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Exposure to bisphenol A induces dysfunction of insulin secretion and apoptosis through the damage of mitochondria in rat insulinoma (INS-1) cells

Y Lin[1,3], X Sun[1,3], L Qiu[1], J Wei[2], Q Huang[1], C Fang[1], T Ye[1], M Kang[1], H Shen[1] and S Dong*,[1]

Bisphenol A (BPA) is widely used in plastic products, through which humans are exposed to it. Accumulating evidence suggests that BPA exposure is associated with β-cell dysfunction. Mitochondrial defects can cause impairment and failure of β cells, but there is little information about the effects of BPA on the mitochondrial function of β cells. In this study, we assessed the role of mitochondria-mediated mechanisms underlying BPA-induced β-cell dysfunction and resulting β-cell apoptosis. INS-1 cells were cultured with 0, 0.0020, 0.020, 0.20, or 2.0 μM BPA. Cell viability, glucose-stimulated insulin secretion (GSIS), and mitochondrial function were examined. The mitochondrial apoptotic pathway was also analyzed at molecular level. We found that BPA suppressed cell viability and disturbed GSIS in a dose-dependent manner. Positive Annexin- propidium iodide (PI) staining and altered expression of Bcl-2 family members and caspases in INS-1 cells indicated that the cells progressively became apoptotic after BPA exposure. Additionally, BPA-induced apoptosis was associated with mitochondrial defects in β cells, as evidenced by depletion of ATP, release of cytochrome c, loss of mitochondrial mass and membrane potential, and alterations in expression of genes involved in mitochondrial function and metabolism. Taken together, these findings provide strong evidence that BPA triggers INS-1 cells dysfunction and apoptosis may be meditated via the mitochondrial pathway.

Bisphenol A (BPA) is an environmental endocrine disruptor extensively used in the manufacture of food and beverage containers, cigarette filters, thermal (carbonless) receipts, and paper currency.[1] The human population is continuously and inevitably exposed to BPA from multiple sources, and 92.6% of the population aged six and older in the United States have detectable levels of BPA in their urine and blood.[2,3] Recently, the potential effects of BPA exposure on the risk of type 2 diabetes and insulin resistance have been of great interest.[4,5] Epidemiological studies using data from the 2003–2004 National Health and Nutrition Examination Survey (NHANES) first reported a positive association between increased levels of urinary BPA and diabetes diagnosis.[4] However, follow-up analysis of the NHANES 2005–2008 report showed an inconsistent association between the prevalence of type 2 diabetes and the concentration of BPA in urine.[6,7] Interestingly, when all three NHANES studies were combined and analyzed together, urinary BPA levels were significantly associated with diabetes mellitus independent of traditional risk factors.[7,8] In China, a community-based investigation conducted between 2008 and 2009 indicated an association between the highest urinary levels of BPA and diabetes but no clear association between diabetes and the presence of BPA in the urine at lower levels.[9] Therefore, more longitudinal and long-term prospective studies are urgently needed to further investigate whether there is a true association between BPA exposure and type 2 diabetes.

Although the role of BPA as a risk factor for diabetes mellitus remains controversial, accumulating studies in animals and in cellular models demonstrate the physiological plausibility of BPA's adverse effect on β-cell function and the whole body glucose homeostasis. As just one example, administration of 100 μg/kg/day BPA for 8 days in adult mice led to insulin resistance.[10] Moreover, significantly increased glucose-stimulated insulin secretion (GSIS) and insulin content were detected in pancreatic β cells of male mice exposed to 100 μg/kg/day BPA for 4 days, implying that BPA directly affected β-cell function in addition to affecting peripheral tissue. Indeed, several published studies confirm that pancreatic β cells are a target of BPA. For instance, glucose-induced Ca^{2+} signals were potentiated, and the transcription factor CREB was activated in freshly isolated islets treated with a low dose of BPA.[11,12] Increased insulin content and release, as well as changes in Insulin gene transcription, had also been demonstrated in an ERα-dependent manner in BPA-treated isolated islets.[13] In a more recent study, this group further highlighted the impact of BPA on β cells, indicating that BPA induced a rapid reduction in K_{ATP} channel activity and induced insulinotropic effects in mice and human islets through nuclear ERβ.[14] All of these

data confirmed that BPA could regulate insulin biosynthesis and secretion in β cells via nuclear ER, but other molecular and cellular pathways involved in BPA-induced β-cell toxicity must be further elucidated.

One of the key organelles in pancreatic β cells is the mitochondrion, which plays a central role in the generation of ATP and other factors linking glucose metabolism to insulin secretion.[15–17] In a previous study, mitochondrial damage was observed in β cells at weaning in Wistar rat offspring exposed perinatally to 50 μg/kg/day of BPA. Furthermore, the early mitochondrial defects observed in BPA-exposed offspring worsened with age, causing progressive loss of pancreatic islet function, development of insulin resistance, and pre-diabetes later in life, even though BPA exposure was discontinued at weaning.[18] In the case of isolated rat islets, BPA exposure also induced remarkable swelling of the mitochondria with loss of distinct crista structure, and these changes were accompanied by decreases in ATP and the mitochondrial respiratory enzyme COX IV.[19] These findings suggest that BPA-induced mitochondrial defects may play a critical role in the pathology of glucometabolic disorders.

Considering that a large amount of cellular material from β cells was factored into biochemical determinations, the present study used a rat insulinoma (INS-1) cell model[20] to explore the hypothesis that exposure of β cells to BPA leads to mitochondrial dysfunction, subsequently attenuates insulin secretion, and ultimately triggers apoptosis.

Results

BPA decreases cell viability in INS-1 cells. INS-1 cells exposed to 0.0020 or 0.020 μM BPA for 12 h did not exhibit any significant change in cell viability, whereas at higher concentrations (0.20 or 2.0 μM), cell viability was significantly decreased to 85.1 or 55.1% of that observed in control cells, respectively (Figure 1a). When the incubation time was extended to 24 h, 0.020, 0.20, or 2.0 μM BPA significantly reduced the viability of INS-1 cells to 83.4, 69.3, or 47.1% of that observed in unstimulated control cells, respectively. A further decrease in cell viability was observed after 48 h of BPA incubation at all concentrations.

BPA impairs GSIS and alters genes expression of components of classic GSIS pathway in INS-1 cells. As shown in Figure 1b, insulin secretion in response to glucose in INS-1 cells differed between cells treated with low and high levels of BPA. Incubating cells for 48 h with 0.0020 μM BPA did not significantly affect basal insulin release (5.6 mM glucose); whereas the secretion response evoked by 16.7 mM glucose was significantly elevated in BPA-exposed cells. Compared with untreated control cells, cells exposed to 0.020 μM BPA displayed a 122% increase in basal insulin release but exhibited a slight inhibition of the secretory response to 16.7 mM glucose. In contrast to the increased GSIS, basal insulin secretion tended to be abrogated in cells exposed to 2.0 μM BPA. Cells exposed to either 0.20 or 2.0 μM BPA secreted markedly less insulin when cultured in 16.7 mM glucose than did untreated control cells. Consistent with GSIS, the amount of insulin mRNA was found to be

slightly increased in cells exposed to 0.0020 μM BPA, whereas it was significantly decreased in the cells exposed to 0.020, 0.20, or 2.0 μM BPA when compared with controls (Figure 1c). This effect was further confirmed at the protein level by western blot analysis. Treatment of cells with 0.020, 0.20, or 2.0 μM BPA induced progressive decreases in the insulin protein level (Figure 1c).

Next, real-time RT-PCR was used to evaluate the mRNA levels of genes involved in the classic GSIS pathway. Figure 1d and e show that the amounts of mRNA of the major glucose transporter *Glut2* and the metabolic enzyme *Gck* were not affected by 0.0020 or 0.020 μM BPA in INS-1 cells, but they were significantly reduced after exposure to 0.20 or 2.0 μM BPA. Treatment with 0.0020 μM BPA increased the mRNA levels of *Kir6.2* and *Sur*, but they were significantly decreased when the cells were incubated with 0.020, 0.20, or 2.0 μM BPA (Figure 1f and g).

BPA induces mitochondrial dysfunction in INS-1 cells. Because the mitochondrion is one of the most important organelles for β-cell function and survival, its morphology, mass, membrane potential (Δψm), and ATP generation were measured to evaluate the effects of BPA on mitochondrial function. As shown in Figure 2a, mitochondria were abundant and appeared interconnected in a network throughout the cytoplasm in untreated control cells. That network gradually became fragmented and less organized in cells exposed to BPA, as characterized by dispersed staining. As the signal from MitoTracker Green (MTG) also reflected changes in mitochondrial mass, its fluorescence density was measured. Figure 2b revealed that the average density of MTG in BPA-exposed cells was decreased in a dose-dependent manner compared with that measured in controls. Consistent with these findings, cellular ATP levels were reduced to 49.0, 40.3 or 17.3% of that detected in untreated control cells after incubation 0.020, 0.20, or 2.0 μM BPA, respectively. No difference in ATP production was observed between cells treated with 0.0020 μM BPA and unexposed control cells (Figure 2c). Further, the fluorescent mitochondrial probe JC-1 was used to measure mitochondrial Δψm, which is essential for ATP formation and is a marker of mitochondrial membrane integrity. Apoptotic or unhealthy cells with collapsed mitochondria contain mainly green JC-1 monomers rather than red JC-1 clusters. After cells were treated with 0.20 or 2.0 μM BPA, a shift of fluorescence from red to green was observed (Figure 2d), indicating that mitochondrial depolarization was induced by BPA (0.20 and 2.0 μM) in INS-1 cells.

The expression of genes involved in mitochondrial function and metabolism was also evaluated. Figure 3 showed that treatment of INS-1 cells with 0.20 or 2.0 μM BPA for 48 h decreased the expression of *Tfam*, a gene providing a molecular basis for the connection between environmental stimuli and mitochondrial biogenesis and respiration. No difference was detected in the expression levels of the mtDNA-encoded gene *Nd4l* between BPA-exposed and control cells (Figure 3). The expression of *Atp6*, a subunit of the mitochondrial pump ATP synthase, was significantly downregulated in cells exposed to 0.020, 0.20, or 2.0 μM BPA when compared with untreated control cells (Figure 3).
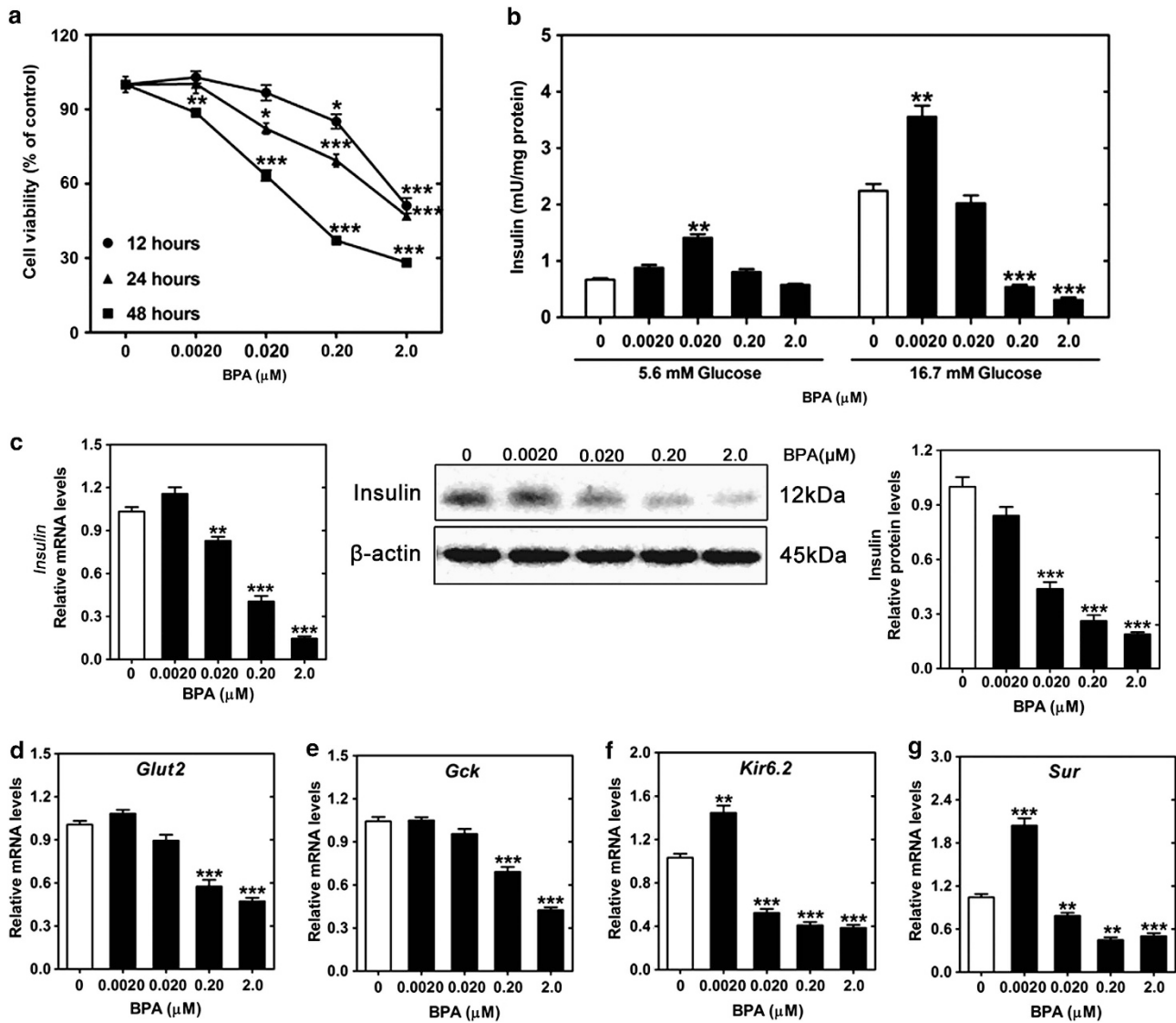
Figure 1 BPA decreases cell viability and disrupts GSIS. (a) Cell viability as determined by MTT assay. INS-1 cells were cultured in the presence or absence of BPA (0.0020–2.0 μM) for 12, 24, or 48 h. The data are expressed as percentages of untreated control cells. (b) Insulin secretion triggered by glucose after 48 h BPA exposure. The levels of secreted insulin were normalized to protein content. (c) Relative mRNA and protein expression of insulin in INS-1 cells exposed to BPA for 48 h. (d–g) Expression of genes involved in the GSIS pathway. The results are expressed as the mean ± S.E.M. of 3–6 replicates from three independent experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared with untreated control cells

Citrate synthase, one of the genes involved in the TCA cycle, was also dramatically suppressed in INS-1 cells after exposure to 0.020, 0.20, or 2.0 μM BPA (Figure 3). Additionally, the expression of *Ucp2* was increased, and the expression of *Ogdh* was suppressed in INS-1 cells in a dose-dependent manner after 48 h BPA treatment (Figure 3).

BPA at higher concentrations triggers apoptosis and the release of apoptogenic factors in INS-1 cells. To examine whether BPA caused cell apoptosis, INS-1 cells were stained with Annexin V-PI and analyzed by flow cytometry. Figure 4 showed that exposure to BPA for 48 h resulted in a dose-dependent increase in the proportion of early apoptotic and apoptotic cells compared with controls. Moreover, the mitochondrial and cytosolic fractions were prepared from

INS-1 cells to investigate whether the release of cytochrome *c* was involved in the apoptotic process induced by BPA. As shown in Figure 5, protein expression of cytochrome *c* was reduced with increasing BPA concentrations in isolated mitochondria. Likewise, cytochrome *c* was significantly elevated in the cytosolic fractions in INS-1 cells exposed to 0.020, 0.20, or 2.0 μM BPA for 48 h. BPA promoted the translocation of cytochrome *c* from the mitochondria into the cytosol in INS-1 cells.

BPA triggers apoptosis through the intrinsic mitochondrial pathway. To further determine whether the mitochondrial pathway mediated BPA-induced apoptosis, the expressions of Bcl-2 family members, Apaf-1, and caspases were evaluated. As shown in Figure 6a, BPA significantly
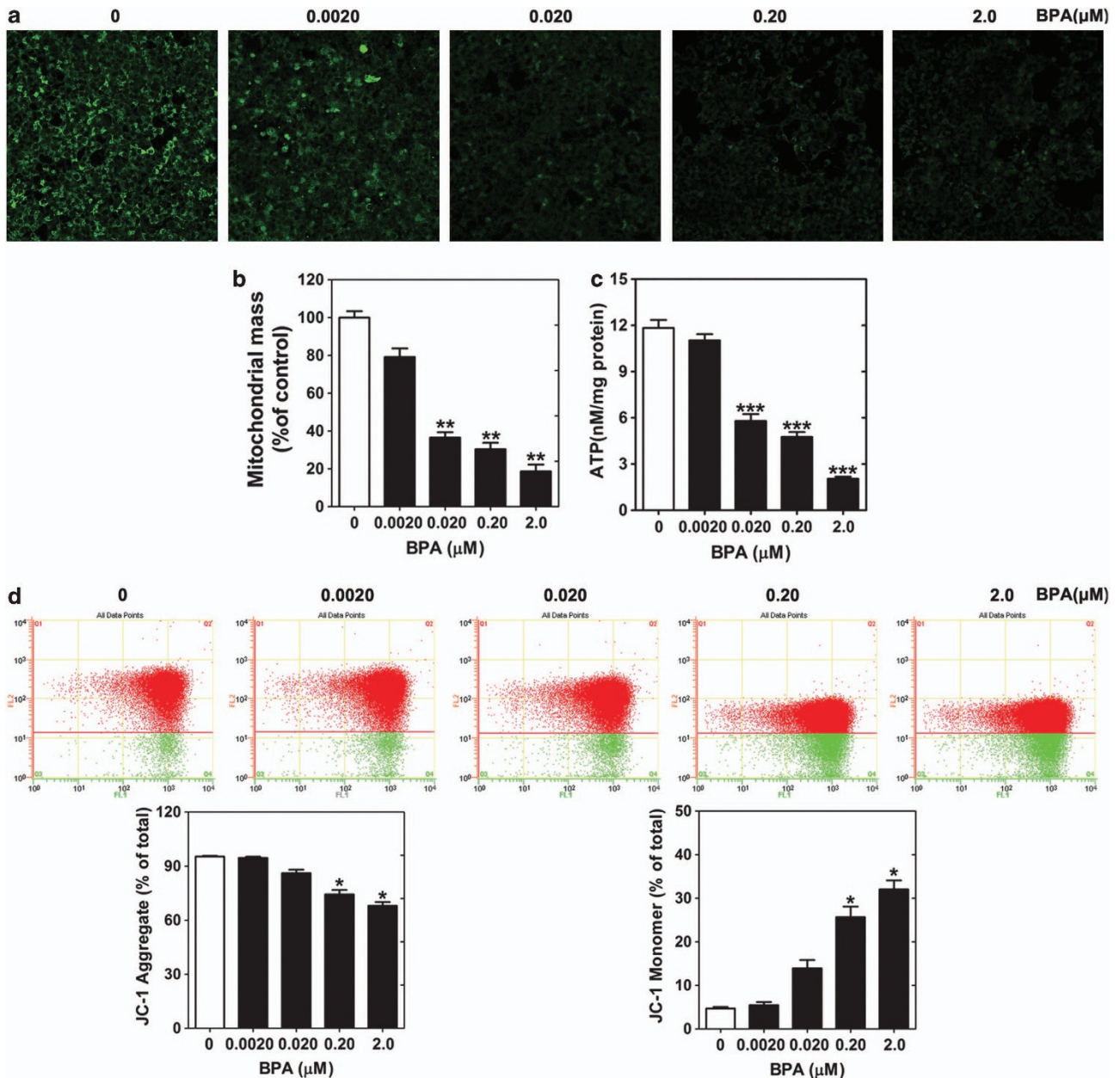
Figure 2 BPA triggers mitochondrial dysfunction. INS-1 cells were cultured in the presence or absence of BPA (0.0020–2.0 μM) for 48 h. (**a**) Representative images of mitochondria in cells stained with MitoTracker Green at 200 × magnification. (**b**) Quantitative results of (**a**). The data are expressed as a percentage of the MitoTracker Green staining in untreated control cells. (**c**) Intracellular ATP content normalized to protein content. (**d**) Mitochondrial $\Delta\psi$m measured by the JC-1 probe. The dot-plot representation of the flow cytometry analysis shows the distribution of JC-1 aggregates (cells emitting red fluorescence in the FLI-2 channel) and JC-1 monomers (cells emitting green JC-1 detected in the FLI-1 channel). The bar graphs show the percentage of JC-1 aggregate-positive and JC-1 monomer-positive cells. The results are expressed as the mean ± S.E.M. of 2–3 replicates from three independent experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared with untreated control cells

increased the expression of the pro-apoptotic protein Bax and moderately decreased the expression of the anti-apoptotic protein Bcl-2 in dose-dependent manners. Additionally, markedly increased levels of Apaf-1 and the 17-kDa cleaved form of caspase-9 were observed in cells treated with 0.020, 0.20, or 2.0 μM BPA. There was no change in the expression of the 40-kDa form of cleaved caspase-9 (Figure 6b). Both the 17-kDa and the 19-kDa form of cleaved

caspase-3 displayed marked dose-dependent increases in BPA-exposed cells compared with controls (Figure 6b).

Discussion

BPA is a ubiquitous environmental pollutant that is reported to be associated with an increased incidence of diabetes mellitus and increased damage to β-cell function. In this study, we
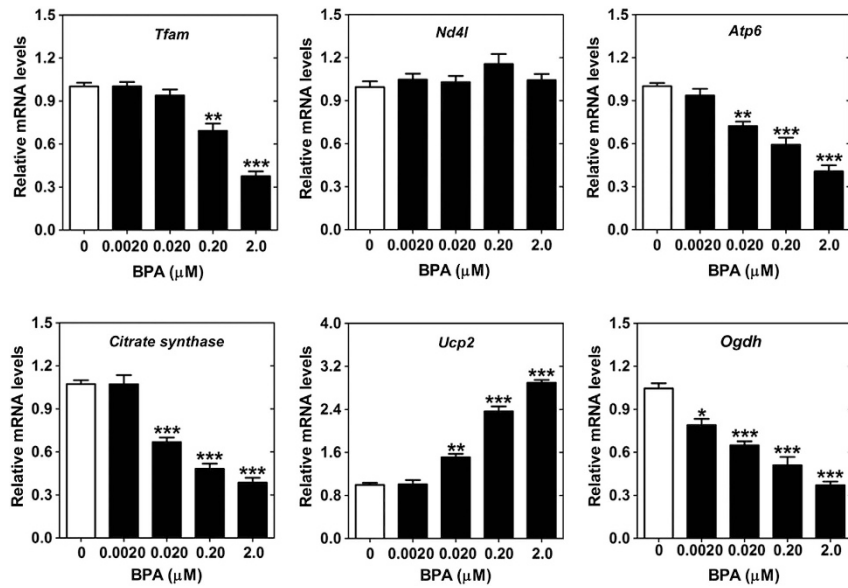
Figure 3 BPA alters the mRNA expression of genes involved in mitochondrial function and metabolism. INS-1 cells were cultured in the presence or absence of BPA (0.0020–2.0 μM) for 48 h. The results are expressed as the mean ± S.E.M. of duplicates from three independent experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared with untreated control cells
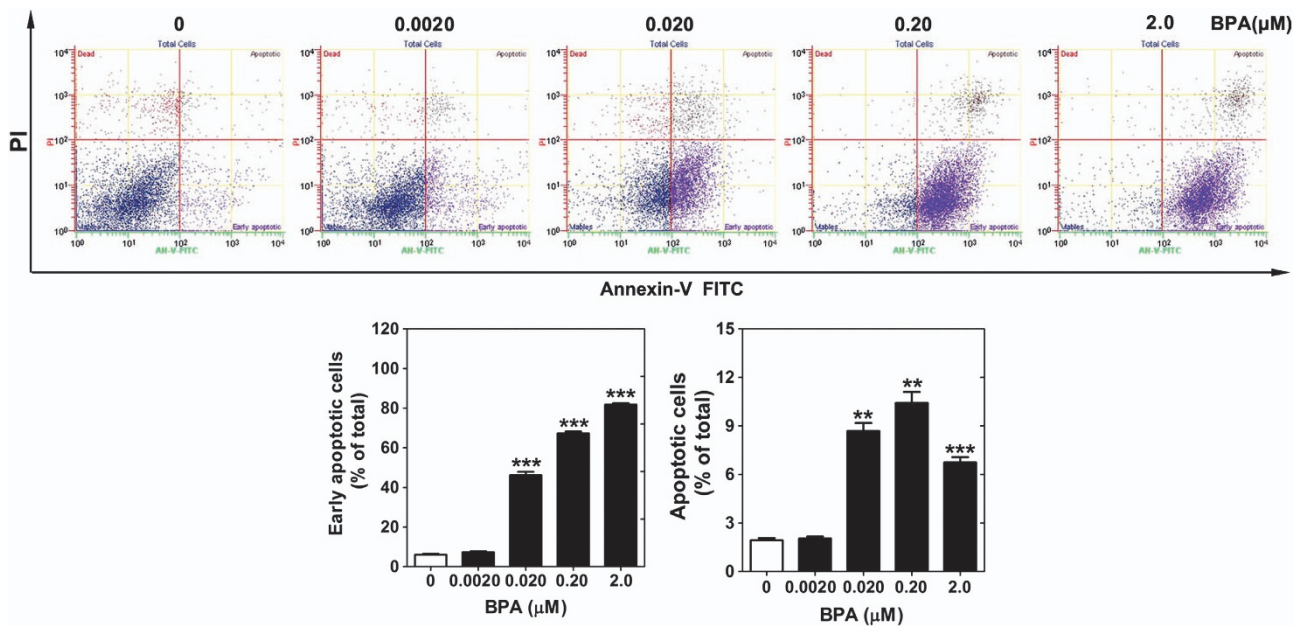

Figure 4 BPA induces dose-dependent apoptosis. INS-1 cells were cultured in the presence or absence of BPA (0.0020–2.0 μM) for 48 h. The cells in the bottom right quadrant (FITC + and PI −) and the upper right quadrant (FITC + and PI +) represent early apoptotic and apoptotic cells, respectively. All data are presented as the mean ± S.E.M. of three independent experiments. **$P < 0.01$; ***$P < 0.001$ compared with untreated control cells

reported that exposure to BPA (0.020–2.0 μM) decreased rat insulinoma (INS-1) cells' viability, disrupted cell function, and, furthermore, increased cell apoptosis in a dose-dependent manner. More importantly, we found that BPA triggered apoptotic signaling in INS-1 cells via a mitochondria-mediated pathway (Figure 7), as shown by mitochondrial fragmentation, the reduction of mitochondrial mass and $\Delta\psi$, the depletion of

ATP, the release of cytochrome *c* from the mitochondria into the cytosol, and the activation of caspases. Despite the fact that the concentration required for these effects was higher than environmentally relevant levels (1 nM) and the mean serum or urine concentrations reported in bio-monitoring studies, it was still within the reported ranges for some populations, such as factory workers exposed to higher levels
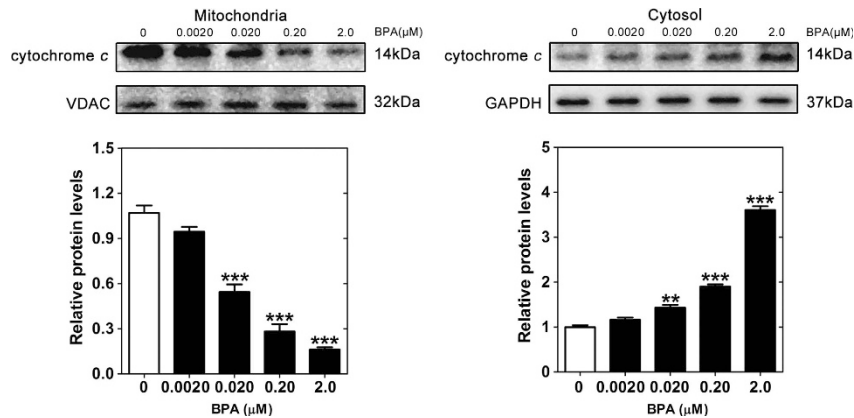
Figure 5 BPA induces the release of cytochrome c from the mitochondria to the cytosol. INS-1 cells were cultured in the presence or absence of BPA (0.0020–2.0 μM) for 48 h. (**a**) Representative western blot for protein expression of cytochrome c in mitochondria. VDAC served as a loading control. (**b**) Representative western blot for protein expression of cytochrome c in the cytosol. GAPDH served as a loading control. All data are presented as the mean ± S.E.M. of duplicates from three independent experiments. **$P < 0.01$; ***$P < 0.001$ compared with untreated control cells
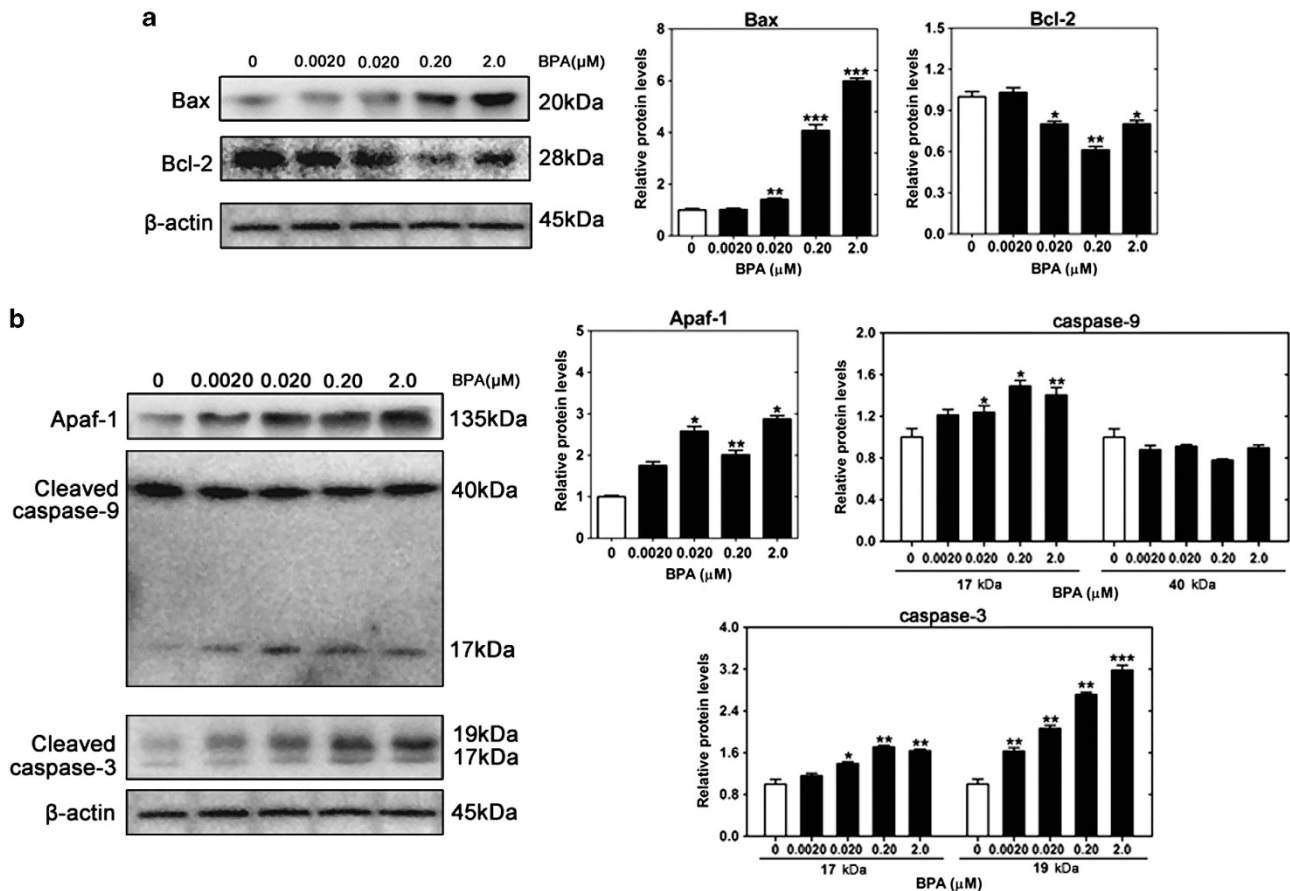
Figure 6 BPA triggers apoptosis through a caspase-dependent mitochondrial pathway. INS-1 cells were cultured in the presence or absence of BPA (0.0020–2.0 μM) for 48 h. (**a**) Western blot analysis of Bax and Bcl-2 expression in cell lysates. (**b**) Western blot analysis of Apaf-1, cleaved caspase-9, and cleaved caspase-3 in cell lysates. All protein expression levels were normalized relative to a β-actin loading control. The data are presented as the mean ± S.E.M. of duplicates from three independent experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared with untreated control cells

of BPA.[21–23] Moreover, the dose chosen in this study can be considered a 'low dose' because estimates of circulating levels of BPA at the LOAEL define an equivalent low-dose concentration as $< 2.19 \times 10^{-7}$ M in vitro culture studies.[24,25]

In this study, the effects of BPA on cell viability were first determined by the MTT assay, which showed that BPA significantly reduced INS-1 cell viability in a dose- and time-dependent manner. We next examined GSIS and found that
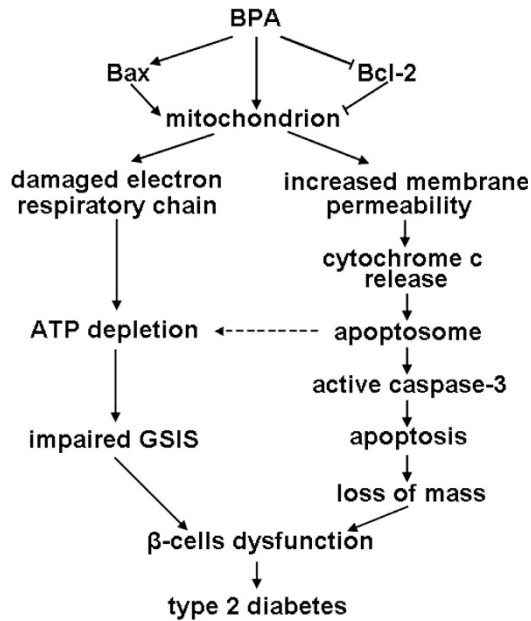
Figure 7 A proposed signaling pathway for BPA-induced β-cell dysfunction and apoptosis. Mitochondrial damage is triggered by BPA that either directly damages the mitochondria or alters the levels of pro- and anti-apoptotic Bcl-2 family proteins. On the one hand, BPA damages the mitochondrial electron respiratory chain complexes, resulting in the depletion of ATP and the impairment of GSIS in β cells. On the other hand, damaged mitochondria release cytochrome *c* to the cytosol. Cytochrome *c* consequently triggers the formation of apoptosomes, the activation of caspases, and finally, the execution of apoptosis in β cells. Arrows in the figure represent stimulative effects, and T lines represent inhibitory effects

cells exposed to 0.20 or 2.0 μM BPA exhibited a significantly diminished ability to secrete insulin in response to glucose stimulation, even though insulin secretion was slightly increased after exposure to 0.0020 or 0.020 μM BPA. The reactive insulin stimulatory effects induced by exposure to lower doses of BPA could be considered cellular adaptive responses to BPA stimulus. However, INS-1 cells were unable to compensate for higher levels of BPA, resulting in significant declines in GSIS as well as decreased mRNA and protein expression of insulin. These observations are in agreement with a prior report that environmentally relevant doses of BPA (1 nM) could stimulate GSIS in human and mouse islets[14] and are highly consistent with a recent study showing that short-term exposure (24 h) to 0.11 or 1.1 μM BPA reduced insulin secretion and insulin content in isolated rat islets.[19] A nonlinear dose–response relationship may exist between BPA and the impairment of GSIS, but this remains to be elucidated in our future research.

In β cells, glucose is transported by Glut2, phosphorylated by Gck, and converted to pyruvate by glycolysis. In the mitochondria, pyruvate enters the TCA cycle and activates ATP formation, which promotes the closure of ATP-sensitive K (K_{ATP}) channels and the depolarization of the plasma membrane and stimulates insulin secretion.[26,27] Impaired GSIS can be mediated by reduced glucose sensing, disrupted mitochondrial metabolism, or a combination of both. In this study, we did not find evidence that exposure to lower concentrations of BPA (0.0020 or 0.020 μM) altered the expression of *Glut2* and *Gck* in INS-1 cells, despite the fact that treatment with 0.20 or 2.0 μM BPA downregulated both genes at the mRNA level. Thus, it was not clear whether BPA impaired GSIS mainly through regulating the rate of glucose uptake and glycolysis. The downregulation of *Gck* and *Glut2* in INS-1 cells treated with the highest concentrations (0.20 or 2.0 μM) of BPA was most likely a consequence rather than a cause of the impaired GSIS observed in BPA-exposed cells. On the other hand, treatment with 0.0020 μM BPA moderately increased the expression of the K_{ATP} channel subunits *Kir6.2* and *Sur*, which may account, at least in part, for the elevated insulin secretion observed in INS-1 cells exposed to BPA at this concentration. Conversely, the expression levels of these two genes were suppressed in INS-1 cells after incubation with as little as 0.020 μM BPA, and this effect was accompanied by a significant depletion of ATP. Because it has been estimated that 98% of the ATP in β cells is produced by mitochondrial oxidative metabolism,[28] we hypothesized that BPA-induced mitochondrial damage might play a critical role in the development of β-cell dysfunction.

Indeed, a recent study indicated that Wistar rat offspring perinatally exposed to 50 μg/kg/day BPA present profound mitochondrial structural defects in β cells that precede any observable alterations in glucose homeostasis.[18] Moreover, exposure of isolated rat islets to 0.11 μM BPA has also been reported to cause mitochondrial swelling, to alter the expression of typical mitochondrial genes, to decrease the activity of the mitochondrial respiratory enzyme COX IV, and to reduce ATP content.[19] In line with these results, we found that several key events involved in the regulation of mitochondrial function and metabolism were damaged by BPA in INS-1 cells. MTG fluorescence showed that BPA destroyed mitochondrial structural integrity and decreased mitochondrial mass in a dose-dependent manner in INS-1 cells. These profound structural defects were accompanied by changes in the regulation of genes that are central to mitochondrial function and energetic metabolism. For example, the expression of *Atp6* (subunit 6 of the mitochondrial ATP synthase) and *Citrate synthase* (one of the genes coding for enzymes involved in rate-limiting steps of the TCA cycle) was significantly decreased in cells exposed to 0.020–2.0 μM BPA, partly explaining the decrease in the capacity of the mitochondria to generate ATP after BPA treatment. Meanwhile, downregulation of *Ogdh* and upregulation of *Ucp2* were observed in BPA-exposed cells. *Ogdh* encodes a rate-limiting enzyme in the mitochondrial TCA cycle, and *Ucp2* has been reported to negatively regulate the sensitivity of insulin secretion to glucose stimulation and the metabolic efficiency of mitochondrial ATP production in β cells.[29–31] Observed changes in the expression of these genes are therefore likely to contribute to the BPA-induced defects in ATP production and GSIS. Tfam encodes a mitochondrial transcription factor that provides a molecular basis for the connection between environmental stimuli and mitochondrial biogenesis.[32] The reduction in the expression of Tfam observed in BPA-exposed cells thus further underlined a negative effect of BPA on mitochondrial function.

One potential consequence of mitochondrial dysfunction is the increase in apoptosis. It has been well documented that BPA can trigger apoptosis in various cells and tissues through

mitochondrial signaling pathways.[24,33] For instance, pubertal BPA exposure resulted in germ cell apoptosis in the testes through not only the Fas/FasL signaling pathway but also the mitochondrial apoptotic pathway.[34] A low dose of BPA was also reported to induce apoptosis in cultured ovarian granulosa cells through the regulation of Bcl-2 family proteins.[35] In the present study, we found that BPA exposure caused apoptosis in INS-1 cells via activation of the mitochondrial apoptotic pathway, that is, the Bcl-2 family and caspases. The Bcl-2 family, including pro-apoptotic and anti-apoptotic members, has been reported to be a central regulator in the mitochondria-dependent apoptotic pathway. Bax is a typical pro-apoptotic protein that can induce mitochondrial membrane permeability and promote cell death by releasing apoptogenic factors from the mitochondria. In contrast, Bcl-2 is an anti-apoptotic protein that can maintain the integrity of the mitochondrial outer membrane.[36,37] In the current study, we found that BPA induced the upregulation of Bax expression and the downregulation of Bcl-2 expression, leading to altered mitochondrial membrane permeability, reduced $\Delta\psi$, the release of mitochondrial cytochrome c to the cytosol and the subsequent activation of caspases (Figure 7). The caspase family is a class of cysteine proteases. After being processed into their active forms in cells undergoing apoptosis, caspases play a crucial role in the transduction of apoptotic signals.[38] Relevant caspases include the initiator caspases (caspases-8, -9, and -10) and the effector caspases (caspases-3, -6, and -7). When INS-1 cells received BPA stimulus, the cells were responsive to Apaf-1 activation in a dose-dependent fashion. Apaf-1 interacts with cytochrome c in the cytoplasm to form an apoptosome complex, which in turn activates the caspases that are responsible for the execution of cell death.[39] Consistent with this hypothesis, BPA triggered the activation of pro-caspase-9, as evidenced by a significant increase in the expression of cleaved (active) caspase-9 in INS-1 cells. Activated caspase-9 remained with the apoptosome as a holoenzyme to maintain its catalytic activity and activated caspase-3, one of the effector caspases involved in the execution of the majority of cellular apoptotic events.[39,40] All of these findings indicated that the mitochondria-mediated apoptotic signaling pathway was involved in the apoptotic process triggered by BPA in INS-1 cells.

In summary, data from the current study overwhelmingly indicate that BPA affects the viability and function of normal β cells and accelerates the rate of apoptosis in INS-1 cells in a dose-dependent manner. Importantly, we suggest that mitochondrial defects and mitochondria-dependent apoptotic signals play vital roles in BPA-induced apoptosis in INS-1 cells (Figure 7). BPA is an important environmental risk factor for diabetes, and precisely defining the mechanisms involved in underlying the toxic action of BPA in β cells requires further investigation.

Materials and methods

Cell culture and treatments. The INS-1 cells derived from rat insulinoma were purchased from Biohermes (Shanghai, China) and were grown in RPMI 1640 medium supplemented with 10% FBS, 5.6 mM glucose, 25 mM HEPES, 2 mM L-glutamine, 50 μM β-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere containing 95% air and 5% CO_2. INS-1 cells were used because they are highly responsive to changes in the glucose concentration and have been widely used to evaluate β-cell function *in vitro*.[20] Culture medium, FBS, and supplements were purchased from Invitrogen (Carlsbad, CA, USA).

For experiments, INS-1 cells were seeded at a density of 6.0×10^3/well in 96-well plates, 2.5×10^5/well in 24-well plates, or 6.0×10^5 cells/well in six-well plates. After 24 h, the medium was removed, and the cells were incubated with fresh medium containing serial dilutions of BPA (0, 0.0020, 0.020, 0.20, or 2.0 μM) for a designated period of time. BPA was dissolved in dimethyl sulfoxide (DMSO) because of its poor water solubility. The final concentration of DMSO in medium was 0.1%, v/v.

MTT viability assay. INS-1 cells were seeded in 96-well plates and stimulated with BPA. At the end of the treatment, the medium was removed, and MTT (0.5 mg/ml) was added to each well for at least 4 h. The purple formazan precipitate was dissolved with DMSO, and the color intensity was measured at 550 nm with a SpectraMAX M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Glucose-stimulated insulin secretion. INS-1 cells were seeded in 24-well plates and exposed to BPA for 48 h. Insulin secretion in response to glucose was assessed as described previously.[18] Briefly, the treated cells were first preincubated with Krebs-Ringer bicarbonate HEPES buffer (KRBH: 115 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 24 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 10 mM HEPES) supplemented with 0.5% BSA and a low level of glucose (3.0 mM) for 1 h. Afterward, the cells were incubated in KRBH buffer containing 5.6 or 16.7 mM glucose for 1 h. The supernatants were collected for the determination of insulin secretion. Insulin concentrations were measured using a Rat Insulin ELISA kit (Millipore, Billerica, MA, USA). The levels of secreted insulin were normalized to the protein content, which was determined with the BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA).

ATP assay. INS-1 cells were seeded in six-well plates and incubated with BPA for 48 h as described above. At the end of the treatment, the cells were collected, and cellular ATP concentrations were determined using an ATP luminometric assay kit (Beyotime Inst. Biotech, Peking, China) following the manufacturer's instructions. Briefly, the cells were mixed with luciferase, which were centrifuged at $10\,000 \times g$ for 2 min at 4 °C. The resulting supernatant was then mixed with luciferase reagent, which catalyzed the light production from ATP and luciferin. Luminance (RLU) was linearly related to the ATP concentration and was measured using a SpectraMAX M5 microplate reader. ATP levels were expressed as nmol/mg protein.

Measurements of mitochondrial morphology and mass. After treatment, the cells were mixed with 100 nM MTG fluorescent probe (MTG, Beyotime Inst. Biotech) for 30 min at 37 °C. The probe can be taken up into the mitochondria and will then produce a fluorescence signal. Fluorescence images of cells were obtained with an LSM 710 Laser Scanning Confocal Microscope (Zeiss, Jena, Germany) using a 490-nm laser for excitation and a 510-nm filter for emission. The fluorescence intensity of cells was analyzed with a SpectraMAX M5 microplate reader.

Determination of mitochondrial membrane potential ($\Delta\psi$m). The $\Delta\psi$m in INS-1 cells was measured using 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetra-ethylimidacarbocyanine iodide fluorescent probe (JC-1) (Cayman Chemical, Ann Arbor, MI, USA). The treated cells in six-well plates were harvested and incubated with JC-1 at 37 °C for 15–20 min. Next, the staining solution was removed. The cells were washed and resuspended in phosphate-buffered saline (PBS) and then analyzed with a flow cytometer (Quanta SC, Beckman Coulter, Brea, CA, USA). The loss of $\Delta\psi$m was reflected by the increased green fluorescence from JC-1 monomers as well as the loss of red fluorescence from JC-1 aggregates.

Apoptosis assay. An Annexin V-fluorescein isothiocyanate (FITC) staining assay was performed to measure apoptosis in INS-1 cells. The cells were seeded in six-well plates and exposed to BPA for 48 h. The cells were then collected by trypsinization, washed with PBS, and stained with Annexin V-FITC and PI (BioVision, Mipitas, CA, USA) for 15 min at room temperature. Positive cells were detected with flow cytometry.

Table 1 Primer sequences for quantiative real-time PCR

Gene name	Size (bp)	5′-Primer	3′-Primer	Accession number
Insulin	166	CTACAGTCGGAAACCATCAGCA	CCACCAAGTGAGAACCACAAAG	NM_019130.2
Glut2	151	CAGCTGTCTCTGTGCTGCTTGT	GCCGTCATGCTCACATAACTCA	NM_012879.2
Gck	72	CACCCCAGAAGGCTCAGAAG	AGCATCACTCTGAAGTTGGTTCCT	NM_012565.1
Kir6.2	142	TGTCCCGAAAGGGCATTATC	ATGTTCTTGTGGGCGACGTT	NM_031358.3
Sur	142	CAAGGTCGTAAACCGCAAGC	AGGGGTCCAGGTGAAGAAGC	NM_013039.2
Tfam	118	GAAGAGCAAATGGCTGAAGTT	GTGCCCAATCCCAATGAC	NM_031326.1
Nd4l	103	TCCCAATTACCATTCTAGTTTT	AGGTTTTGTACGTAGTCTGTTCCGT	EU104720
Atp6	164	GCGTCTGGAGGACCTGTTGA	CCTCAGGACTGGGGTTTGT	BC059121
Citrate synthase	103	GCCCTCAACAGTGAAAGCA	TGGCAATCAGGTCCATACAG	NM_130755
Ucp2	143	TGTGGTAAAGGTCCGCTTCC	TTCGGGCAACATTGGGAG	NM_019354.2
Ogdh	138	CCGTGCCCGCTGACATTAT	TCTCCCGAAGAGGAAGTGC	NM_001017461.1
36B4	93	CAGCAGGTGTTTGACAATGGC	TGAGGCAACAGTCGGGTAGC	NM_022402.1

Abbreviations: *Glut2*, glucose transporter member 2; *Gck*, glucokinase; *Kir6.2*, potassium inwardly rectifying channel, subfamily J, member 11; *Sur*, ATP-binding cassette, subfamily C (CFTR/MRP), member 8; *Tfam*, transcription factor A, mitochondrial; *Nd4l*, NADH-ubiquinone oxireductase subunit 4 L; *Atp6*, ATP synthase subunit 6; *Ucp2*, Uncoupling protein 2; *Ogdh*, oxoglutarate (α-ketoglutarate) dehydrogenase (lipoamide).

Total RNA preparation and real-time PCR. INS-1 cells were cultured and treated as described above. Total RNA was extracted using the High Pure RNA kit (Roche, Mannheim, Germany) and was reverse transcribed using PrimeScript TM RT-PCR Kit (Takara, Dalian, China) according to the manual. Real-time PCR was carried out using SYBR Premix Ex Taq II kit (Takara) following the manufacturer's instructions on a LightCycler 480 Instrument (Roche Applied Science). All quantifications were performed with *36B4* as an internal standard, and the relative amount of mRNA was calculated using the $2^{-\Delta\Delta CT}$ method. The primer sequences are shown in Table 1.

Western blot analysis. After treatment, INS-1 cells were quickly harvested, rinsed thoroughly with PBS, and then lysed in ice-cold RIPA lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% SDS, 1 mM EDTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride) for 30–60 min. The lysates were collected and centrifuged at 13 000 r.p.m. for 10 min at 4 °C. The supernatants were transferred to a new tube, and protein concentrations were measured using a BCA Protein Assay Kit described above. To separate the mitochondrial and cytosolic fractions, a Mitochondria/Cytosol Fractionation Kit (Beyotime Inst. Biotech) was used according to the manufacturer's instructions. For western blots, equal amounts of protein (40 μg) were resolved with SDS-PAGE and transferred to PVDF membranes (Bio-Rad, Hercules, CA, USA). The immunoblot membranes were incubated with blocking solution consisting of 5% milk in TBST for 2 h at room temperature and then incubated at 4 °C on a shaker overnight with the primary antibodies. The next day, the primary antibodies were removed, and the membranes were incubated with 1 : 2000 horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology, Beverly, MA, USA) at 37 °C for 1–2 h. Antibody-binding bands were visualized with enzyme-catalyzed chemiluminescence (ECL, Beyotime Inst. Biotech). The density of the bands were quantified with Image Pro Plus version 6.0 software (Media Cybernetics, Bethesda, MD, USA) and normalized against a loading control. The following primary antibodies (Cell Signaling Technology or Santa Cruz Biotechnology, Santa Cruz, CA, USA) and dilutions were used: Insulin (sc-9168, 1 : 500), Bax (#2772, 1 : 2000), Bcl-2 (#2876, 1 : 2000), Apaf-1 (#8723S, 1 : 1000), caspase-9 (#9506, 1 : 1000), caspase-3 (#9662, 1 : 1000), cytochrome *c* (#4272, 1 : 1000), β-actin (#4976, 1 : 2000), GAPDH (#2118 : 1 : 2000) and VDAC (#4866 : 1 : 2000).

Statistical analyses. All data are expressed as the mean ± S.E.M. The differences between the untreated control and BPA-exposed groups were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's T3 test. Differences with $P < 0.05$ (*), $P < 0.01$ (**), or $P < 0.001$ (***) considered statistically significant. Analyses were conducted with SPSS (Statistical Package for the Social Sciences) software v.17.0 (SPSS Inc., Chicago, IL, USA).

Conflict of Interest

The authors declare no conflict of interest.

1. Vandenberg LN, Maffini MV, Sonnenschein C, Rubin BS, Soto AM. Bisphenol-A and the great divide: a review of controversies in the field of endocrine disruption. *Endocr Rev* 2009; **30**: 75–95.
2. Calafat AM, Ye X, Wong LY, Reidy JA, Needham LL. Exposure of the US population to bisphenol A and 4-tertiary-octylphenol: 2003–2004. *Environ Health Perspect* 2008; **116**: 39.
3. Vandenberg LN, Hauser R, Marcus M, Olea N, Welshons WV. Human exposure to bisphenol A (BPA). *Reprod Toxicol* 2007; **24**: 139–177.
4. Lang IA, Galloway TS, Scarlett A, Henley WE, Depledge M, Wallace RB *et al.* Association of urinary bisphenol A concentration with medical disorders and laboratory abnormalities in adults. *JAMA* 2008; **300**: 1303–1310.
5. Wang T, Li M, Chen B, Xu M, Xu Y, Huang Y *et al.* Urinary bisphenol A (BPA) concentration associates with obesity and insulin resistance. *J Clin Endocrinol Metab* 2012; **97**: E223–E227.
6. Melzer D, Rice NE, Lewis C, Henley WE, Galloway TS. Association of urinary bisphenol A concentration with heart disease: evidence from NHANES 2003/06. *PLoS One* 2010; **5**: e8673.
7. Silver MK, O'Neill MS, Sowers MFR, Park SK. Urinary bisphenol a and type-2 diabetes in US adults: data from NHANES 2003–2008. *PLoS One* 2011; **6**: e26868.
8. Shankar A, Teppala S. Relationship between urinary bisphenol A levels and diabetes mellitus. *J Clin Endocrinol Metab* 2011; **96**: 3822–3826.
9. Ning G, Bi Y, Wang T, Xu M, Xu Y, Huang Y *et al.* Relationship of urinary bisphenol A concentration to risk for prevalent type 2 diabetes in Chinese adults. *Ann Intern Med* 2011; **155**: 368–374.
10. Batista TM, Alonso-Magdalena P, Vieira E, Amaral MEC, Cederroth CR, Nef S *et al.* Short-term treatment with bisphenol-A leads to metabolic abnormalities in adult male mice. *PLoS One* 2012; **7**: e33814.
11. Nadal A, Ropero AB, Laribi O, Maillet M, Fuentes E, Soria B. Nongenomic actions of estrogens and xenoestrogens by binding at a plasma membrane receptor unrelated to estrogen receptor α and estrogen receptor β. *Pro Natl Acad Sci* 2000; **97**: 11603–11608.
12. Quesada I, Fuentes E, Viso-León MC, Soria B, Ripoll C, Nadal A. Low doses of the endocrine disruptor bisphenol-A and the native hormone 17ß-estradiol rapidly activate transcription factor CREB. *FASEB J* 2002; **16**: 1671–1673.
13. Alonso-Magdalena P, Ropero AB, Carrera MP, Cederroth CR, Baquie M, Gauthier BR *et al.* Pancreatic insulin content regulation by the estrogen receptor ERα. *PLoS One* 2008; **3**: e2069.
14. Soriano S, Alonso-Magdalena P, García-Arévalo M, Novials A, Muhammed SJ, Salehi A *et al.* Rapid Insulinotropic action of low doses of bisphenol-A on mouse and human islets of langerhans: role of estrogen receptor β. *PLoS One* 2012; **7**: e31109.
15. Lowell BB, Shulman GI. Mitochondrial dysfunction and type 2 diabetes. *Science* 2005; **307**: 384–387.
16. Szendroedi J, Phielix E, Roden M. The role of mitochondria in insulin resistance and type 2 diabetes mellitus. *Nat Rev Endocrinol* 2011; **8**: 92–103.
17. Maechler P, De Andrade P. Mitochondrial damages and the regulation of insulin secretion. *Biochem Soc Trans* 2006; **34**: 824–827.
18. Wei J, Lin Y, Li Y, Ying C, Chen J, Song L *et al.* Perinatal exposure to bisphenol A at reference dose predisposes offspring to metabolic syndrome in adult rats on a high-fat diet. *Endocrinology* 2011; **152**: 3049–3061.

19. Song L, Xia W, Zhou Z, Li Y, Lin Y, Wei J *et al.* Low-level phenolic estrogen pollutants impair islets morphology and β-cells function in isolated rat islets. *J Endocrinol* 2012; **215**: 303–311.
20. Skelin M, Rupnik M, Cencic A. Pancreatic beta cell lines and their applications in diabetes mellitus research. *Altex* 2010; **27**: 105–113.
21. Vandenberg LN, Chahoud I, Heindel JJ, Padmanabhan V, Paumgartten FJR, Schoenfelder G. Urinary, circulating, and tissue biomonitoring studies indicate widespread exposure to bisphenol A. *Environ Health Perspect* 2010; **118**: 1055–1070.
22. Makaji E, Raha S, Wade MG, Holloway AC. Effect of environmental contaminants on beta cell function. *Int J Toxicol* 2011; **30**: 410–418.
23. Wang F, Hua J, Chen M, Xia Y, Zhang Q, Zhao R *et al.* High urinary bisphenol A concentrations in workers and possible laboratory abnormalities. *Occup Environ Med* 2012; **69**: 679–684.
24. Wetherill YB, Akingbemi BT, Kanno J, McLachlan JA, Nadal A, Sonnenschein C *et al. In vitro* molecular mechanisms of bisphenol A action. *Reprod Toxicol* 2007; **24**: 178–198.
25. Welshons WV, Nagel SC, vom Saal FS. Large effects from small exposures. III. Endocrine mechanisms mediating effects of bisphenol A at levels of human exposure. *Endocrinology* 2006; **147**: s56–s69.
26. Henquin JC. Regulation of insulin secretion: a matter of phase control and amplitude modulation. *Diabetologia* 2009; **52**: 739–751.
27. Rorsman P, Renström E. Insulin granule dynamics in pancreatic beta cells. *Diabetologia* 2003; **46**: 1029–1045.
28. MacDonald PE, Joseph JW, Rorsman P. Glucose-sensing mechanisms in pancreatic β-cells. *Philos Trans R Soc B Bio Sci* 2005; **360**: 2211–2225.
29. Polonsky KS, Semenkovich CF. The pancreatic ß cell heats up: UCP2 and insulin secretion in diabetes. *Cell* 2001; **105**: 705–707.
30. Zhang CY, Baffy G, Perret P, Krauss S, Peroni O, Grujic D *et al.* Uncoupling protein-2 negatively regulates insulin secretion and is a major link between obesity, [beta] cell dysfunction, and type 2 diabetes. *Cell* 2001; **105**: 745–755.
31. Chan CB, De Leo D, Joseph JW, McQuaid TS, Ha XF, Xu F *et al.* Increased uncoupling protein-2 levels in β-cells are associated with impaired glucose-stimulated insulin secretion. *Diabetes* 2001; **50**: 1302–1310.
32. Hock MB, Kralli A. Transcriptional control of mitochondrial biogenesis and function. *Annu Rev Physiol* 2009; **71**: 177–203.
33. vom Saal FS, Akingbemi BT, Belcher SM, Birnbaum LS, Crain DA, Eriksen M *et al.* Chapel Hill bisphenol A expert panel consensus statement: integration of mechanisms, effects in animals and potential to impact human health at current levels of exposure. *Reprod toxicol* 2007; **24**: 131–138.
34. Wang Q, Zhao XF, Ji YL, Wang H, Liu P, Zhang C *et al.* Mitochondrial signaling pathway is also involved in bisphenol A induced germ cell apoptosis in testes. *Toxicol Lett* 2010; **199**: 129–135.
35. Xu J, Osuga Y, Yano T, Morita Y, Tang X, Fujiwara T *et al.* Bisphenol A induces apoptosis and G2-to-M arrest of ovarian granulosa cells. *Biochem Biophys Res Commun* 2002; **292**: 456–462.
36. Kroemer G, Reed JC. Mitochondrial control of cell death. *Nat Med* 2000; **6**: 513–519.
37. Youle RJ, Strasser A. The BCL-2 protein family: opposing activities that mediate cell death. *Nat Rev Molr Cell Biol* 2008; **9**: 47–59.
38. Earnshaw WC, Martins LM, Kaufmann SH. Mammalian caspases: structure, activation, substrates, and functions during apoptosis. *Annu Rev Biochem* 1999; **68**: 383–424.
39. Lakhani SA, Masud A, Kuida K, Porter Jr GA, Booth CJ, Mehal WZ *et al.* Caspases 3 and 7: key mediators of mitochondrial events of apoptosis. *Science* 2006; **311**: 847–851.
40. Zimmermann KC, Bonzon C, Green DR. The machinery of programmed cell death. *Pharmacol Ther* 2001; **92**: 57–70.