Low-level phenolic estrogen pollutants impair islet morphology and β -cell function in isolated rat islets

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Abstract

Phenolic estrogen pollutants, a class of typical endocrine-disrupting chemicals, have attracted public attention due to their estrogenic activities of imitating steroid hormone 17 β -estradiol (E_2) effects. Exposure to these pollutants may disrupt insulin secretion and be a risk factor for type 2 diabetes. In this study, we investigated the direct effects of phenolic estrogen diethylstilbestrol (DES), octylphenol (OP), nonylphenol (NP), and bisphenol A (BPA) on rat pancreatic islets *in vitro*, whose estrogenic activities were DES > NP > OP > BPA. Isolated β -cells were exposed to E_2 , DES, OP, NP, or BPA (0, 0.1, 0.5, 2.5, 25, and 250 μ g/l) for 24 h. Parameters of insulin secretion, content, and morphology of β -cells were measured. In the glucose-stimulated insulin secretion test, E_2 and DES increased insulin secretion in a dose-dependent

manner in a 16.7 mM glucose condition. However, for BPA, NP, or OP with lower estrogenic activity, the relationship between the doses and insulin secretion was an inverted U-shape. Moreover, OP, NP, or BPA (25 μ g/l) impaired mitochondrial function in β -cells and induced remarkable swelling of mitochondria with loss of distinct cristae structure within the membrane, which was accompanied by disruption of mRNA expression of genes playing a key role in β -cell function (*Glut2* (*Slc2a2*), *Gck*, *Pdx1*, *Hnf1 α* , *Rab27a*, and *Snap25*), and mitochondrial function (*Ucp2* and *Ogdh*). Therefore, these phenolic estrogens can disrupt islet morphology and β -cell function, and mitochondrial dysfunction is suggested to play an important role in the impairment of β -cell function.

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Introduction

Type 2 diabetes, characterized by high blood glucose in the context of insulin resistance and defective insulin secretion, is rising dramatically all over the world. In addition to genetic effects and a high-fat diet, increased exposure to environmental pollutants has been closely linked to the increasing incidence of diabetes mellitus, supported by several laboratory and animal researches as well as epidemiological studies (Carpenter 2008, Hectors *et al.* 2011).

Endocrine-disrupting chemicals (EDCs) are a class of typical environmental pollutants that were widely dispersed in the environment and to which people are exposed virtually in daily life (Diamanti-Kandarakis *et al.* 2009, Casals-Casas & Desvergne 2011). In recent decades, a variety of EDCs have been found to interfere with glucose metabolism (Cranmer *et al.* 2000, Alonso-Magdalena *et al.* 2010, Hectors *et al.* 2011, Lin *et al.* 2011). Physiological levels of estrogen are involved in maintaining normal insulin sensitivity, but an excess of estrogen, such as exposure to chemicals with estrogenic activity at an inappropriate concentration, would increase the risk of developing insulin resistance and diabetes

(Zhang *et al.* 2002, Margolis *et al.* 2004, Godsland 2005). One of the most striking examples is that adult male mice injected with a daily dose of 100 μ g/kg per day bisphenol A (BPA) or 17 β -estradiol (E_2) for 4 days showed higher insulin secretion, thereby resulting in insulin resistance and post-prandial hyperinsulinemia (Alonso-Magdalena *et al.* 2006). Our previous studies also have demonstrated that perinatal exposure to 50 μ g/kg per day BPA led to severe glucose and insulin intolerance in adult rat offspring along with progressive damage of β -cells (Wei *et al.* 2011).

Pancreatic β -cells are programmed to produce and secrete insulin, so the factors involved in initiation, acceleration, and exacerbation of β -cell dysfunction would disrupt glucose homeostasis and contribute to the development of diabetes. More importantly, recent studies in rodents suggest that endocrine pancreas is the target of some phenolic estrogens (Alonso-Magdalena *et al.* 2011). For example, long-term exposure to BPA or E_2 (between 100 and 1 nM) has been reported to disrupt pancreatic insulin gene expression, insulin content, and secretion in isolated mice islets (Adachi *et al.* 2005, Alonso-Magdalena *et al.* 2008). The stimulation of insulin release under both hypoglycemic and hyperglycemic

conditions was also observed in beta TC-6 cells treated with 100 µg/l BPA (Makaji *et al.* 2011). In addition to BPA and E₂, long-term exposure to nonylphenol (NP) has been confirmed to increase insulin secretion via cytosolic/nuclear estrogen receptors (Adachi *et al.* 2005).

Human exposure to EDC pollutants is ubiquitous. Among the EDCs, alkyl phenols (for example, NP and octylphenol (OP)), phenolic estrogen mimics (such as BPA), and phenolic estrogens (such as diethylstilbestrol (DES)) are often studied as a group of environmental phenolic estrogens due to their structural similarity and estrogenic activity (Tapiero *et al.* 2002, Lin *et al.* 2008). These phenolic estrogens can act by mimicking the action of the sex hormone E₂ to bind to the classical ERs, ER α and ER β (Newbold 2004), and ERs are involved in important aspects of β -cell physiology, including the regulation of insulin biosynthesis and release (Wozniak *et al.* 2005, Alonso-Magdalena *et al.* 2008). However, little is known about whether the potencies of phenolic estrogen binding to ERs are correlated with their effects on insulin secretion in isolated rat islets.

In this study, we chose E₂ and four phenolic estrogen pollutants (DES, OP, NP, and BPA), whose potencies of binding to ERs were E₂ > DES > NP > OP > BPA (Nakada *et al.* 2004), to investigate their effects on β -cell function. To further understand the disruptive effects of different phenolic estrogen pollutants on the isolated islets, morphology and ultrastructure of β -cells were observed. The mRNA expression levels of genes playing a key role in β -cell function and mitochondrial function were also studied.

Materials and Methods

Animals

Male Sprague Dawley (SD) rats were obtained from the Center for Animal of Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China), and housed under specific pathogen-free conditions. Rats were kept under controlled temperature (22 ± 2 °C) and light conditions (12 h light:12 h darkness cycle) with *ad libitum* access to a normal diet and water. Glass water bottles and polypropylene cages were used in the study. All animal experiments were approved by the Ethics Committee of Tongji Medical College, in accordance with the guidelines for the care and use of animals established by Tongji Medical College, Huazhong University of Science and Technology.

Cell culture and treatments

Islets were isolated from pancreas of rats by collagenase V digestion and Ficoll 400 step density gradient separation as described (Cherif *et al.* 1998, Carter *et al.* 2009, Li *et al.* 2009). Briefly, collagenase V (Sigma–Aldrich) at 1.0 mg/ml was injected into the common bile duct of rats. The whole pancreas was then excised and digested in Hanks buffer at 37 °C for

12 min. After digestion, pancreatic tissue was rinsed using pre-cooled Hanks buffer several times and the undigested fragments were carefully removed. Isolated cell pellets were resuspended in Ficoll 400 (GE Healthcare, Bucks, UK) and purified from acinar and ductal tissue by density gradient. Purified islets appeared as round or oval structures. A calibrated grid in the eyepiece was used to measure the diameter of the islets. Islets with 150 µm diameter were defined as an islet equivalent quantity (IEQ). Thirty IEQs were handpicked and cultured at 37 °C with 5% CO₂ in RPMI 1640 complete medium with 20% fetal bovine serum (FBS) and 11.1 mM glucose for 24 h before experiments. When indicated, isolated islets were treated with E₂, BPA, NP, OP, DES, or vehicle (DMSO, 0.1% v/v) for 24 h. Culture media and FBS were purchased from Invitrogen. E₂, DES, BPA, NP, and OP were obtained from Sigma.

3-(4,5-Dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay

At the end of the treatments with phenolic estrogens, the medium of cell pellets was aspirated and 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was added. After 4 h, medium was removed and 150 µl DMSO was added to dissolve the purple formazan. Absorbance was determined at 570 nm with a microplate reader. Results were expressed as percent (%) cytotoxicity of the control.

Insulin secretion and content measurement

To measure the amount of insulin secretion in isolated islets after exposure to phenolic estrogens, glucose-stimulated insulin secretion (GSIS) was performed as described (Cherif *et al.* 1998). Briefly, islets were incubated with phenolic estrogens or vehicle for 24 h. Later, the media were switched to Krebs–Ringer/bicarbonate–HEPES (KRBH) buffer containing 0.5% BSA/3.0 mM glucose and incubated for 30 min. Next, media were removed and replaced with KRBH buffer containing 3.0 and 16.7 mM glucose for an additional 60 min. Supernatants were collected and insulin was measured by commercially available RIA Kits (Linco Research, Millipore, Billerica, MA, USA).

Cellular insulin content was determined by acid–ethanol extracts (Nguyen *et al.* 2012). After islets were exposed to E₂ or phenolic estrogen for 24 h, the islets were lysed in 200 µl 70% ethanol plus HCl (1.5% vol/vol) and then sonicated for 15 s. Extraction was allowed to proceed overnight at 4 °C followed by centrifugation at 12 000 g, and the supernatant was used to determine insulin content.

Transmission electron microscopy

Islets treated with E₂ or phenolic estrogens were fixed in 2.5% glutaraldehyde for 2 h at 4 °C, then were postfixed in 1% OsO₄, dehydrated in a graded series of ethanol, and embedded in Epon. Ultrathin sections were stained with

Table 1 Real-time PCR primer list for determination of gene expression

Gene	Primers	Gene ID
<i>Glut2</i>	Forward: AGCACATACGACACCAGACGC Reverse: AAGAGGGCTCCAGTCAACG	NM_012879.2
<i>Gck</i>	Forward: TGTGCTCCGACGACTAGCT Reverse: GGTGATTCGACAGTTGGGTGT	NM_012565.1
<i>Pdx1</i>	Forward: GTGCCAGAGTTCAGTGCTAATCC Reverse: GGACTTCCCTGTTCCAGCGT	NM_022852.3
<i>Hnf1α</i>	Forward: GGTGCCACAGGGCTTGACT Reverse: GCCGGTGTCTACTGCTGGC	NM_012669.1
<i>Rab27a</i>	Forward: GAACGTAAACAGATGGCGGC Reverse: GTGGGCTGAACCACGGATAA	NM_017317.2
<i>Snai2</i>	Forward: CTGGCATCAGGACTTTGGTT Reverse: ATTATTGCCCCAGGCTTTTT	NM_030991.3
<i>Ucp2</i>	Forward: TGTGGTAAAGGTCCGCTTCC Reverse: TTCGGGCAACATTGGGAG	NM_019354.2
<i>Ogdh</i>	Forward: GTGTCGGCGCCTCACTCGG Reverse: CAGTCTGGGAGGCTGTCAATGGC	NM_001017461.1

uranyl acetate and lead citrate and were examined with FEI Tecnai 12G² transmission electron microscope (TEM; FEI, Eindhoven, The Netherlands). The number of insulin granules, the area, and density of mitochondria in β -cells were manually quantified by analyzing 13 500 $\times$ magnification micrographs. Quantification was performed on ten sections from eight islets, which was analyzed by Image Pro Plus Version 6.0 Software (Media Cybernetics, Silver Spring, MD, USA).

Mitochondrial function assay

Mitochondrial function was assessed by detecting Complex IV (cytochrome *c* oxidase (COX)) activity and ATP content. At the end of treatment, cells were trypsinized, rinsed with PBS, and resuspended in 10 mM KH₂PO₄/K₂HPO₄ buffer (pH 7.4) containing 20 mM succinate. Then, cells were lysed in Triton X-100 and reduced cytochrome *c* (20 μ M) was added to lysates. Complex IV activity was measured by the rate and extent of enzymatic cytochrome *c* oxidation at 550 nm. Assays were performed using u.v. spectrophotometry.

Cells were precultured and preincubated as described earlier. Cells were lysed and ATP contents were measured with an ATP bioluminescence assay kit (Roche) according to the manufacturer's instructions. Measurement of the protein concentration in cell lysates was performed using the Bradford assay. The ATP content was calculated as nanomoles ATP per microgram protein.

Real-time PCR analysis

After 24-h culture with phenolic estrogens, total RNA was extracted from isolated rat islets by TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Total RNA was reverse transcribed using Fermentas Revert Aid First-Strand cDNA Synthesis Kit (ON, Burlington, Canada). Real-time PCR was performed using an Applied Biosystems model 7900HT Fast Real-Time PCR System. Specific mRNA

content was normalized to 36B4 mRNA and was presented as the fold increase or decrease compared with control. Corresponding primer sequences used in the study are listed in Table 1.

Statistical analysis

Results were expressed as mean $\pm$ S.E.M. Statistical analysis was performed using SPSS 13.0 (SPSS, Inc., Chicago, IL, USA). Differences among groups were determined using one-way ANOVA followed by Dunnett's *post hoc* test, as indicated. Values with $P < 0.05$ were considered statistically significant.

Results

Cytotoxic effects of phenolic estrogens on isolated islets

MTT assay was performed to examine the cytotoxic effect of phenolic estrogens on isolated islets from rat. After isolated islets were treated with E₂, DES, NP, or OP for 24 h, cell viability was significantly decreased at doses 25 and 250 μ g/l (Fig. 1A, B, C and D). However, BPA significantly attenuated cell viability at a dose of 2.5 μ g/l (Fig. 1E).

In addition, the diameter of islets (an indicator of islet mass) was measured. As shown in Fig. 2, compared with control, the diameter of islets was significantly increased in a concentration-dependent manner after exposure to E₂ or DES between 2.5 and 250 μ g/l for 24 h. Similarly, the diameter of islets treated with 2.5 and 25 μ g/l NP, OP, or BPA was increased. However, 250 μ g/l BPA significantly decrease the diameter of islets.

Effects of phenolic estrogens on insulin secretion and content in isolated islets

To evaluate the effect of phenolic estrogens on β -cell function, GSIS was detected with a basal (3.0 mM) or

Figure 1 Effect of phenolic estrogen pollutant administration on isolated islet viability. Islet cell viability was analyzed 1 day after islet isolation in groups of hand-picked islets by MTT. (A) E₂, (B) DES, (C) OP, (D) NP, and (E) BPA. Results are expressed as mean $\pm$ S.E.M. ($n=5$). * $P<0.05$ compared with control, ** $P<0.01$ compared with control.

a stimulatory (16.7 mM) concentration of glucose for 1 h (Fig. 3). In Fig. 3A, after 24-h phenolic estrogen treatment, basal insulin secretion at 3.0 mM glucose was increased by E₂ (25 and 250 µg/l), DES (250 µg/l), OP (25 and 250 µg/l), NP (25 and 250 µg/l), or BPA (2.5, 25, and 250 µg/l) compared with control. When isolated islets were incubated with 16.7 mM glucose medium to mimic the increased glucose conditions in diabetes (Fig. 3B), increased insulin secretion was exhibited dose dependently after exposure to E₂ and DES. However, insulin secretion showed an inverted U-shaped dose-response curve to different concentrations of BPA, NP, and OP. Particularly, insulin secretion was significantly increased by BPA treatment at a dose as low as 0.1 µg/l, while it was significantly decreased at 25 and 250 µg/l.

To investigate whether insulin secretion was changed by phenolic estrogen because of changed insulin stores, insulin content was determined after isolated islets were cultured in the presence of 11 mM glucose and increasing concentrations of E₂ or phenolic estrogens. As shown in Fig. 3C, E₂ and phenolic estrogen increased pancreatic insulin content in a non-monotonic trend. Insulin content was significantly increased in β -cells treated with E₂ (0.5–250 µg/l), DES (2.5 and 25 µg/l), OP (2.5 µg/l), NP (2.5 µg/l), or BPA (0.5 and 2.5 µg/l). However, 250 µg/l BPA, NP, or OP treatment significantly decreased insulin content.

Effects of phenolic estrogens on β -cell morphology and mitochondrial function in isolated islets

Effects of phenolic estrogens' exposure on ultrastructural alterations on β -cells were observed using a TEM. As shown in Fig. 4A, B, C, D, E, F and G, all the tested estrogen treatments (25 µg/l) reduced the proportion of filled insulin granule in β -cells compared with control. Additionally, E₂ or DES exposure significantly increased the proportion of immature granules. Although no difference was displayed in the proportion of immature granules in islets after treated with OP, NP, or BPA, a significant increase in the proportion of empty granules compared with control was observed.

Ultrastructure abnormalities in mitochondrial morphology were also observed in β -cells treated with 25 µg/l E₂ or phenolic estrogens. For E₂- or DES-treated β -cells, mitochondria were mildly enlarged but maintained almost normal cristae structure (Fig. 4B and C). Further quantitative analysis revealed that there was no significant difference in density of mitochondria among control, E₂-, and DES-treated β -cells (Fig. 4H and I). The mitochondria in β -cells treated with NP, OP, or BPA were remarkably swollen and most of the mitochondria in β -cells exhibited loss of structural integrity with abnormal cristae relative to control (Fig. 4D, E and F). The average area and optical density of the mitochondria

Figure 2 Comparison between mean morphometric data measured from control and treated isolated islets. Results are expressed as mean $\pm$ S.E.M. ($n=5$). * $P<0.05$ compared with control, ** $P<0.01$ compared with control.

Figure 3 Effect of phenolic estrogen on insulin secretion and content *in vitro*. (A) Glucose-induced insulin secretion from isolated islets treated with E₂, DES, OP, NP, or BPA (0, 0.1, 0.5, 2.5, 25, or 250 µg/l) for 24 h at basal 3.0 mM glucose. (B) Glucose-induced insulin secretion from isolated islets treated with E₂, DES, OP, NP, or BPA (0, 0.1, 0.5, 2.5, 25, or 250 µg/l) for 24 h with 16.7 mM glucose stimulation. (C) Insulin content of islets exposed to increasing doses of E₂, DES, OP, NP, or BPA (0, 0.1, 0.5, 2.5, 25, or 250 µg/l) for 24 h. Results are expressed as mean $\pm$ S.E.M. ($n=5$). * $P<0.05$ compared with control, ** $P<0.01$ compared with control.

in NP-, OP-, or BPA-treated β -cells were significantly higher than those of control (Fig. 4H and I).

Pancreatic complex IV mitochondrial respiratory chain enzyme (COX) activity, an indicator of mitochondrial function, was significantly decreased in 25 µg/l NP-, OP-, and BPA-treated islets relative to control (Fig. 4J), but that did not differ among control, E₂, and DES groups. In addition, NP-, OP-, or BPA-treated islets had lower cytosolic ATP level than control (Fig. 4K).

Effects of phenolic estrogens on mRNA levels of genes involved in β -cell function

To investigate the possible mechanisms leading to defects in β -cells, mRNA expression levels of genes playing a key role

in β -cell function were measured by quantitative real-time PCR. As shown in Fig. 5, exposure to 25 µg/l E₂ or phenolic estrogens for 24 h significantly decreased mRNA expression of glucose transporter 2 (*Glut2* (*Slc2a2*)), glycolytic enzyme glucokinase (*Gck*), insulin promoter factor 1 (*Pdx1*), and hepatocyte nuclear factor 1 α (*Hnf1 α*). A significant increase in mRNA levels of *Rab27a* was observed in E₂- or DES-treated islets, and the mRNA levels of synaptosome-associated protein of 25 kDa (*Snap25*) was downregulated by BPA, NP, or OP treatment.

An increase in the mRNA expression of uncoupling protein 2 (*Ucp2*), a gene that participates in mitochondrial biogenesis, was found by 25 µg/l BPA, NP, or OP treatment. The mRNA expression of 2-oxoglutarate dehydrogenase (*Ogdh*), another gene associated with mitochondrial function, was reduced in BPA-, NP-, or OP-treated islets. But the mRNA levels of both *Ucp2* and *Ogdh* in isolated islets were unchanged after 25 µg/l E₂ or DES treatment for 24 h.

Discussion

In this study, we have demonstrated that the phenolic estrogens impaired islet morphology and disrupted insulin secretion in isolated rat pancreatic islets, and OP, NP, or BPA (25 µg/l) induced remarkable swelling of mitochondria in β -cells, which was accompanied by disruption of mRNA expression of genes playing a key role in β -cell function and mitochondrial function.

To examine the cytotoxic effects of E₂ and the phenolic estrogens on β -cells, 0.1, 0.5, and 2.5 µg/l were chosen as lower doses to mimic the real levels of human exposure to environmental estrogen (Vandenberg *et al.* 2010, Asimakopoulos *et al.* 2011). From the MTT results, the viability of β -cells was decreased with increasing chemical concentration. E₂, DES, OP, and NP decreased β -cell viability significantly at doses of 25 and 250 µg/l. However, BPA significantly attenuated cell viability from the dose of 2.5 µg/l, which is similar to a previous study demonstrating that 10 µg/l BPA exposure can affect the viability of β -cells (Adachi *et al.* 2005).

Estrogen administration in rodents can induce islet hypertrophy, which may be crucial in the development of diabetes (Rosmalen *et al.* 2001). In this study, the diameter of islets was increased in a concentration-dependent manner after exposure to E₂ or the phenolic estrogen chemicals. While it was decreased by 250 µg/l OP, NP, or BPA treatment, which may be explained by remarkably cytotoxic effects on β -cells at such high dose.

Next, we examined insulin secretion and content in β -cells following exposure to E₂ or phenolic estrogens. At 16.7 mM glucose, E₂ and the phenolic estrogens were demonstrated to result in a greater change of insulin response than that at the basal glucose concentration. These data are consistent with a previous report that exposure to BPA or NP for 24 h significantly changes insulin secretion in isolated rat islets with 16.7 mM glucose stimulation (Adachi *et al.* 2005).

Figure 4 Morphological analysis of β -cells in isolated islets. Representative electron microscopy photomicrographs of control (A), E₂-exposed β -cells (B), DES-exposed β -cells (C), OP-exposed β -cells (D), NP-exposed β -cells (E), and BPA-exposed β -cells (F) at a dose of 25 $\mu\text{g/l}$ illustrated the typical insulin granules and mitochondrial pattern. N, nucleus; Gr, secrete granulation. Swollen mitochondria are indicated by black boxes. Black arrows show immature secretory granules and black arrowheads show empty granules. Photographs were taken at 13 500 $\times$ magnification. Mitochondria were quantified within the β -cells and presented as the average mitochondrial area (H) and the average mitochondrial optical density (I). Higher optical density values (a measure of brightness) was an indication of organelle swelling. (G) Manual quantifications of the percentage of total number of granules (filled, immature, and empty). (J) Complex IV enzyme activity (an indicator of mitochondrial electron transport chain function). (K) ATP content. Results are expressed as mean $\pm$ S.E.M. * $P < 0.05$ compared with control, ** $P < 0.01$ compared with control.

For BPA, NP, and OP with lower estrogen activity, the relationship between the doses and insulin secretion was an inverted U-shape, namely increased at first and then declined. E₂ and phenolic estrogen below 2.5 $\mu\text{g/l}$ increased insulin secretion along with increased insulin content in the experiments. These results are consistent with the concept that increased insulin secretion in response to stimulatory glucose in phenolic-treated islets may be a consequence of their higher insulin content (Alonso-Magdalena *et al.* 2008). But the disruption of insulin secretion by phenolic estrogen can be partly explained by the change of insulin content, as insulin content presented a tendency of decrease after the islets were treated with 25 $\mu\text{g/l}$ BPA, NP, or OP. In the past decades, EDCs have been recognized to exhibit a lack of linear dose-dependent relationship with endocrine abnormalities, showing instead inverted U-shaped curves

(Alonso-Magdalena *et al.* 2011). In a recent report from Wei *et al.* (2011), perinatal exposure to BPA (50 $\mu\text{g/kg}$ per day) induces hyperinsulinemia and impaired glucose regulation in SD rat offspring. Interestingly, the effects are not observed at higher doses. These reports and our results in this study propose a non-monotonic dose response that low dose exposure of some EDCs might be more effective on altering β -cell function. But there is no ready explanation for this phenomenon, suggesting the existence of two independent mechanisms for low doses and high doses (vom Saal *et al.* 2007).

Note that BPA, with the lowest estrogenic activity in the four phenolic estrogens, was able to significantly increase insulin release at a dose as low as 0.1 $\mu\text{g/l}$ with 16.7 mM glucose stimulation, which means that BPA would affect insulin secretion within the environmental concentration range. These data suggest that BPA has more effect on

Figure 5 Comparison of the mRNA levels of islet-associated transcription factor and β -cell function in isolated islets after exposed to E₂, DES, OP, NP, or BPA at 25 μ g/l. Results are expressed as mean $\pm$ S.E.M. determined of five replicates. The mRNA levels were compared by real-time PCR. Each gene was normalized to the 36B4 internal control and gene expression values were normalized to control islet values. * $P < 0.05$ compared with control, ** $P < 0.01$ compared with control.

disrupting insulin secretion and content in isolated β -cells than those with higher estrogenic activity, which also indicates that the ability of the phenolic estrogens to disrupt insulin secretion is not positively correlated with their estrogenic activity.

E₂ or phenolic estrogen treatment induced downregulation of key genes important for β -cell function in β -cells, such as *Glut2*, *Gck*, *Pdx1*, and *Hnf1α*. Glucose is the predominant regulator of insulin secretion and biosynthesis. In pancreatic β -cells, glucose enters the cell through GLUT2 transporter phosphorylated by GCK, and then glucose is metabolized to generate ATP, which promotes insulin secretion. Therefore, the reduction in mRNA levels of *Gck* and *Glut2* induced by E₂ or phenolic estrogen treatment partly contributes to the impaired insulin secretion to glucose in isolated islets, and the mRNA levels of *Glut2* and *Gck* might be downregulated by the decreased expression levels of *Pdx1* and *Hnf1α* in phenolic estrogen-treated islets. *Pdx1* is considered as a β -cell master regulator gene, which plays a critical role in the maintenance of mature β -cell function (Chakrabarti & Mirmira 2003). Downregulation of *Pdx1* would inhibit *Glut2* transcription and *Gck* promoter activity in β -cells (Waeber *et al.* 1996, Lee *et al.* 2009), finally leading to β -cell dysfunction. *Hnf1α* is also required for *Glut2* transcription; the expression pattern of *Glut2* is quite similar to that of *Hnf1α* (Boj *et al.* 2001, Shih *et al.* 2001). Also, *Pdx1* and *Hnf1α* regulate expression of the insulin gene and other components of the GSIS pathway (Shih *et al.* 2001, Chakrabarti & Mirmira 2003), so *Pdx1* and

Hnf1α deficiency would lead to reduced insulin content and impaired insulin secretion. Moreover, *Rab27a* and *Snap25* are necessary plasma membrane factors that mediate glucose signals for the exocytosis of insulin granules in pancreatic β -cells (Takahashi *et al.* 2004, Kasai *et al.* 2005). The change in mRNA levels of *Rab27a* and *Snap25* induced by phenolic estrogen treatment are partly responsible for the change of secretory granules in β -cells and the impaired insulin release. But how the phenolic estrogens affect the process of insulin secretory granules is unclear and remains to be demonstrated.

Mitochondria are critical for the regulation of β -cell mass and maintenance of β -cell function through the coupling of glucose stimulus to insulin release (Lowell & Shulman 2005, MacDonald *et al.* 2005, Maechler & de Andrade 2006). Mitochondrial dysfunction has been implicated as a key factor in the development of type 2 diabetes (Lu *et al.* 2010). In our study, the ultrastructure of β -cells was observed with abnormal alteration after exposure to E₂ or phenolic estrogens (25 μ g/l), characterized by swollen mitochondria and a modest deregulation in β -cells. Particularly, administration of OP, NP, or BPA induced remarkable swelling of mitochondria with loss of distinct cristae structure within the membrane, offering an explanation for the blunted insulin secretion stimulated by 16.7 mM glucose in BPA, as well as NP- and OP-treated islets. So mitochondrial swelling is suggested to be a key initiating event in the mitochondrial mediated defects in GSIS.

In addition, BPA, NP, and OP significantly decreased COX activity in isolated rat islets, indicating that mitochondrial respiratory enzyme function had been damaged. As the enzymes in the electron transport chain are located in the inner membrane of the mitochondria, they would be affected by impaired mitochondrial structure (Bruin *et al.* 2010). COX is the last enzyme in the respiratory electron transport chain of mitochondria, helping to establish a transmembrane difference of proton electrochemical potential to synthesize ATP, so decreased COX activity may cause defects in glucose-stimulated ATP production. Consistent with COX activity reduction, cellular ATP content was reduced in islets treated with 25 µg/l NP, OP, or BPA relative to control. Thus, estrogen pollutants differentially affect mitochondrial function, resulting in defective insulin secretion.

To further study whether phenolic estrogen treatment impairs the mRNA expression of genes that are critical for mitochondrial function, we measured mRNA levels of typical mitochondrial genes, including *Ogdh* and *Ucp2*. Data from this study demonstrated that BPA, NP, or OP exposure (25 µg/l) resulted in reduced mRNA expression of *Ogdh* and increased mRNA expression of *Ucp2* in isolated islets. *Ogdh* constitutes the rate-limiting enzyme in the mitochondria tricarboxylic acid cycle, so the reduction in *Ogdh* mRNA expression may be partially responsible for defective mitochondrial function and consequently impaired insulin secretory responses to glucose induced by BPA, NP, and OP treatment. UCP2 has been shown to promote proton leakage across the mitochondrial membrane (Rial *et al.* 1999) and play a critical role in insulin resistance, glucose utilization, and the regulation of reactive oxygen species (Arsenijevic *et al.* 2000). Overexpression of UCP2 could reduce the efficiency of mitochondrial ATP generation and inhibit GSIS in cultured β-cell lines (Chan *et al.* 1999, 2001). So we suggest that increased mRNA expression of *Ucp2* in islets treated with BPA, NP, or OP induces mitochondrial dysfunction and thereby limits β-cell function in response to hyperglycemia.

But interestingly, E₂ and DES (25 µg/l) treatment of islets *in vitro* did not significantly affect mitochondrial structure and function of β-cells in this study. The mRNA levels of *Ogdh* and *Ucp2* were also comparable among E₂-, DES-, and control-treated islets. DES, with higher estrogen activity, impairs islet morphology and β-cell function in a similar way as E₂. As a result, we propose E₂ and DES impair β-cell function through different mechanisms, and the detailed mechanisms remain to be elucidated in further studies.

In conclusion, data in this study show that low-level exposure to DES, OP, NP, or BPA impairs islet morphology and β-cell function in isolated rat islets. BPA with the lowest estrogenic activity among the four phenolic estrogens can disrupt insulin secretion in isolated β-cells at a dose as low as 0.1 µg/l, and β-cell dysfunction induced by OP, NP, or BPA is correlated with mitochondrial dysfunction. Mitochondrial swelling induced by OP, NP, or BPA is suggested to be a key initiating event for defects in GSIS.

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.

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