Puerarin protects pancreatic β-cell survival via PI3K/Akt signaling pathway

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Abstract
Pancreatic β-cell loss because of apoptosis is the major cause of type 1 diabetes (T1D) and late stage T2D. Puerarin possesses anti-diabetic properties; whether it acts directly on pancreatic β-cell is not clear. This study was designed to investigate the effects of puerarin on pancreatic β-cell survival and function. Diabetes was induced in male C57BL/6 mice by a single peritoneal injection of streptozotocin (STZ). Pancreatic β-cell survival and function were assessed in diabetic mice by measuring β-cell apoptosis, β-cell mass, pancreatic insulin content, and glucose tolerance, and in cultured islets and clonial MIN6 β-cells by measuring β-cell viability and apoptosis and glucose-stimulated insulin secretion. We found that pre-treatment with puerarin decreased the incidence of STZ-induced diabetes. Puerarin increased pancreatic β-cell mass via β-cell apoptosis inhibition in diabetic mice, and increased serum insulin, whereas it decreased blood glucose levels and improved glucose tolerance. In cultured islets and MIN6 cells, puerarin protected β-cell from cobalt chloride (CoCl2)-induced apoptosis and restored the impaired capacity of glucose-stimulated insulin secretion. Puerarin protection of β-cell survival involved the phosphoinositide 3-kinase (PI3K) /Akt signaling pathway. In conclusion, puerarin protects pancreatic β-cell function and survival via direct effects on β-cells, and its protection of β-cell survival is mediated by the PI3K/Akt pathway. As a safe natural plant extraction, puerarin might serve as a preventive and/or therapeutic approach for diabetes.

Introduction
The incidence of diabetes has been increasing at an alarming rate in the past decade and is now becoming a major epidemic in China and worldwide (Xu et al. 2013). Although type 1 diabetes mellitus (T1D) is characterized by loss of pancreatic islet β-cell mass by immune attack, while T2D is characterized by insulin resistance and β-cell failure resulted from reductions in β-cell function and mass, they do share a common consequence, which is progressive β-cell failure due to apoptosis – the main form of β-cell death in the two forms of the disease (Cnop et al. 2005). Current therapeutic approaches are mainly focused on increasing serum insulin levels by direct insulin administration or other agents that promote insulin secretion and/or improving insulin sensitivity in the
target tissues. Life-threatening side effects and the occurrence of inevitable complications due to poor insulin management happen frequently, making more physiological treatment for diabetes increasingly appealing. Islet transplantation has been proposed as the most physiological treatment for T1D, but its application is greatly hampered due to the shortage of donors and the rapid β-cell loss post transplantation (Robertson 2010). For T2D, although the potential regenerative influences of incretin therapies on preserving pancreatic β-cells are intriguing, the marked expansion of the exocrine and endocrine pancreatic compartments with the risk of carcinogenesis require more strict and convincing investigations (Butler et al. 2013). Thus, so far the therapeutic approaches that can safely and efficaciously prevent pancreatic β-cell loss and maintain its mass and function are absent.

Puerarin, the main isoflavone glycoside and the major active ingredient extracted from the traditional Chinese medicine Radix puerariae, has beneficial effects in protecting various organs survival and possesses anti-oxidative stress (Hwang & Jeong 2008, Bebrevska et al. 2010, Jin et al. 2012, Zhang et al. 2012), anti-inflammation (Huang et al. 2012, Jin et al. 2012, Zhang et al. 2012), and anti-apoptosis (Li et al. 2012, Hsu et al. 2003) properties. It has been reported that puerarin improved insulin resistance and glucose tolerance (Prasain et al. 2012), protected pancreatic islet from hydrogen peroxide-induced β-cell damage (Xiong et al. 2006) and lowered blood glucose level in mice challenged with streptozotocin (STZ) (Hsu et al. 2003), via promoting glucose uptake by skeletal muscle and adipocytes (Hsu et al. 2003, Lee et al. 2010) and attenuating glucose and insulin response (Prasain et al. 2012). However, there is a lack of understanding of whether puerarin can act on insulin-producing β-cell and improve its function and survival. Thus this study was designed to investigate the effects of puerarin on β-cell function and survival in vivo in pancreatic islet of diabetic mice and in vitro in cultured islet and clonal MIN6 β-cells, thus to elucidate the effects and mechanisms of puerarin in controlling blood glucose and diabetes.

Materials and methods

Animals and the induction of experimental diabetes
Male C57BL/6 mice at the age of 8 weeks (20–22 g) were purchased from Shanghai Center for Experimental Animals, Chinese Academy of Sciences (Shanghai, People’s Republic of China). Mice were housed under controlled temperature (22 ± 1°C) and humidity with a 12 h light:12 h darkness cycle, and had free access to water and standard chow pellets. The mice were divided into three groups: control, diabetic, and diabetic with puerarin treatment. Diabetes was induced by a single i.p. injection of STZ (80 mg/kg). Blood glucose was measured every 48 h following STZ injection with One Touch Ultra Glucose Monitor. Mice with random blood glucose exceeding 250 mg/dl were considered diabetic. Puerarin, with a purity of more than 99%, was obtained from Shanghai Winherb Medical Science Co. Ltd (Shanghai, China) and dissolved in 5% propanediol. Puerarin was administrated intraperitoneally once daily staring 3 days before STZ injection at a dose of 100 mg/kg body weight. At day 8, after STZ injection, the mice were killed after the collection of serum and pancreata under anesthetization. All animal experiments were approved by Xiamen University Animal Care and Use Committee.

Cell viability assay
The mouse insulinoma MIN6 cells (passage 22–30) were maintained routinely in DMEM supplemented with 10% fetal bovine serum (FBS). The cells were kept at 37°C in a humidified atmosphere with 5% CO2 and 95% air. MIN6 cells were pretreated with various concentrations of puerarin (0.1, 1, and 10 M) for 4 h, followed by incubation with vehicle or 400 μM cobalt chloride (CoCl2) for 16 h. The cell viability was assessed by the Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan). Briefly, after cultivation, 10 μl CCK-8 solution was added to each sample. After 2 h of incubation at 37°C, the absorbance at 450 nm was determined using a microplate reader (BioTek, Synergy H1, Winooski, VT, USA). Data are reported as relative values to the vehicle.

Caspase-3/7 activity assay
Caspase-3/7 activity was measured using the Caspase-3/7 Assay Kit (Promega Co.) according to the manufacturer’s instruction. Briefly, after cultivation, the 96-well plate was equilibrated to room temperature, 100 μl caspase-3/7 reagent was added to each well, then the contents were gently mixed, and the plate was allowed to incubate at room temperature in darkness for 1 h. Luminescence (RLU) was measured by a microplate reader (BioTek, Synergy H1). Data are reported as relative values to the vehicle.

Measurement of apoptosis by nuclear morphology
The nuclear morphological change was assessed using the Hoechst 33258 (Beyotime, Nantong, China). The cells
were incubated with 1 μg/ml Hoechst 33258 for 10 min at room temperature and apoptotic cells with condensed or fragmented nuclei were visualized using an Olympus Microscope with a WU excitation filter.

**Measurement of reactive oxygen species generation**

DCFH-DA diffuses through the cell membrane and is enzymatically hydrolyzed by intracellular esterases to the nonfluorescent DCFH, which can be rapidly oxidized to the highly fluorescent DCF, the fluorescent product, in the presence of reactive oxygen species (ROS). Briefly, treated cells were incubated with DCFH-DA (Beyotime) for 30 min at 37 °C in darkness, washed with PBS three times, and then imaged using a fluorescent microscope (Olympus). Data are reported as relative values to the vehicle.

**Measurement of insulin concentration**

Pancreata were collected, weighed, and homogenized in acid/ethanol. Insulin levels were examined by ELISA (Millipore, Billerica, MA, USA).

**Intraperitoneal glucose tolerance test**

Intraperitoneal glucose tolerance test (IPGTT) was carried out after 12 h fasting. Glucose was administrated by i.p. injection (1 g glucose/kg body weight). Blood glucose was measured at time points of 0, 15, 30, 60, 90, and 120 min post glucose administration. Glucose areas under the curve values during the IPGTT were calculated using the trapezoidal rule.

**Islet isolation**

Mouse islets were isolated by collagenase digestion. Briefly, the pancreas was injected with collagenase V (2 mg/ml, Sigma), dissected out, and incubated at 37 °C for 15 min. The digested pancreas was centrifuged at 4 °C. The supernatant was discarded, and the pellet was re-suspended and rinsed three times with HBSS. After passing through a wire mesh, the islets were separated by density gradient using Histopaque (Sigma). After several washes with HBSS and PBS, islets were hand picked under a dissection microscope and then cultured in phenol-red free RPMI medium, containing 11 mM glucose, 10% charcoal-stripped FBS, 1 mM glutamine, penicillin (100 U/ml), and streptomycin (100 mg/ml).

**RNA extraction and RT-PCR**

Total RNA was extracted using the RNA simple Total RNA Kit (Tiangen Biotech, Beijing, China). RT of total RNA to cDNA was carried out with the PrimeScript RT Reagent Kit with gDNA eraser (Takara, Dalian, China) in a MyCycler Thermal Cycler (Bio-Rad) following the manufacturer’s instructions. Real-time quantitative PCR was carried out with LightCycler 480 SYBR Green I Master Mix (Roche Diagnostics GmbH, Mannheim, Germany) in a Light Cycler 480 System. The primers used were manganese superoxide dismutase (MnSod) (Sod2): forward, 5′-CAGACCTGCCTTACGACTATGG-3′ and reverse, 5′-CTCGGTGGCGTTGAGATTGTT-3′; glutathione peroxidase 1 (Gpx1): forward, 5′-AGTCCACCGTG-TATGCTTCT-3′ and reverse, 5′-GAGACGGACATT-CTCAATGA-3′; Bcl2: forward, 5′-GGGAGAACAGGTTAT-GATAACCG-3′ and reverse, 5′-TAGGCCCTCTGAGCACA-GCTTA-3′; Bax: forward, 5′-AGACAGGCGGCTTTTGTGCTAC-3′ and reverse, 5′-ATTCGCGCGAGACACTCG-3′; and Gapdh: forward, 5′-TGACACCAGTCATGCCATC-3′ and reverse, 5′-GACGGACACATTGGGGTAG-3′.

**Western blotting analysis**

Whole-cell protein was extracted and 30 μg protein was loaded and separated by SDS–PAGE, and then transferred to

![Image](http://jme.endocrinology-journals.org)
the PVDF transfer membranes. Expression of BCL2, BAX, phos-AKT, AKT (Cell Signaling, Danvers, MA, USA), and β-actin was determined by immunoblotting the membranes with antibodies at 4 °C. After incubation with the peroxidase-conjugated goat anti-rabbit or anti-mouse secondary antibodies, the immunoreactivity was visualized using an ECL Kit (Amersham Pharmacia Biotech).

**Immunohistochemistry**

Pancreata from five mice of each group were fixed and processed for immunohistochemistry. For insulin staining, sections were incubated with rabbit anti-mouse insulin antibody (Cell Signaling) and then imaged by fluorescence microscope. For TUNEL staining, sections were pretreated with proteinase K and then labeled by TUNEL (Promega Co.) followed by staining with anti-insulin antibody. The ratios of TUNEL positive to total islet β-cells were calculated. Pancreatic β-cell mass was determined by using the following equation: β-cell mass = (insulin positive area (μm²)/total pancreas area (μm²)) × pancreas weight (mg). Data are reported as relative values to the vehicle.

Immunofluorescent-stained sections were visualized using fluorescence microscope (Olympus). Morphometric analysis was conducted using Image J program (Image J, National Institutes of Health, Bethesda, MD, USA).

**Statistical analysis**

Data are expressed as means ± S.E.M. Data were analyzed by one-way ANOVA. Statistical analysis of cumulative diabetes incidence was determined by log-rank test. A value of P<0.05 was considered statistically significant.

**Results**

**Puerarin protected mice from STZ-induced experimental diabetes**

Puerarin has no effects on body weight and blood glucose of normal mice (data not shown), while when
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Pre-administered to mice treated with pancreatic β-cell toxic STZ, it significantly lowered blood glucose and decreased the incidence of diabetes in C57BL/6 male mice, showing a potent anti-diabetic effect (Fig. 1A and B). In the IPGTT, puerarin significantly improved glucose clearance compared with the vehicle, implying a beneficial effect in improving glucose-dependent insulin secretion (Fig. 1C and D).

**Puerarin preserved pancreatic β-cell mass and insulin content**

STZ treatment resulted in dramatic disruption of pancreatic islet structure (Fig. 2A). Pancreatic β-cell mass was reduced in vehicle-treated diabetic mice, while puerarin treatment retained a relatively normal islet structure and thus preserved a larger β-cell mass and higher pancreatic insulin concentration (Fig. 2A, B and C). Accordingly, diabetic mice exhibited higher blood glucose and lower plasma insulin levels, which was significantly reversed by puerarin (Fig. 2D).

**Puerarin protected pancreatic islet survival by preventing β-cell apoptosis**

TUNEL is a commonly used method for detecting the DNA fragmentation resulting from apoptosis. With STZ administration, the number of TUNEL-positive apoptotic β-cells was increased in vehicle-treated diabetic mice, which was reversed by puerarin treatment (Fig. 3A and B), suggesting an anti-apoptosis effect of puerarin in insulin-producing β-cells.

**Puerarin acted directly on pancreatic β-cell protecting function and survival**

We have shown that puerarin prevented β-cell apoptosis in STZ-treated diabetic mice, here we tested the effects of puerarin on cell viability and apoptosis in CoCl2-treated clonial MIN6 β-cells and cultured pancreatic islet. Exposure to CoCl2 (400 µM) decreased β-cell viability and increased cell apoptosis, which were significantly attenuated by puerarin treatment in a dose-dependent manner.

**Figure 4**

Puerarin prevented CoCl2-induced β-cell apoptosis and dysfunction in MIN6 cells and mouse islet. (A and D) Cell viability; (B and E) caspase-3/7 activity; (C) condensed or fragmented apoptotic cells; and (F) glucose-stimulated insulin secretion. Results are representative of five independent experiments and values represent the mean ± S.E.M. (n = 5). *P < 0.05 and **P < 0.01 vs vehicle only group; ***P < 0.001 vs CoCl2 only group. The cells were treated with puerarin for 24 h, followed by exposure to CoCl2 (400 µM) for the last 16 h.
(Fig. 4A, B and C), these were true in cultured pancreatic islets (Fig. 4D and E). In healthy MIN6 β-cells, glucose dose-dependently provoked a robust insulin secretion, which was blunted in the presence of CoCl2, while with 10^{-6} M puerarin treatment, the impaired capacity of insulin secretion was significantly restored (Fig. 4F).

Puerarin inhibited the generation of ROS and activated anti-apoptotic factors

CoCl2, a commonly used hypoxia-like response inducer, provoked a robust generation of ROS when administered to β-cells for 16 h (Fig. 5A and B). We found that puerarin significantly decreased ROS generation, which might be mediated via increased gene expression of ROS scavengers – MnSod and Gpx1 (Fig. 5C). Furthermore, CoCl2 induced a dramatic decrease in the ratio of anti-apoptotic Bcl2 to pro-apoptotic Bax (Bcl2/Bax) at both mRNA and protein levels, which were significantly reversed by puerarin treatment (Fig. 5D, E and F).

Puerarin protected pancreatic β-cell survival through phosphoinositide 3-kinase/Akt signaling pathway

The phosphoinositide 3-kinase (PI3K)/Akt signaling pathway is the key regulator in β-cell function and survival. We observed that puerarin rapidly activated AKT phosphorylation (Fig. 6A and B). CoCl2 largely decreased AKT phosphorylation, which could be significantly attenuated by puerarin. When the PI3K/Akt pathway was inhibited by LY294002 inhibitor, puerarin effects on AKT phosphorylation was blocked (Fig. 6C and D). We then investigated if PI3K/Akt is involved in the puerarin protection of β-cell survival. We found that puerarin protection of β-cell survival, shown by increased cell viability and decreased apoptosis, were abolished when the PI3K inhibitor was present (Fig. 6E and F).

Discussion

Puerarin, the major component of Radix puerariae, possesses systemic anti-diabetic properties mainly through ameliorating insulin resistance. Here, by studying the puerarin actions on β-cells, we found that: i) puerarin preserved pancreatic β-cell mass in diabetic mice, mainly through inhibiting apoptosis in insulin-producing β-cells; ii) puerarin acted directly on cultured pancreatic islet and clonal MIN6 β-cells, protecting β-cell survival from CoCl2-induced apoptosis and restored the impaired glucose stimulated insulin secretion; and iii) puerarin protection of β-cell survival, shown by increased cell viability and decreased apoptosis, were abolished when the PI3K inhibitor was present (Fig. 6E and F).

Figure 5

Puerarin inhibited CoCl2-induced ROS accumulation and increased anti-oxidant and anti-apoptotic factors. (A) Green fluorescence represents the amounts of ROS, stained with DCFH-DA, scale bar: 200 μm. (B) Quantitative analysis of A, (C and D) mRNA expression of MnSod, Gpx1, and Bcl2/Bax in MIN6 cells, (E) protein expression of BCL2 and BAX, and (F) quantitative analysis of E. Results are representative of five independent experiments and values represent the mean ± S.E.M. (n=5). **P<0.01 and ***P<0.001 vs vehicle group and *P<0.05 vs CoCl2 only group.
of β-cell survival from CoCl₂-induced apoptosis involved the PI3K/Akt signaling pathway.

Previous studies have demonstrated that puerarin could lower blood glucose level and ameliorate glucose and insulin tolerance in both STZ-induced diabetic and obese animals (Hsu et al. 2003, Prasain et al. 2012), and its currently known anti-hyperglycemia effects mainly centralize on anti-insulin resistance, mainly via promoting glucose utilization by upregulating the mRNA and protein levels of glucose transporter 4, insulin receptors, and peroxisome proliferators-activated receptor α (Hsu et al. 2003, Wu et al. 2013), while whether it can directly act on pancreatic β-cell remains poorly clarified. In addition to the leading cause of T1D, reduced insulin-producing β-cell mass and impaired insulin secretion have been reported as the pivotal contributors to the pathogenesis of T2D, which currently lacks efficacious intervention (Cnop et al. 2005). Here, we found that puerarin could act directly on pancreatic β-cells, maintaining the β-cell mass and insulin secretion. Pancreatic islets are vulnerable to the oxidative stress and puerarin could sufficiently inhibit the hydrogen peroxide-induced β-cell apoptosis (Xiong et al. 2006). Here, we confirmed the inhibitory effect of puerarin on the ROS generation induced by CoCl₂. Furthermore, we showed that puerarin could block CoCl₂-induced hypoxic damage on β-cell function and survival, because CoCl₂ is also commonly used as a hypoxia-like inducer and the puerarin protection was mediated through upregulating the anti-oxidant

Figure 6
Puerarin protected β-cell survival via PI3K/Akt pathway. (A) AKT phosphorylation, (B) quantitative analysis of A, (C) AKT phosphorylation in CoCl₂-treated MIN6 β-cells, (D) quantitative analysis of C, (E) cell viability, and (F) caspase-3/7 activity. Results are representative of five independent experiments and values represent the mean ± S.E.M. (n = 5), and data are reported as relative values to the vehicle. ***P < 0.001 vs the vehicle group; *P < 0.05 and **P < 0.01 vs the CoCl₂ group (*P < 0.05 vs 0 min for B); and ΔΔP < 0.01 vs the puerarin only group.

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http://jme.endocrinology-journals.org
DOI: 10.1530/JME-13-0302
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Journal of Molecular Endocrinology
Sod2 and Gpx1, and the anti-apoptotic Bcl-2, while decreasing the pro-apoptotic Bax.

Puerarin has beneficial effects in various tissues, including hepatocyte, neural cells, kidney, endothelial cells, and osteoblastic cells, protecting the cell survival from a variety of damage, such as β-amyloid, lead, 1-methyl-4-phenylpyridinium iodide, cisplatin, especially oxidative stress, in which PI3K/Akt played a pivotal role (Hwang & Jeong 2008, Hwang et al. 2011, Xing et al. 2011, Liu et al. 2012, Zhang et al. 2012, Zhu et al. 2012, Wang et al. 2013). Here, in clonal MIN6 β-cells, we observed a rapid activation of Akt phosphorylation by puerarin. The Akt phosphorylation was blunted when CoCl2 was present, which was restored by puerarin. By investigating β-cell viability and apoptosis in the presence of PI3K/Akt inhibitor LY294002, we found that puerarin protection of β-cell survival was PI3K/Akt dependent. As a phytoestrogen, puerarin was shown to exhibit weak estrogenic activity in female rats and protected various cells from apoptosis via estrogen receptor (ER)-dependent PI3K signaling (Hwang & Jeong 2008, Hwang et al. 2011, Wang et al. 2013). In deed, estrogen is a potent protector of pancreatic β-cell function and survival (Liu et al. 2009, 2013, Wong et al. 2010). Although we have shown that puerarin protected β-cells in a manner similar to that of estrogens, whether its protection is ER-dependent needs further investigation.

Current therapeutic approaches for diabetes are mainly focusing on direct administration of insulin or insulin sensitizers, and rare options for retaining β-cell mass and function, such as glucagon-like peptide 1 and its modulators still need further evaluation due to arising concerns about safety and side effects (Cernea & Raz 2011, Butler et al. 2013). Puerarin, the major component of Radix puerariae (which has been used for thousands of years in traditional Chinese medicine), was shown to possess protective effects against apoptosis, inflammation, and oxidative stress, which are commonly considered as pathogenic contributors to the development of diabetes and its complications. Indeed, besides the anti-hyperglycemia and β-cell protection effects, puerarin was shown to have beneficial actions in ameliorating diabetic complications, including diabetic retinopathy (Teng et al. 2009, Hao et al. 2012), nephropathy (Shen et al. 2009), vascular endothelial cell damage induced by high-glucose (Chen et al. 2012), and diabetic osteoporosis (Chen et al. 2012). In addition, puerarin is also prescribed for cardiovascular disease in China, and could accelerate peripheral nerve regeneration, and thus might be beneficial to diabetic neuropathy (Hsiang et al. 2011) as well. With those pleiotropic benefits on insulin-producing β-cell and its target tissues concomitantly, puerarin may serve as a therapeutic option for diabetes and its complications.

In conclusion, in addition to its reported beneficial effects on insulin sensitivity and diabetic complications, puerarin has potent and direct protection on β-cell survival and insulin secretion, and thus might be developed as a therapeutic approach for diabetes and its complications.

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 grants from National Natural Science Foundation to S L (81270901), S Y (30973912), and X L (81073113); Key Project of Fujian Provincial Science and Technology Planning programs (2012D60) and Xiamen Innovation Program for Outstanding Youth Scientist (201150446) to S L, Xiamen Science and Technology Bureau (Xiamen Research Platform for Systems Biology of Metabolic Disease, 3502Z20100001).

Author contribution statement
The author(s) have made the following declarations about their contributions: S L and S Y designed the experiments; Z L, Z S, and J W performed the experiments; Y L and X L participated in the critical discussions; S L and Z L analyzed data; and S L and Z L wrote the article.

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Received in final form 20 April 2014
Accepted 13 May 2014
Accepted Preprint published online 14 May 2014