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ARTICLE

Prostaglandin E_2 regulates Foxo activity via the Akt pathway: implications for pancreatic islet beta cell dysfunction

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Abstract
Aims/hypothesis Prostaglandin E_2 (PGE_2) is a well-recognised inhibitor of glucose-stimulated insulin secretion (GSIS). The aim of this study was to investigate the signalling pathway of PGE_2 in beta cell function regulation in HIT-T15 cells and isolated rat islets.
Materials and methods mRNA levels of the prostaglandin E receptor 3 (*Ptger3*) were measured by real-time PCR. Western blot analysis was used to detect changes in the levels of PTGER3, phosphorylated and total Akt, phosphorylated and total forkhead box 'Other' (Foxo). Transient transfection and reporter assays were used to measure Foxo transcriptional activity. The biological significance of PGE_2 in beta cell function was analysed using MTT, flow cytometry and GSIS assays.
Results We found that treating HIT-T15 cells with exogenous PGE_2 stimulated *Ptger3* gene expression specifically, and diminished cAMP generation. These were accompanied by the downregulation of Akt and Foxo phosphorylation in HIT-T15 cells and isolated rat islets. Moreover, PGE_2 upregulated basal and partially reversed constitutively active Akt-inactivated Foxo transcriptional activity. Furthermore, GSIS was impaired in PGE_2-treated HIT-T15 cells and isolated islets. However, the dosage used in the above experiments did not affect beta cell viability and apoptosis. In addition, insulin-like growth factor 1 (IGF-1) pretreatment reversed the effects of PGE_2, and wortmannin treatment abolished the preventive effects of IGF-1.
Conclusions/interpretation Our observations strongly suggest that PGE_2 can induce pancreatic beta cell dysfunction through the induction of *Ptger3* gene expression and inhibition of Akt/Foxo phosphorylation without impacting beta cell viability. These results shed light on the mechanisms of PGE_2 actions in pancreatic beta cell dysfunction.

Keywords Akt · Foxo · GSIS · Islets · Pancreatic beta cell · PGE_2

Abbreviations

CA-Akt	constitutively active Akt
COX	cyclooxygenase
FBS	fetal bovine serum
FKHR	forkhead transcription factor Foxo1
FKHRL1	forkhead transcription factor Foxo3a
Foxo	forkhead box 'Other'
GSIS	glucose-stimulated insulin secretion
IGF-1	insulin-like growth factor-1
PGE_2	prostaglandin E_2
PI3K	phosphatidylinositol 3-kinase
PTGER	prostaglandin E receptor
RPMI-1640	Roswell Park Memorial Institute-1640

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Introduction

Types 1 and 2 diabetes mellitus are characterised by autoimmune destruction and functional impairment of insulin-secreting beta cells in the pancreatic islets of Langerhans [1–3]. Although the initial events leading to the development of diabetes mellitus are not well characterised, proinflammatory prostaglandins, including prostaglandin E_2 (PGE_2) appear to play an important role. Three isoforms of cyclooxygenase (COX) have been characterised to date, including two constitutive subtypes, COX-1 and COX-3, and one inducible isoform, COX-2 [4]. In contrast to most mammalian cells, beta cells constitutively and dominantly produce the COX-2 isoform of PGE_2-generating enzymes rather than COX-1 [5]. Prior studies have demonstrated that PGE_2 inhibits glucose-stimulated insulin secretion (GSIS) in clonal beta cells and isolated islets [6, 7], and that selective inhibition of COX-2 attenuates the development of diabetes in the low-dose streptozotocin mouse model and protects rat islets from cytokine-induced inhibition of GSIS [8, 9]. Despite the established role of PGE_2 in pancreatic beta cells, the exact molecular mechanisms of PGE_2-mediated inhibition of insulin secretion remain poorly understood.

PGE_2 exerts its actions on cells by interacting with one or more of its four G protein-coupled prostaglandin E receptor (PTGER) subtypes named PTGER1, PTGER2, PTGER3 and PTGER4, all of which are coupled to signal transduction systems that involve phosphoinositide hydrolysis, calcium or adenylate cyclase activity. However, only PTGER3 has been shown to have post-receptor activities that result in a decrease in cAMP levels [10, 11].

Akt, also known as protein kinase B, is a serine–threonine kinase that is activated by phosphatidylinositol 3-kinase (PI3K) at Thr^{308} and/or Ser^{473}. Accumulating evidence indicates that upon activation in response to many different growth factors, hormones and external stresses, Akt serves as a pivotal regulator of glucose transport, glycolysis, protein production, lipogenesis, glycogen synthesis, suppression of gluconeogenesis, cell survival, determination of cell size and cell-cycle progression as well as insulin synthesis and secretion [12–15]. However, the precise role of Akt in the beta cell dysfunction associated with diabetes mellitus remains controversial [15, 16].

One downstream target of the PI3K/Akt pathway that could mediate the PGE_2 effects is the forkhead box 'Other' (Foxo) class of transcription factors, a subfamily of the large group of forkhead transcription factors. Mammalian cells contain three members of this family, Foxo1 (FKHR), Foxo3a (FKHRL1) and Foxo4, whose functions are blocked by Akt via the phosphorylation of three conserved residues, which leads to their sequestration in the cytoplasm away from target genes. However, dephosphorylation of Foxo transcription factors leads to nuclear entry and modulates their targeted gene expression [17, 18]. There is increasing evidence that Foxo transcription factors play an important role in mediating the effects of hormone and growth factors on diverse physiological functions, including cell proliferation, apoptosis, metabolism and insulin synthesis [14, 19]. Nevertheless, the exact role of Foxo in beta cell dysfunction is ambiguous.

In the present study, we chose to test the hypothesis that Akt and Foxo are involved in PGE_2-mediated pancreatic islet beta cell dysfunction. Using the glucose-responsive beta cell line HIT-T15 and isolated rat islets, we found that PGE_2 can induce pancreatic beta cell dysfunction through the induction of *Ptger3* gene expression and the upregulation of Foxo activity in a PI3K/Akt pathway-dependent manner without affecting beta cell viability.

Materials and methods

Reagents Roswell Park Memorial Institute-1640 (RPMI-1640) medium, glucose-free DMEM and the Lipofectamine Plus transfection kit were obtained from Invitrogen Life Technologies (Grand Island, NY, USA). FBS was purchased from Hyclone (Logan, UT, USA). PGE_2 and wortmannin were purchased from Sigma Aldrich (St Louis, MO, USA). Human recombinant insulin-like growth factor-1 (IGF-1) was manufactured by R&D Systems (Minneapolis, MN, USA). Rabbit polyclonal antibody against PTGER3 was purchased from Cayman Chemical (Ann Arbor, MI, USA). Rabbit polyclonal antibodies against Ser^{256}-phosphorylated FKHR, Thr^{24}-phosphorylated FKHRL1, Thr^{308}-phosphorylated Akt, Ser^{473}-phosphorylated Akt, total FKHR and total Akt were purchased from Cell Signaling Technology (New England Biolabs, Beverly, MA, USA). Horseradish peroxidase-conjugated anti-rabbit IgG was obtained from Amersham Pharmacia Biotech (Piscataway, NJ, USA). The Detergent Compatible (DC) Protein Assay kit was purchased from Bio-Rad Laboratories (Hercules, CA, USA). The RNeasy Mini Kit was from Qiagen (Hilden, Germany). The Luciferase Assay System was obtained from Promega (Madison, WI, USA). The TaqMan One-step PCR Master Mix Reagents kit and Assays-on-Demand gene expression products were purchased from ABI (Applied Biosystems, Foster City, CA, USA). The firefly luciferase reporter construct pGL3-FKHR (containing three FKHR-binding sites) was a kind gift from M. J. Anderson (La Jolla, CA, USA). The constitutively active Akt (CA-Akt) construct was kindly provided by J. Zieg (Boston, MA, USA).

Cell culture HIT-T15 cells were kindly provided by R. P. Robertson (Seattle, WA, USA). The cells were grown in a

humidified atmosphere containing 95% air and 5% CO_2, and maintained in RPMI-1640 medium (11.1 mmol/l of glucose) supplemented with 10% FBS, as described previously [20]. Before treatment, the cells were depleted in RPMI-1640 medium containing 0.5% BSA and 3 μg/ml indomethacin for 8 h. The cells were then washed in PBS, and the depletion medium was reintroduced. At that time, wortmannin and/or IGF-1 were added in certain experiments before the addition of PGE_2. For all compounds prepared in alcohol or DMSO, the final concentration of alcohol and DMSO in the culture medium was kept less than 0.1%. Vehicle controls were prepared for all treatments.

Islet isolation and culture All animal studies were performed according to guidelines established by the Research Animal Care Committee of Nanjing Medical University, China. Male Sprague–Dawley rats (250–300 g, purchased from Shanghai Laboratory Animal Centre, Chinese Academy of Sciences, Shanghai, China) were used. Islet isolation and culturing techniques have been described previously [21]. Freshly isolated islets were transferred to sterile six-well dishes and cultured in DMEM containing 11.1 mmol/l glucose supplemented with 10% FBS, 10 mmol/l HEPES, 100 U/ml penicillin and 100 μg/ml streptomycin. The islets were allowed to equilibrate for 3 h, at which point they were counted and re-picked into static incubation tubes (ten islets per tube) and cultured overnight at 37°C. The next morning, the islets were depleted in DMEM containing 0.5% BSA and 3 μg/ml indomethacin for 4 h. Then the islets were washed in PBS, and depletion medium with the corresponding combination of wortmannin (300 nmol/l), IGF-1 (100 ng/ml) or PGE_2 (1 μmol/l) was reintroduced. GSIS studies were performed 24 h later. For western blot analysis, aliquots of about 600 islets were transferred into six-well dishes and cultured overnight in DMEM as described above. The next morning, the islets were depleted in DMEM containing 0.5% BSA and 3 μg/ml indomethacin for 4 h. Then the islets were treated without (control) or with PGE_2 (1 μmol/l) in the depletion medium for 0.5, 1, 2, 4 and 8 h, after which they were collected and lysed.

Real-time PCR assay Cells were cultured and treated with PGE_2 as described above. The total RNA samples were extracted from HIT-T15 cells treated without (control) or with PGE_2 (1 μmol/l) for 0.5, 1, 2 and 4 h using Qiagen RNeasy Mini Kits. A TaqMan ABI Prism 7000 Sequence Detection System (Applied Biosystems) was used for the analysis. One-step real-time PCR was conducted with a TaqMan One-step PCR Master Mix Reagents kit. The TaqMan probes and primers were acquired by Assays-on-Demand (Applied Biosystems). The primer and probe sequences are given in Electronic supplementary material (ESM) Table 1. All data were analysed using the values of the β-actin gene levels as a reference.

Western blot analysis HIT-T15 cells and isolated rat islets were cultured and treated as described above, and lysed with ice-cold lysis buffer containing: 50 mmol/l Tris–HCl, pH 7.4; 1% NP-40; 150 mmol/l NaCl; 1 mmol/l EDTA; 1 mmol/l phenylmethylsulfonyl fluoride; and complete proteinase inhibitor mixture (one tablet per 10 ml; Roche Molecular Biochemicals, Indianapolis, IN, USA). After protein content determination using a DC Protein Assay kit, western blotting was performed as described [7].

cAMP determination assay Cells were harvested by centrifugation at 2,000 *g* for 10 min, and 0.1% trichloroacetic acid in 95% ethanol was added to the cell pellets. After 0.5 h extraction, the supernatants were recovered by centrifugation at 5,000 *g* for 10 min and dried. The cAMP levels were determined by RIA. Protein pellets were assayed using a DC Protein Assay kit to normalise the cAMP concentrations.

Transient transfection and luciferase reporter assay FKHR transcriptional activity was assessed in HIT-T15 cells using the FKHR luciferase reporter construct pGL3-FKHR. We used a plasmid containing the β-galactosidase gene driven by the cytomegalovirus promoter (Clontech Laboratories, Palo Alto, CA, USA) as an internal control. The HIT-T15 cells grown in 24-well dishes were cotransfected with two (pGL3-FKHR and β-galactosidase) or three plasmids (CA-Akt, pGL3-FKHR and β-galactosidase) using the Lipofectamine Plus transfection kit, according to the manufacturer's instructions. Twenty-four hours after transfection, the cells were cultured in serum-free RPMI-1640 medium containing 0.5% BSA for 4 h before treatment. The cells were then gently washed in PBS, and the depletion medium was reintroduced. At that time, wortmannin (300 nmol/l) was added to the medium in certain experiments 0.5 h before the addition of IGF-1 (100 ng/ml) and PGE_2 (1 μmol/l). Then cells were incubated for an additional 12 h, and harvested for luciferase reporter assays. Luciferase activity was measured with a luminometer (TD-20/20; Turner Designs, CA, USA) using the Luciferase Assay System. β-Galactosidase activity was detected to normalise any variations in the transfection efficiency.

MTT assay Cell viability was determined using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assays. Briefly, the cells were seeded in 96-well dishes at 1×10^4 to 2×10^4 cells per well, and treated without (control) or with different concentrations of PGE_2, as described above, for 24 h. Then each well was supplemented with 10 μl MTT (Sigma Aldrich) and incubated for

4 h at 37°C. The medium was then removed, and 150 µl DMSO (Sigma Aldrich) were added to solubilise the MTT formazan. The optical density was read at 570 nm.

Flow cytometry analysis HIT-T15 cells (1.5×10^6 cells per well) were cultured in six-well dishes and treated without (control) or with 1, 2, 5, 10 µmol/l PGE_2 for 24 h. The cells of each well were then harvested and fixed with 1 ml 75% ice-cold ethanol at −20°C overnight. After fixation, the cells were washed in PBS and stained with 500 µl propidium iodide solution (50 µg/ml in PBS) containing 25 µg/ml RNase. The cells were incubated at room temperature for 0.5 h in the dark, and analysed using a FACSCalibur flow cytometer and Cellquest Pro software (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) for data acquisition and analysis.

GSIS assay HIT-T15 cells (5×10^5 cells per well) were seeded into 1 ml RPMI-1640 medium with standard glucose concentration (11.1 mmol/l) in 12-well dishes, and treated with corresponding drugs for 24 h as described above. Following preincubation for 1 h in glucose-free RPMI-1640 medium and drug solutions, the cells were treated for 1 h in RPMI-1640 medium and drug solutions with low (0.2 mmol/l) or stimulatory (11.1 mmol/l) glucose concentrations [22]. Isolated rat islets were cultured and treated as described above. Then the islets were preincubated for 1 h in glucose-free DMEM with the appropriate drug combinations, and treated for 1 h in DMEM containing drug solutions and basal (3 mmol/l) or stimulatory (17 mmol/l) concentrations of glucose. After the static incubation, the supernatants were obtained and frozen at −70°C for subsequent insulin concentration determination. The insulin levels were measured using RIA as described previously [23], or a rat/mouse insulin ELISA kit (Linco Research, St Louis, MO, USA).

Statistical analysis Comparisons were performed using Student's *t* test between two groups, or ANOVA in multiple groups. Results are presented as means±SEM. A *p* value of less than 0.05 was considered to be statistically significant.

Results

PGE2 stimulates Ptger3 gene expression and protein production and decreases cAMP synthesis in HIT-T15 cells Using one-step real-time PCR assays, we demonstrated that treating HIT-T15 cells with PGE_2 (1 µmol/l) for 2 h led to a dramatic increase in *Ptger3* gene expression ($p<0.01$, Fig. 1a). However, the expression levels of *Ptger1*, *Ptger3* or *Ptger4* were not detected with RT-PCR (results not shown). To further confirm that the protein level of PTGER3 is also upregulated by PGE_2, western blot analysis was performed after the HIT-T15 cells were treated with PGE_2 (1 µmol/l) for the indicated periods of time (Fig. 1b). Consistent with the real-time PCR results, PGE_2 significantly increased the protein production of PTGER3 in HIT-T15 cells. It has been reported previously that among the four PTGER subtypes only PTGER3 possesses a post-receptor action that reduces cAMP levels [24]. Therefore, we analysed the functional activation of the PTGER3 in pancreatic beta cells by cAMP determination following treatment with PGE_2. PGE_2 significantly diminished cAMP production (ESM Fig. 1), indicating that the PTGER3 might be responsible for mediating the effects of PGE_2 on pancreatic islet beta cells.

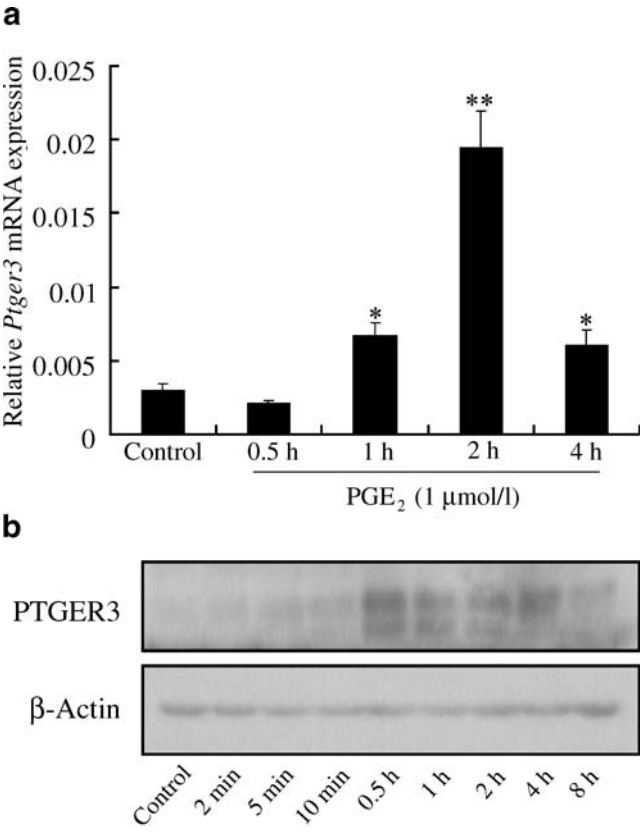

Fig. 1 PGE_2 stimulates *Ptger3* gene expression and protein production. HIT-T15 cells were treated without (*Control*) or with PGE_2 (1 µmol/l) for the indicated periods of time, followed by one-step real-time PCR assays and western blot analysis to determine the mRNA (**a**) and protein levels (**b**) of PTGER3 and β-actin. **a** Relative levels of the *Ptger3* mRNA to β-actin are shown as means±SEM of three separate experiments. **b** Representative immunoblots of three separate experiments. *$p<0.05$ and **$p<0.01$ vs control

PGE_2 decreases phosphorylation of Akt in HIT-T15 cells and isolated rat islets, which is reversed by IGF-1 The PI3K-dependent Ser/Thr kinase Akt, a key mediator of multiple signalling pathways, plays a central role in

metabolism, cell survival and insulin synthesis and secretion. To assess the effect of PGE_2 on Akt activity, we investigated the levels of Ser^{473}- and Thr^{308}-phosphorylated Akt by western blot analysis. The HIT-T15 cells treated with PGE_2 (1 μmol/l) showed a time-dependent decline in Thr^{308}- and Ser^{473}-phosphorylated Akt levels (Fig. 2a). The same results were observed from PGE_2-treated (1 μmol/l) isolated rat islets. As expected, PGE_2 treatment reduced the level of Ser^{473}-phosphorylated Akt in the isolated rat islets in a time-dependent manner (Fig. 2b). To further confirm the relationship between reduced Akt activity and PGE_2 effects, we used IGF-1, a strong stimulator of Akt in beta cells. As shown in Fig. 2c, pretreatment of HIT-T15 cells with IGF-1 (100 ng/ml) for 1 h increased the level of

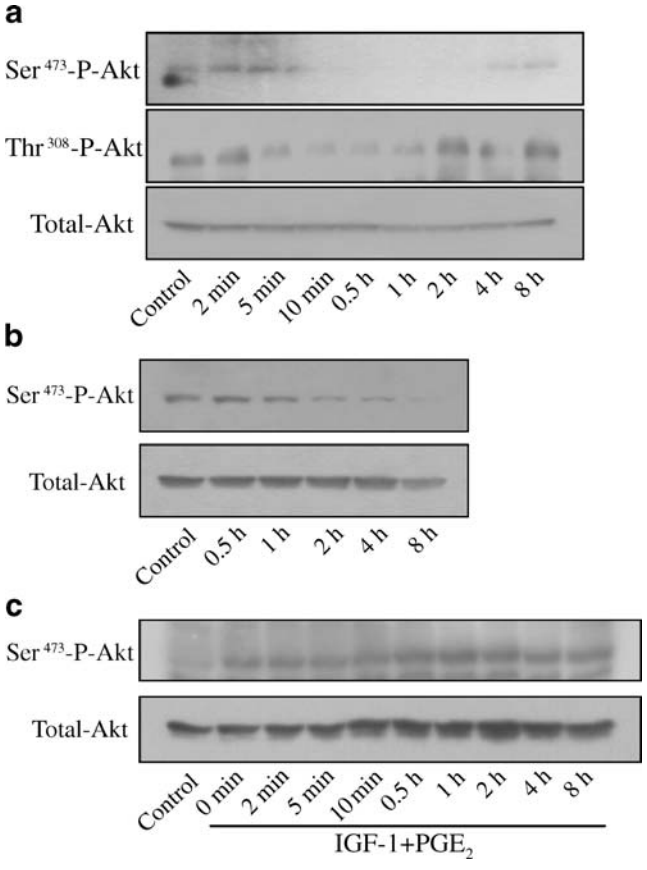

Fig. 2 Effects of PGE_2 on the phosphorylation levels of Akt. PGE_2 decreased the phosphorylation levels of Akt in HIT-T15 cells (**a**) and isolated rat islets (**b**). HIT-T15 cells and isolated rat islets were treated without (*Control*) or with PGE_2 (1 μmol/l) for the indicated periods of time; the cells were then harvested and lysed for western blot analysis to determine Ser^{473}-phosphorylated Akt (*Ser^{473}-P-Akt*), Thr^{308}-phosphorylated Akt (*Thr^{308}-P-Akt*) and total Akt (*Total-Akt*, as a loading control). **c** IGF-1 reversed the effect of PGE_2. After preincubation without (*Control*) or with IGF-1 (100 ng/ml) for 1 h, HIT-T15 cells were treated with PGE_2 (1 μmol/l) for the indicated periods of time, the cell lysates were obtained for western blot analysis to assess Ser^{473}-phosphorylated Akt (*Ser^{473}-P-Akt*) and total Akt (*Total-Akt*, as a loading control). Immunoblots are representative of three separate experiments

Ser^{473}-phosphorylated Akt, and reversed PGE_2-induced reduction of Akt phosphorylation. However, neither IGF-1 nor PGE_2 had an effect on the levels of total Akt, which served as an internal control (Fig. 2a–c).

PGE_2 decreases phosphorylation of Foxo in HIT-T15 cells and isolated rat islets, which is reversed by IGF-1 We next investigated what downstream targets of PI3K/Akt might be involved in the PGE_2 regulation pathway. There is growing evidence indicating that Foxo proteins, the downstream targets of PI3K/Akt, play an important role in mediating the effects of hormone and growth factors on diverse physiological functions [19], as well as insulin gene expression [14]. To further explore the involvement of Foxo in the PGE_2 regulation pathway, we examined the effect of PGE_2 on Foxo protein phosphorylation in HIT-T15 cells and isolated rat islets. As shown in Fig. 3a,b, the levels of Ser^{256}-phosphorylated FKHR and Thr^{24}-phosphorylated FKHRL1 were markedly decreased in a time-dependent manner in HIT-T15 cells treated with PGE_2 (1 μmol/l) and isolated rat islets. In addition, we found that pretreatment of HIT-T15 cells with IGF-1 for 1 h increased the levels of Ser^{256}-phosphorylated FKHR and Thr^{24}-phosphorylated FKHRL1, and blocked the PGE_2-induced reduction of phosphorylation of Foxo proteins. In contrast, total FKHR levels were not affected by IGF-1 and PGE_2, and served as an internal control (Fig. 3a–c).

The effects of PGE_2 on FKHR transcriptional activity Dephosphorylation of Foxo transcription factors leads to nuclear entry and modulates the expression of their target genes [17, 18]. Thus, to evaluate the effects of PGE_2 on FKHR transcriptional activity, we performed transient transfection and luciferase reporter assays. The HIT-T15 cells were transiently cotransfected with the firefly luciferase reporter construct pGL3-FKHR and the β-galactosidase plasmid, and were analysed for luciferase activity after exposure to PGE_2. As shown in Fig. 4a, PGE_2 stimulated the FKHR luciferase activity in a dose-dependent manner, with the maximum induction occurring at the concentration of 1 μmol/l (116% increase, $p<0.01$).

To examine whether the effect of PGE_2 on FKHR transcriptional activity is mediated by Akt, we transiently cotransfected HIT-T15 cells with or without the CA-Akt expression plasmid and pGL3-FKHR (all cells were transfected with β-galactosidase plasmid simultaneously as an internal control). Consistent with previous results [17, 18], FKHR luciferase activity was decreased significantly (by 46% vs control, $p<0.01$; Fig. 4b) by CA-Akt expression. However, the addition of PGE_2 (1 μmol/l) to the CA-Akt-cotransfected cells partially reversed the inhibitory effect of CA-Akt on the FKHR luciferase reporter activity (98% increase, $p<0.01$; Fig. 4b).

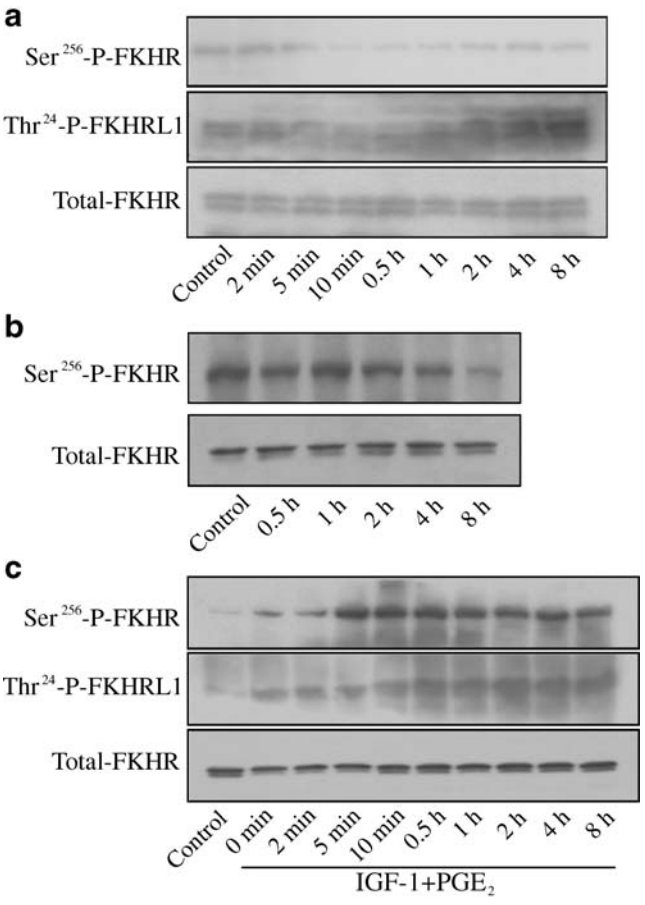

Fig. 3 Effects of PGE_2 on the phosphorylation levels of Foxo proteins. The phosphorylation levels of Foxo were markedly down-regulated by PGE_2 in HIT-T15 cells (**a**) and isolated rat islets (**b**). HIT-T15 cells and isolated rat islets were treated without (*Control*) or with PGE_2 (1 μmol/l) for the indicated periods of time and the cell lysates were obtained for western blot analysis to determine Ser^{256}-phosphorylated FKHR (*Ser^{256}-P-FKHR*), Thr^{24}-phosphorylated FKHRL1 (*Thr^{24}-P-FKHRL1*) and total FKHR (*Total-FKHR*, as a loading control). **c** IGF-1 reversed the effect of PGE_2. After preincubation without (*Control*) or with IGF-1 (100 ng/ml) for 1 h, HIT-T15 cells were treated with PGE_2 (1 μmol/l) for the indicated periods of time and cell lysates were obtained for western blot analysis to assess Ser^{256}-phosphorylated FKHR (*Ser^{256}-P-FKHR*), Thr^{24}-phosphorylated FKHRL1 (*Thr^{24}-P-FKHRL1*) and total FKHR (*Total-FKHR*, as a loading control). Immunoblots are representative of three separate experiments

To further examine the relationship between reduced Akt activity and PGE_2 effects on FKHR transcriptional activity, we treated the transfected cells with wortmannin, IGF-1 and PGE_2, as described in Materials and methods. IGF-1 attenuated the stimulatory effects of PGE_2 on FKHR luciferase activity ($p<0.01$; Fig. 4c). Furthermore, wortmannin, a well-known PI3K inhibitor, reversed the attenuation effect of IGF-1 ($p<0.01$; Fig. 4c).

The effects of PGE_2 on HIT-T15 cell viability and apoptosis Both Akt and Foxo have been shown to play important roles in regulating cell survival, apoptosis and

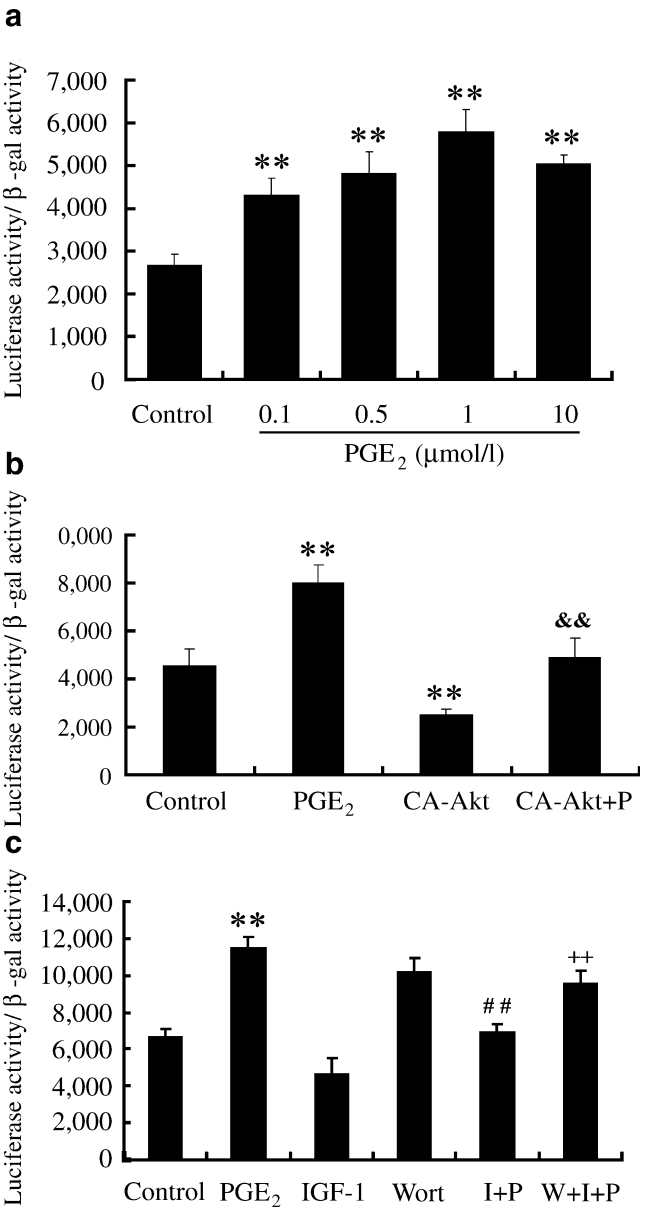

Fig. 4 Effects of PGE_2 on FKHR transcriptional activity. HIT-T15 cells were transiently transfected with the firefly luciferase reporter construct pGL3-FKHR and β-galactosidase (β-gal) plasmid (as an internal control). Twenty-four hours after transfection, the cells were treated without (*Control*) or with the indicated concentrations of PGE_2 (**a**). Following transient cotransfection with pGL3-FKHR, β-galactosidase, and without or with CA-Akt construct, the HIT-T15 cells were treated without (*Control*, *CA-Akt*) or with PGE_2 (1 μmol/l; *PGE_2*, *CA-Akt+P*) (**b**). **c** The pGL3-FKHR- and β-galactosidase-transfected HIT-T15 cells were treated without (*Control*) or with PGE_2 (1 μmol/l), IGF-1 (100 ng/ml) or wortmannin (300 nmol/l) (*Wort*) alone; or IGF-1 (100 ng/ml) and PGE_2 (1 μmol/l) (*I+P*); or wortmannin (300 nmol/l), IGF-1 (100 ng/ml) and PGE_2 (1 μmol/l) (*W+I+P*) as indicated, where wortmannin was added 0.5 h before the addition of others. Then all the transfected cells were incubated for an additional 12 h and harvested for luciferase reporter assays. The relative values of FKHR luciferase activity to β-galactosidase are shown as means±SEM of three independent experiments. $^{**}p<0.01$ vs control; $^{\&\&}p<0.01$ vs CA-Akt-cotransfected only (*CA-Akt*); $^{\#\#}p<0.01$ vs PGE_2-treated alone; $^{++}p<0.01$ vs IGF-1 and PGE_2 cotreated (*I+P*)

metabolism [12, 17, 19]. Therefore, we next evaluated the potential effects of PGE_2 on pancreatic beta cell viability using MTT assays. Surprisingly, as shown in Fig. 5a, PGE_2 did not significantly inhibit HIT-T15 cell viability at 0.2–5 μmol/l ($p=0.064$), although PGE_2 could decrease cell viability in a dose-dependent manner at increasing concentrations (>5 μmol/l).

To confirm that the PGE_2 dose (1 μmol/l) used in this study did not induce any obvious impairment of cell survival, we assessed the effects of PGE_2 on apoptosis and cell-cycle progression using flow cytometry analysis. Consistent with our MTT assay results, we observed no significant apoptosis or cell-cycle phase alteration in HIT-T15 cells treated with PGE_2 in the range of 1–10 μmol/l (Fig. 5b), suggesting that PGE_2 induces beta cell dysfunction without affecting cell survival.

PGE_2 inhibits GSIS via the PI3K/Akt signalling pathway To ascertain the involvement of PI3K/Akt in PGE_2-induced pancreatic beta cell dysfunction, we treated HIT-T15 cells and isolated rat islets with wortmannin, IGF-1 and PGE_2, and conducted GSIS assays. As shown in Fig. 6a, 1 μmol/l PGE_2 significantly diminished insulin secretion from HIT-T15 cells after stimulation with 11.1 mmol/l glucose, relative to non-treated controls ($p<0.01$). This effect was reversed by pretreating the cells with IGF-1 (100 ng/ml, $p<0.01$ vs PGE_2 treatment alone). However, the protective effect of IGF-1 was abolished in the presence of wortmannin (300 ng/ml, $p<0.01$). Furthermore, similar changes were observed in isolated rat islets (Fig. 6b). Taken together, these results suggest that the PI3K/Akt signalling pathway plays a central role in the inhibitory effects of PGE_2 on beta cells.

Discussion

The production of PGE_2, as a consequence of COX-2 gene induction, has long been known to impair pancreatic beta cell function. However, the potential signalling pathway by which PGE_2 induces beta cell dysfunction has not been resolved. PGE_2 was shown to bind to a single class of specific receptors in beta cells whose post-receptor activities activate G proteins and decrease adenylate cyclase activity and cAMP synthesis [25]. In addition, Tran et al. [26] demonstrated that the PTGER3 agonist misoprostol mimicked the inhibitory action of PGE_2 on GSIS. In the present study, we found that PGE_2 specifically stimulated *Ptger3* mRNA expression and protein production. Since no PTGER3 antagonist is available for further confirmation of the involvement of PTGER3 in the effect of PGE_2 on GSIS, and it has been reported that only PTGER3 has post-receptor activities resulting in a decrease in cAMP [10, 11], we chose to assess the effect of PGE_2 on cAMP generation. In agreement with previous studies, our results demonstrated that PGE_2 diminished cAMP generation in HIT-T15 cells. Previous studies have indicated that the second messenger cAMP plays an important role in insulin metabolism; specifically it may potently enhance insulin secretion and stimulate gene expression through cAMP response elements in the insulin gene promoters [14, 27]. In addition to these effects, which require an acute elevation of intracellular cAMP, a basal level of cAMP is required to maintain pancreatic beta cells in a glucose-competent state [27]. In the present study, the intracellular cAMP level was significantly decreased by PGE_2, suggesting that PTGER3 and cAMP may partially mediate the PGE_2-induced pancreatic beta cell dysfunction (Fig. 7).

Accumulating evidence indicates that Akt plays a central role in the regulation of glucose transport, glycolysis,

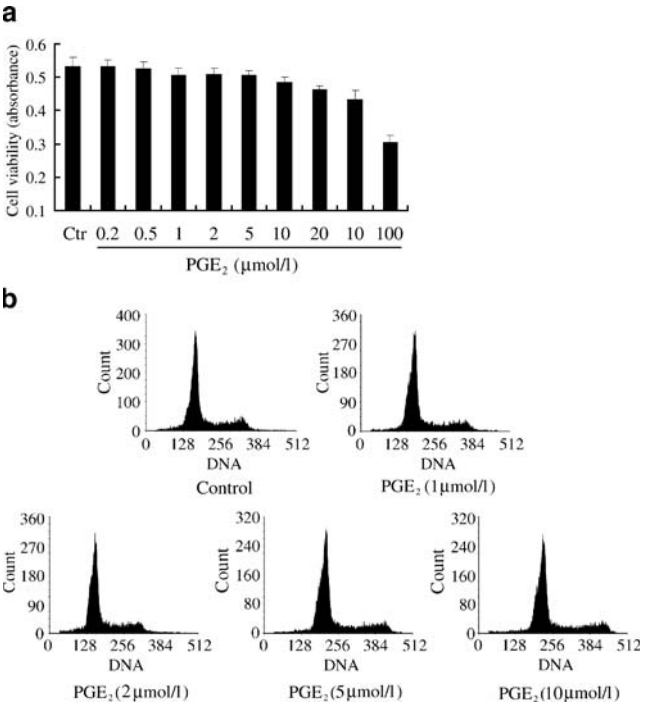

Fig. 5 Effects of PGE_2 on HIT-T15 cell viability and apoptosis. **a** After treating HIT-T15 cells without (control: *Ctr*) or with the indicated concentrations of PGE_2 for 24 h, MTT assays were performed to evaluate the cell viability. The data shown are means±SEM of three separate experiments. **b** HIT-T15 cells (1.5×10^6 cells per well) were cultured in six-well dishes and treated without (*Control*) or with the indicated concentrations of PGE_2 for 24 h. Cells were then harvested and fixed with 1 ml 75% ice-cold ethanol at −20°C overnight. The next morning, cells were washed in PBS and stained with 500 μl propidium iodide solution (50 μg/ml in PBS) containing 25 μg/ml RNase. Then cell-cycle progression and apoptosis were investigated using flow cytometry analysis as described in Materials and methods. Graphs are representative of three separate experiments

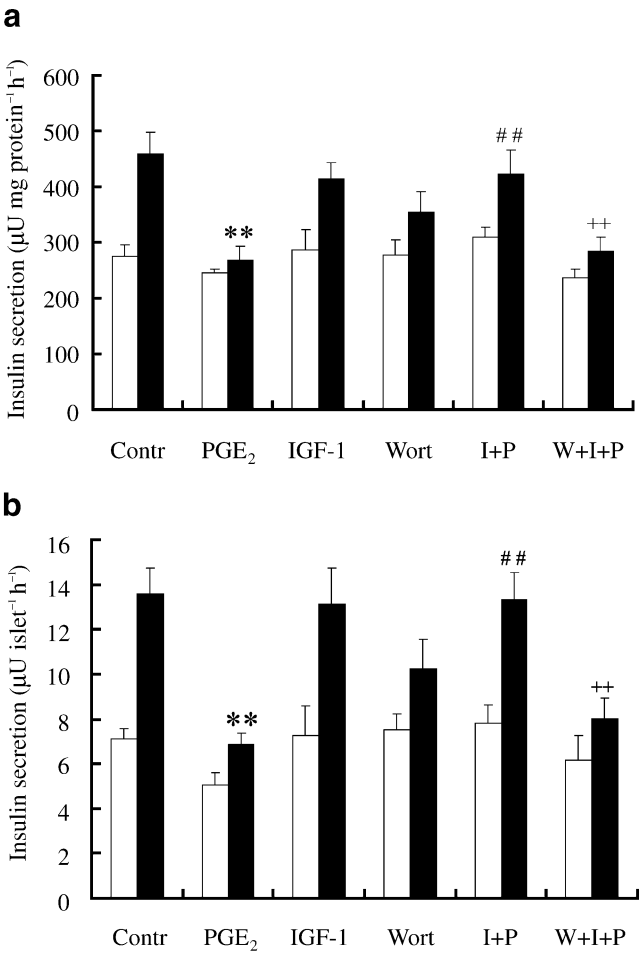

Fig. 6 PGE_2 inhibits GSIS via the PI3K/Akt signalling pathway. HIT-T15 cells (5×10^5 cells per well) and isolated rat islets (ten islets per well) were treated without (control: *Contr*) or with certain drugs as described in Fig. 4c for 24 h. Following preincubation for 1 h in glucose-free medium and drug solutions, HIT-T15 cells were treated for 1 h in RPMI-1640 medium and drug solutions with low (0.2 mmol/l) or stimulatory (11.1 mmol/l) concentrations of glucose (**a**), and isolated rat islets were treated for 1 h in DMEM medium and drug solutions with basal (3 mmol/l) or stimulatory (17 mmol/l) glucose concentrations (**b**). After the static incubation, supernatant fractions were obtained for insulin concentration determination as described in Materials and methods. Values are means±SEM of more than three individual experiments. $^{**}p<0.01$ vs control; $^{\#\#}p<0.01$ vs PGE_2-treated alone; $^{++}p<0.01$ vs IGF-1 and PGE_2 cotreated (*I*+*P*). *Wort* and *W*, wortmannin

protein production, lipogenesis, glycogen synthesis, suppression of gluconeogenesis, cell survival, determination of cell size and cell-cycle progression [12]. Many reports have also demonstrated that Akt activation plays an important role in promoting pancreatic beta cell survival and preserving beta cell function [16, 28]. In this study, we demonstrated that PGE_2 decreased the levels of Ser^{473}- and Thr^{308}-phosphorylated Akt in both HIT-T15 cells and isolated rat islets. Moreover, IGF-1 reversed the inhibitory effects of PGE_2 (1 μmol/l) on GSIS in HIT-T15 cells and isolated rat islets. Meanwhile wortmannin, a PI3K inhibitor, abolished the protective effect of IGF-1. These data suggest that suppression of the PI3K/Akt pathway is involved in PGE_2-induced beta cell dysfunction. Importantly, our demonstration using MTT and flow cytometry analysis that this concentration of PGE_2 (1 μmol/l) did not affect beta cell viability indicates that PGE_2 may exert its inhibitory effect on beta cells without affecting cell survival. Consistent with our observations, recent studies have revealed that reducing Akt activity in beta cells resulted in dysregulation of insulin secretion without affecting beta cell mass and development [13, 15], and Akt has also been shown to play a key role in insulin synthesis [14]. Hence, we presume that dysregulation of insulin synthesis and secretion resulting from diminished Akt activity may account for PGE_2-induced beta cell dysfunction, although the underlying mechanisms of this dysregulation have yet to be resolved.

Foxo transcription factors, a subfamily of the large group of forkhead transcription factors, are phosphorylated and regulated by Akt and play crucial roles in mediating the effects of insulin and growth factors on diverse physiological functions, including cell proliferation, apoptosis and metabolism [19, 29–31]. We found in this study that the levels of Ser^{256}-phosphorylated FKHR and Thr^{24}-phosphorylated FKHRL1 were decreased in response to PGE_2 treatment. The effect of PGE_2 on intracellular signalling was further investigated with a luciferase reporter gene system. We observed that PGE_2 stimulated FKHR transcriptional activity markedly in pancreatic beta cells. The addition of exogenous PGE_2 partially reversed CA-Akt-inactivated FKHR luciferase activity. Furthermore, IGF-1 attenuated the stimulatory effects of PGE_2 on FKHR luciferase activity, while wortmannin reversed the attenuation effect of IGF-1. Collectively, these results further confirm the functional association of PI3K/Akt in PGE_2-mediated beta cell dysfunction. Previous studies have shown that FKHR is a negative regulator of insulin synthesis that acts by decreasing PDX1 production [32]. In addition to its important roles in the development and differentiation of pancreatic islets and in beta cell specific gene expression [33], PDX1, an important downstream target of Foxo transcription factors, functions as an essential mediator of the glucose effect on insulin gene expression on differentiated beta cells [14]. In accord, our study demonstrated that PGE_2 could dephosphorylate Foxo transcription factors, prompting them to enter the nucleus and modulate the expression of target genes. For example, Foxo could induce the downregulation and nucleocytoplasmic translocation of PDX1, resulting in a reduction of insulin expression [32]. The precise mechanisms mediating this effect, however, remain to be elucidated; specifically further evidence is needed to confirm the role of this pathway in PGE_2-induced beta cell dysfunction.

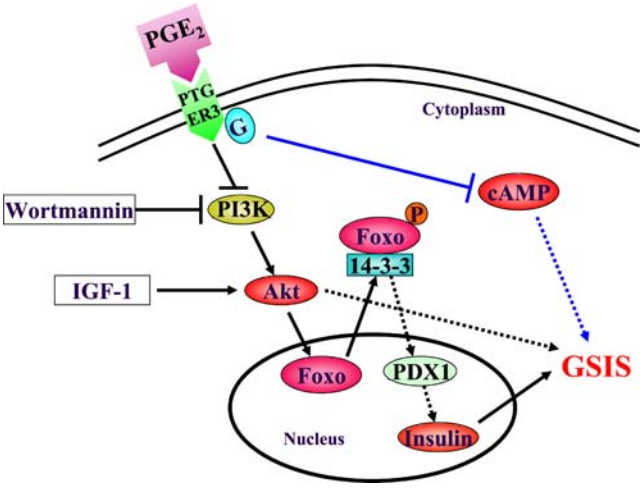

Fig. 7 Scheme illustrating possible signalling pathways and target transcription factors involved in PGE_2-induced pancreatic beta cell dysfunction. In the present study, we demonstrated that PGE_2 could stimulate *Ptger3* gene expression. Following binding to G protein-coupled PTGER3, on the one hand, PGE_2 diminished intracellular cAMP generation (*blue lines*); on the other hand, PGE_2 dephosphorylated and inactivated Akt resulting in dephosphorylation and activation of Foxo transcription factors (*black lines*). Therefore GSIS was inhibited. However, IGF-1 could reverse the inhibitory effect of PGE_2, and wortmannin abolished the preventive action of IGF-1. *Solid-line arrows*, stimulatory effects; ⊣, inhibitory effect; *dashed-line arrows*, tentative effects

In conclusion, we report for the first time that PGE_2 can induce pancreatic beta cell dysfunction through the induction of *Ptger3* gene expression, inhibition of intracellular cAMP generation and upregulation of Foxo activity via suppression of the PI3K/Akt signalling pathway, without affecting beta cell viability. This finding is best illustrated in Fig. 7, which shows Akt and Foxo as key regulators in PGE_2-mediated dysfunction in pancreatic beta cells. Our studies contribute to the understanding of the underlying mechanisms by which PGE_2 regulates pancreatic beta cell function and provide important clues for intervention in the diabetes mellitus disease course.

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Duality of interest The authors declare that they have no duality of interest.

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