 by western blot analysis. In correspondence with the previous result, INS-1 cells cultured in high-glucose media with testosterone or losartan displayed significantly decreased levels of the active form of caspase 3 when compared with those cultured in basal-glucose media. The results of Annexin V/PI staining also indicated that there was no difference in the level of pancreatic β -cell apoptosis for the INS-1 cells cultured in basal-glucose medium with or without testosterone, losartan, or both. Hence, it is possible that testosterone and losartan may exploit the same pathway to protect against glucotoxicity. Another interesting finding was that Ang II did not induce β -cell apoptosis in cells cultured in basal-glucose medium. This result indicates that expression of AGTR1 may be an important factor in Ang II-increased β -cell apoptosis. To verify this observation, expression of AGTR1 in the INS-1 cells cultured in basal-glucose medium with or without Ang II was determined. AGTR1 was found to be expressed at similar levels in the INS-1 cells cultured in basal-glucose medium or in basal-glucose medium with Ang II. This result indicates that addition of Ang II to basal-glucose medium does not increase pancreatic β -cell apoptosis as it does not influence the AGTR1 signaling pathway. While the addition of Ang II to high-glucose medium increased AGTR1 expression and testosterone decreased AGTR1 expression under the same extent in both conditions that was induced by high-glucose medium and high-glucose medium with the addition of Ang II. Again, expression of AGTR1 correlated with β -cell apoptosis, which supports the hypothesis that both testosterone and losartan attenuate the AGTR1 signaling pathway. Altogether, our results indicate that expression of AGTR correlates with pancreatic β -cell apoptosis while Ang II has an effect on

pancreatic β -cell apoptosis when AGTR1 is upregulated. Similarly, in separate experiments, expression of AGTR1 mRNA and protein were increased in both INS-1 cells and isolated male mouse islets when they were cultured under high-glucose conditions. Interestingly, testosterone ameliorated the effect of high-glucose-medium-induced AGTR1 expression in pancreatic β -cells but had no effect on AGTR1 expression in cells-cultured in basal-glucose medium. These results indicate that testosterone reduces both AGTR1 mRNA and protein levels in high-glucose medium.

The mechanistic effect of AGTR1 activation on pancreatic β -cell death is the induction of NADPH oxidase and ROS generation (Shao *et al.* 2006, Leung 2010). NADPH oxidase subunits in pancreatic β -cells are composed of p22^{phox}, gp91^{phox}, and p47^{phox} (Oliveira *et al.* 2003). The results of this study also indicated that high-glucose medium increased expression of p47^{phox} mRNA and its protein in both a pancreatic β -cell line and isolated male mouse islets and also increased ROS production in the β -cell line. Supplementation of the high-glucose medium with testosterone reduced the expression of p47^{phox} mRNA and protein and production of ROS. These results are consistent with the previous finding that for human pancreatic islets prolonged culture in high-glucose increased expression of p47^{phox} and p22^{phox} (Lastra & Manrique 2007). Also, culturing INS1-E cells in high-glucose medium has been shown to induce expression of p47^{phox} mRNA (Leung & Leung 2008). Furthermore, inhibition of AGTR1 decreased Ang II-induced ROS production in pancreatic β -cells (Hirata *et al.* 2009). Taken together, the results from this study indicate that chronic high levels of glucose induce AGTR1 and NADPH oxidase activation, which produces ROS in pancreatic β -cells, and that testosterone protects against high-glucose-induced β -cell apoptosis through the inhibition of AGTR1-mediated NADPH-oxidase-induced ROS production both *in vitro* and *ex vivo*.

The results from our previous study indicated that testosterone conferred protection against high-glucose-induced pancreatic β -cell apoptosis via decreased endoplasmic reticulum (ER) stress (Hanchang *et al.* 2013). The results of this study indicated that testosterone protected against high-glucose-induced pancreatic β -cell apoptosis via suppression of AGTR1 and its signaling pathway. Thus, there may be a connection between AGTR1 activation and ER stress. This notion was supported by results from a recent study which indicated that high levels of glucose up-regulated the expression of AGTR1 and AGT mRNA in human islets, leading to the activation of islet RAAS (Madec *et al.* 2013). AGTR1 was activated through phospholipase C and caused the depletion of ER calcium. The reduction in ER calcium

levels induced ER stress, which then induced β -cell dysfunction and apoptosis. Losartan showed a protective effect against glucotoxicity via inhibition of expression of AGTR1 and reduction of ER stress (Madec *et al.* 2013). All of these lines of evidence indicated that testosterone and losartan inhibit similar aspects of high-glucose activated AGTR1. To verify this notion, induction of AGTR1 silencing was performed. High-glucose medium induced cleaved caspase 3. In contrast, cleaved caspase 3 was diminished by siRNA against AGTR1 but not si-control in INS-1 cultured in high-glucose medium. Thus, testosterone and losartan protect pancreatic β -cells against apoptosis due to glucotoxicity through reduction of expression of AGTR1 and ER stress.

Conclusion

Our results indicated that testosterone protects against glucotoxicity-induced β -cell apoptosis by means of a decrease in the action of the AGTR1 signaling pathway both *in vitro* and *ex vivo*. The molecular mechanism by which testosterone inhibits the activation of AGTR1 is still unknown. Identification of the molecular mechanism by which testosterone reduces AGTR1 signaling merits further investigation.

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.

Funding

This work was supported by Siriraj Research Development Grant (to S K) and Siriraj Chalearmprakiat Fund (to S K, T L, and P Y), Mahidol University Grant (to S K), Siriraj Graduate Thesis Scholarship (to W H), TRF Mid-Career Research Grant (to T L), and TRF Senior Research Scholar Grant (to P Y).

Acknowledgements

The authors thank to Malika Churintaraphan and Samarn Onreabroi for their technical assistances.

References

- Chu KY & Leung PS 2009 Angiotensin II in type 2 diabetes mellitus. *Current Protein & Peptide Science* **10** 75–84. (doi:10.2174/138920309787315176)
- Chu KY, Lau T, Carlsson PO & Leung PS 2006 Angiotensin II type 1 receptor blockade improves β -cell function and glucose tolerance in a mouse model of type 2 diabetes. *Diabetes* **55** 367–374. (doi:10.2337/diabetes.55.02.06.db05-1022)
- Evans J, Goldfine I, Maddux B & Grodsky G 2002 Oxidative stress and stress-activated signaling pathways: a unifying hypothesis of type 2 diabetes. *Endocrine Reviews* **23** 599–622. (doi:10.1210/er.2001-0039)
- Federici M, Hribal M, Perego L, Ranalli M, Caradonna Z, Perego C, Usellini L, Nano R, Bonini P, Bertuzzi F *et al.* 2001 High glucose causes apoptosis in cultured human pancreatic islets of Langerhans: a potential role for regulation of specific Bcl family genes toward an apoptotic cell death program. *Diabetes* **50** 1290–1301. (doi:10.2337/diabetes.50.6.1290)
- Forbes JM & Cooper ME 2013 Mechanisms of diabetic complications. *Physiological Reviews* **93** 137–188. (doi:10.1152/physrev.00045.2011)
- Gotoh M, Maki T, Kiyozumi T, Satomi S & Monaco A 1985 An improved method for isolation of mouse pancreatic islets. *Transplantation* **40** 437–438. (doi:10.1097/00007890-198510000-00018)
- Grillo ML, Jacobus AP, Scalco R, Amaral F, Rodrigues DO, Loss ES & Wassermann GF 2005 Testosterone rapidly stimulates insulin release from isolated pancreatic islets through a non-genomic dependent mechanism. *Hormone and Metabolic Research* **37** 662–665. (doi:10.1055/s-2005-870575)
- Grossmann M, Panagiotopolous S, Sharpe K, MacIsaac RJ, Clarke S, Zajac JD, Jerums G & Thomas MC 2009 Low testosterone and anaemia in men with type 2 diabetes. *Clinical Endocrinology* **70** 547–553. (doi:10.1111/j.1365-2265.2008.03357.x)
- Hanchang W, Semprasert N, Limjindaporn T, Yenchitsomanus P & Kooptiwut S 2013 Testosterone protects against glucotoxicity-induced apoptosis of pancreatic β -cells (INS-1) and male mouse pancreatic islets. *Endocrinology* **154** 4058–4067. (doi:10.1210/en.2013-1351)
- Hasegawa G, Fukui M, Hosoda H, Asano M, Harusato I, Tanaka M, Shiraishi E, Senmaru T, Sakabe K, Yamasaki M *et al.* 2009 Telmisartan, an angiotensin II type 1 receptor blocker, prevents the development of diabetes in male spontaneously diabetic Torii rats. *European Journal of Pharmacology* **605** 164–169. (doi:10.1016/j.ejphar.2009.01.001)
- Hirata AE, Morgan D, Oliveira-Emilio HR, Rocha MS, Carvalho CR, Curi R & Carpinelli AR 2009 Angiotensin II induces superoxide generation via NAD(P)H oxidase activation in isolated rat pancreatic islets. *Regulatory Peptides* **153** 1–6. (doi:10.1016/j.regpep.2008.11.004)
- Kapoor D, Goodwin E, Channer KS & Jones TH 2006 Testosterone replacement therapy improves insulin resistance, glycaemic control, visceral adiposity and hypercholesterolaemia in hypogonadal men with type 2 diabetes. *European Journal of Endocrinology* **154** 899–906. (doi:10.1530/eje.1.02166)
- Kapoor D, Clarke S, Stanworth R, Channer KS & Jones TH 2007 The effect of testosterone replacement therapy on adipocytokines and C-reactive protein in hypogonadal men with type 2 diabetes. *European Journal of Endocrinology* **156** 595–602. (doi:10.1530/EJE-06-0737)
- Lacy PE & Kostianovsky M 1967 Method for the isolation of intact islets of Langerhans from the rat pancreas. *Diabetes* **16** 35–39. (doi:10.2337/diab.16.1.35)
- Lam KY & Leung PS 2002 Regulation and expression of a renin–angiotensin system in human pancreas and pancreatic endocrine tumours. *European Journal of Endocrinology* **146** 567–572. (doi:10.1530/eje.0.1460567)
- Lastra G & Manrique C 2017 The expanding role of oxidative stress, renin angiotensin system, and β -cell dysfunction in the cardiometabolic syndrome and type 2 diabetes mellitus. *Antioxidants & Redox Signaling* **9** 943–954. (doi:10.1089/ars.2007.1615)
- 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. (doi:10.1007/s00125-003-1295-1)
- Leung PS 2007 The physiology of a local renin–angiotensin system in the pancreas. *Journal of Physiology* **580** 31–37. (doi:10.1113/jphysiol.2006.126193)
- Leung PS 2010 Current research of the RAS in pancreatitis and pancreatic cancer. *Advances in Experimental Medicine and Biology* **690** 179–199. (doi:10.1007/978-90-481-9060-7_10)
- Leung PS & Carlsson PO 2001 Tissue renin–angiotensin system: its expression, localization, regulation and potential role in the pancreas. *Journal of Molecular Endocrinology* **26** 155–164. (doi:10.1677/jme.0.0260155)

- Leung KK & Leung PS 2008 Effects of hyperglycemia on angiotensin II receptor type 1 expression and insulin secretion in an INS-1E pancreatic β -cell line. *Journal of the Pancreas* **9** 290–299.
- Lin Y & Sun Z 2010 Current views on type 2 diabetes. *Journal of Endocrinology* **204** 1–11. (doi:10.1677/JOE-09-0260)
- Lonn E, Bosch J, Yusuf S, Sheridan P, Pogue J, Arnold J, Ross C, Arnold A, Sleight P, Probstfield J *et al.* 2005 Effects of long-term vitamin E supplementation on cardiovascular events and cancer: a randomized controlled trial. *Journal of the American Medical Association* **293** 1338–1347. (doi:10.1001/jama.293.11.1338)
- Madec AM, Cassel R, Dubois S, Ducreux S, Vial G, Chauvin MA, Mesnier A, Chikh K, Bosco D, Rieusset J *et al.* 2013 Losartan, an angiotensin II type 1 receptor blocker, protects human islets from glucotoxicity through the phospholipase C pathway. *FASEB Journal* **27** 5122–5130. (doi:10.1096/fj.13-234104)
- Muthusamy T, Dhevika S, Murugesan P & Balasubramanian K 2007 Testosterone deficiency impairs glucose oxidation through defective insulin and its receptor gene expression in target tissues of adult male rats. *Life Sciences* **81** 534–542. (doi:10.1016/j.lfs.2007.06.009)
- Nakayama M, Inoguchi T, Sonta T, Maeda Y, Sasaki S, Sawada F, Tsubouchi H, Sonoda N, Kobayashi K, Sumimoto H *et al.* 2005 Increased expression of NAD(P)H oxidase in islets of animal models of type 2 diabetes and its improvement by an AT1 receptor antagonist. *Biochemical and Biophysical Research Communications* **332** 927–933. (doi:10.1016/j.bbrc.2005.05.065)
- Oliveira HR, Verlengia R, Carvalho CR, Britto LR, Curi R & Carpinelli AR 2003 Pancreatic β -cells express phagocyte-like NAD(P)H oxidase. *Diabetes* **52** 1457–1463. (doi:10.2337/diabetes.52.6.1457)
- Palomar-Morales M, Morimoto S, Mendoza-Rodriguez C & Cerbon M 2010 The protective effect of testosterone on streptozotocin-induced apoptosis in β cells is sex specific. *Pancreas* **39** 193–200. (doi:10.1097/MPA.0b013e3181c156d9)
- Rhoden EL, Ribeiro EP, Teloken C & Souto CA 2005 Diabetes mellitus is associated with subnormal serum levels of free testosterone in men. *BJU International* **96** 867–870. (doi:10.1111/j.1464-410X.2005.05728.x)
- Scheen AJ 2004 Renin–angiotensin system inhibition prevents type 2 diabetes mellitus. Part 1. A meta-analysis of randomised clinical trials. *Diabetes & Metabolism* **30** 487–496. (doi:10.1016/S1262-3636(07)70146-5)
- Shao J, Iwashita N, Ikeda F, Ogihara T, Uchida T, Shimizu T, Uchino H, Hirose T, Kawamori R & Watada H 2006 Beneficial effects of candesartan, an angiotensin II type 1 receptor blocker, on β -cell function and morphology in db/db mice. *Biochemical and Biophysical Research Communications* **344** 1224–1233. (doi:10.1016/j.bbrc.2006.04.011)
- Singh VP, Le B, Khode R, Baker KM & Kumar R 2008 Intracellular angiotensin II production in diabetic rats is correlated with cardiomyocyte apoptosis, oxidative stress, and cardiac fibrosis. *Diabetes* **57** 3297–3306. (doi:10.2337/db08-0805)
- Tajiri Y, Moller C & Grill V 1997 Long-term effects of aminoguanidine on insulin release and biosynthesis: evidence that the formation of advanced glycosylation end products inhibits B cell function. *Endocrinology* **138** 273–280. (doi:10.1210/endo.138.1.4851)
- Weir GC, Laybutt DR, Kaneto H, Bonner-Weir S & Sharma A 2001 β -cell adaptation and decompensation during the progression of diabetes. *Diabetes* **50**(Suppl 1) S154–S159. (doi:10.2337/diabetes.50.2007.S154)
- Wolff S & Dean R 1987 Glucose autooxidation and protein modification. The potential role of 'autooxidative glycosylation' in diabetes. *Biochemical Journal* **245** 243–250.
- Xiang F, Lu X, Strutt B, Hill D & Feng Q 2010 NOX2 deficiency protects against streptozotocin-induced β -cell destruction and development of diabetes in mice. *Diabetes* **59** 2603–2611. (doi:10.2337/db09-1562)
- van der Zijl NJ, Moors CC, Goossens GH, Blaak EE & Diamant M 2012 Does interference with the renin–angiotensin system protect against diabetes? Evidence and mechanisms *Diabetes, Obesity & Metabolism* **14** 586–595. (doi:10.1111/j.1463-1326.2012.01559.x)

Received in final form 1 December 2014

Accepted 15 December 2014

Accepted Preprint published online 15 December 2014