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Perfluorooctanoic acid induces oxidative damage and mitochondrial dysfunction in pancreatic β-cells

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Abstract. Several environmental contaminants have been linked to the development of diabetes and increased diabetes-associated mortality. Perfluorooctanoic acid (PFOA) is a widely used perfluoroalkane found in surfactants and lubricants, and in processing aids used in the production of polymers. Furthermore, PFOA has been detected in humans, wildlife and the environment. The present study investigated the toxic effects of PFOA on rat pancreatic β-cell-derived RIN-m5F cells. Cell viability, apoptosis, reactive oxygen and nitrogen species, cytokine release and mitochondrial parameters, including membrane potential collapse, reduced adenosine triphosphate levels, cardiolipin peroxidation and cytochrome *c* release were assessed. PFOA significantly decreased RIN-m5F cell viability and increased apoptosis. Exposure to PFOA increased the formation of reactive oxygen species, mitochondrial superoxide, nitric oxide and proinflammatory cytokines. Furthermore, PFOA induced mitochondrial membrane potential collapse and reduced adenosine triphosphate levels, cardiolipin peroxidation and cytochrome *c* release. These results indicate that PFOA is associated with the induction of apoptosis in RIN-m5F cells, and induces cytotoxicity via increased oxidative stress and mitochondrial dysfunction.

Introduction

Diabetes is a complex metabolic disorder characterized by abnormalities in glucose homeostasis and insulin sensitivity (1). The increasing prevalence of metabolic syndrome indications, including dyslipidemia, obesity and insulin resistance, has stimulated rising interest in the role environmental pollutants serve in such diseases (2). Environmental exposure to persistent organic pollutants is associated with diabetes (3). Perfluorooctanoic acid (PFOA) is one of the most common perfluoroalkyl acids (PFAAs), which has been detected in humans and wildlife due to environmental exposure. Substantial progress has been made in the toxicological research of these compounds, particularly in the areas of developmental toxicity, immunotoxicity, hepatotoxicity and the potential associated modes of action (4); PFOA is not metabolized and has an estimated human half-life of 2.3-3.4 years (5). Notably, PFOA is water-soluble and migrates readily from soil to groundwater, and human exposure can occur via contaminated drinking water, with other sources of exposure including food, food packaging, treated fabrics, house dust and air (4). Previous research has suggested that PFOA is associated with risk factors for type 2 diabetes, including glucose homeostasis and metabolic disorder (6). Furthermore, PFOA-exposed employees at manufacturing facilities presented an increased risk of mortality from type 2 diabetes (7), and in a longitudinal prospective cohort study of pregnant women followed from pre-conception to delivery, Zhang *et al* (8) revealed a significant positive association between serum PFOA concentrations and the risk of gestational diabetes. Although several epidemiological studies identified no association between PFOA and other PFAAs and the incidence of diabetes, the evidence suggested that these compounds influenced glucose metabolism (2,9,10). Notably, experimental studies have reported that PFOA is able to induce oxidative stress and apoptosis in primary cultured tilapia hepatocytes (11), and in human hepatoma HepG2 cells (12).

Oxidative stress is considered to be a key risk factor for the development and progression of various chronic degenerative diseases, including diabetes (13). Oxidative stress in pancreatic β-cells reduces the mitochondrial membrane potential (MMP), which promotes the release of apoptogenic factors that activate downstream death programs (14). Although the exact mechanism underlying pancreatic β-cell dysfunction is not currently known, the enhanced production of mitochondrial reactive oxygen species (ROS) that occurs under hyperglycemic and

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hyperlipidemic conditions is a major contributing factor to the disruption of β-cell function in type 2 diabetes (15). Pancreatic β-cells are particularly sensitive to oxidative damage; therefore mitochondrial oxidative damage may underlie the marked loss of β-cell function (16). Apoptosis is the predominant mode of pancreatic β-cell death in diabetes (17) and serves a crucial role in the pathogenesis of diabetes (18). The apoptotic process occurs via two pathways: The 'extrinsic' or death receptor-initiated pathway, and the 'intrinsic' or mitochondrial-initiated pathway (19). The intrinsic apoptotic pathway involves fatal alterations to mitochondrial homeostasis, this occurs via the loss of outer mitochondrial membrane integrity and the subsequent release of cytochrome *c*. Mitochondrial pore-formation and the release of cytochrome *c* are believed to irreversibly commit a cell to apoptosis (20).

Proinflammatory cytokines, including interleukin-1β (IL-1β) and tumor necrosis factor α (TNF-α), moderate the expression and activity of antioxidant enzymes, further stimulating an imbalance in the redox status of insulin-producing cells (21). In addition, proinflammatory cytokines stimulate inducible nitric oxide synthase (iNOS) expression, which promotes nitric oxide (NO) formation (21). NO has been widely implicated in nitrosative stress, an event that is linked to cell damage. Therefore, NO and ROS are considered crucial elements in proinflammatory cytokine-mediated pancreatic β-cell death (22). During apoptosis, pancreatic β-cell mitochondria may be influenced by proinflammatory cytokines, and this occurs via activation of the intrinsic or extrinsic pathways (23). Furthermore, cytokines may impair the MMP and disturb adenosine triphosphate (ATP) homeostasis, leading to the release of cytochrome *c* and activation of β-cell apoptosis. The present study therefore investigated the *in vitro* cytotoxic effects of PFOA on pancreatic β-cells and its underlying mechanisms, using the RIN-m5F rat pancreatic β-cell line.

Materials and methods

Materials. PFOA (Fig. 1) was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Fetal bovine serum (FBS) and RPMI-1,640 medium were purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Other reagents were of the highest commercial grade available and were purchased from Sigma-Aldrich (Merck KGaA).

Cell culture. RIN-m5F cells derived from rat pancreatic β-cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained in RPMI-1,640 supplemented with 10% FBS and 1% penicillin/streptomycin, at 37˚C and saturated humidity with 5% CO_2. Cells were seeded into a 24-well plate ($2x10^4$ cells/well) and cultured for 48 h, and subsequently treated with 0-500 μM PFOA at 37˚C for 48 h.

Cell viability. Cell viability was measured using an EZ-Cytox kit (Daeil Lab Service Co. Ltd., Seoul, South Korea). Cells were treated with water-soluble tetrazolium (WST) reagent and incubated at 37˚C for 2 h. Live cells metabolized the WST reagent, resulting in an orange-colored product, the intensity of which was measured at 450 nm using a Zenyth 3100 multimode detector spectrofluorometer (Anthos Labtec Instruments GmbH, Salzburg, Austria). The data were expressed as a percentage of the control [% control=100x (absorbance of experimental group/absorbance of control group)].

Measurement of apoptosis. Apoptosis was assessed using a Cell Death Detection ELISA^{PLUS} kit (Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's protocol. The assay was based on the quantitative sandwich-enzyme-immunoassay-principle, using DNA- and histone-directed mouse monoclonal antibodies, and allowed the specific determination of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates (24).

Measurement of intracellular ROS. ROS was measured using 2',7'-dichlorofluorescin diacetate (DCFH-DA). Oxidation of the non-fluorescent DCFH-DA yields dichlorofluorescin (DCF), a highly fluorescent product that detects reactive oxygen intermediates in intact cells (25). Cells were treated with 10 μM DCFH-DA at 37˚C for 1 h. Following washing with Dulbecco's PBS, ROS levels were determined by measuring the fluorescence intensity at an excitation wavelength of 485 nm and an emission wavelength of 530 nm, using a Zenyth 3100 multimode detector spectrofluorometer.

Measurement of mitochondrial superoxide. Mitochondrial superoxide levels were detected using MitoSOX™ Red mitochondrial superoxide indicator (Invitrogen; Thermo Fisher Scientific Inc.), a fluorogenic dye used for the highly selective detection of mitochondrial superoxide (26). Briefly, cells were incubated with 5 μM MitoSOX™ Red at 37˚C for 20 min, according to the manufacturer's protocol. The cells were subsequently washed by Dulbecco's PBS, and then MitoSOX™ Red fluorescence was measured at an excitation wavelength of 510 nm, and an emission wavelength of 580 nm.

Measurement of intracellular NO production. A sensitive fluorescent indicator of NO, 2',7'-difluorofluorescein (DAF-FM) diacetate, was used to detect intracellular NO production. DAF-FM diacetate is a cell-permeable derivative of DAF-FM, which is converted to the less permeable DAF-FM by cellular esterases when it enters the cell, thus preventing signal loss due to diffusion of the molecule from the cell. In the presence of oxygen, DAF-FM reacts with NO to yield the highly fluorescent triazolofluorescein. Cells were incubated with 5 μM DAF-FM diacetate (Invitrogen; Thermo Fisher Scientific, Inc.) for 2 h at 37˚C (27). Following the removal of excess probe, DAF-fluorescence intensity was measured at an excitation wavelength of 495 nm and an emission wavelength of 515 nm.

Cytokine (TNF-α and IL-1β) immunoassay. Cell extracts were prepared using cell lysis buffer (Cell Signaling Technology, Inc., Danvers, MA, USA) and centrifuged at 10,000 x *g* for 15 min at 4˚C. The cytosolic concentrations of TNF-α (cat. no. RTA00) and IL-1β (cat. no. RBL00) were measured using ELISA kits (R&D System Inc., Minneapolis, MN, USA) according to the manufacturer's protocol. Briefly, cytoplasmic cytokines were bound to antibodies immobilized on a pre-coated microplate. Unbound substances were removed by washing, and a cytokine-specific enzyme-linked polyclonal antibody was added to each well. Unbound antibody-enzyme reagent was removed by washing, and the provided substrate

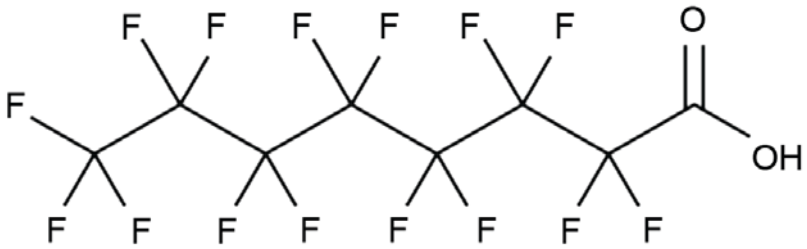

Figure 1. Chemical structure of perfluorooctanoic acid.

was added to each well. The enzyme reaction yielded a blue product, which was converted to a yellow product following addition of the stop solution. The absorbance was measured at 450 nm; the measured color intensity was proportional to the amount of bound cytokine.

Measurement of ATP concentration. Cells were homogenized in ATP assay buffer (BioVision, Inc., Milpitas, CA, USA) Intracellular ATP concentrations were determined using an ATP Colorimetric Assay kit (BioVision, Inc.), which allows rapid measurement of intracellular ATP. The assay was performed according to the manufacturer's protocol. ATP concentrations were normalized to protein content in the samples, protein concentrations were determined using the Bradford Protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) (25). A standard curve using known ATP concentrations was plotted to allow the calculation of nmoles ATP/mg protein. Results are presented as a percentage of the control.

Measurement of the MMP. MMP was measured fluorometrically using the fluorescent probe rhodamine 123 (28). Cells ($1x10^4$) were cultured in black 96-well plates and allowed to adhere overnight. Following adhesion, cells were treated for a further 48 h with PFOA, as aforementioned. The cells were then washed twice with PBS and incubated with 10 μM rhodamine 123 solution, at 37˚C in the dark, for 30 min. Following a further 2 washes with PBS, the fluorescence intensity was measured using a spectrofluorometer at an excitation wavelength of 505 nm and an emission wavelength of 534 nm. The data were analyzed by GraphPad Prism software 4.0 (GraphPad Software, Inc., San Diego, CA, USA).

Cytochrome c release assay. Cells were cultured at $2x10^4$ cells/well onto 24-well plates. Cell extracts were prepared using cell lysis buffer and centrifuged at 10,000 x *g* for 15 min at 4˚C. The supernatants (200 μl) were detected using a cytochrome *c* ELISA kit (cat. no. ab110172; Abcam, Cambridge, MA, USA). The assay was performed according to the manufacturer's protocol. Cytochrome *c* from the conditioned medium was immunocaptured within the wells, and the concentration was determined by adding a cytochrome *c*-specific antibody conjugated to horseradish peroxidase. Following addition of the provided colorless substrate, the peroxidase converted the substrate to a blue end-product. This reaction occurred in a time-dependent manner, which was proportional to the amount of protein captured in the wells. The rate of blue color development was detected at 600 nm. The change in absorbance was expressed as change in milliOD/min.

Measurement of cardiolipin peroxidation. Cardiolipin peroxidation was assessed using 10-N-nonyl-Acridine Orange (NAO) (Molecular Probes; Thermo Fisher Scientific, Inc.), which binds to mitochondria-specific cardiolipin. NAO loses its affinity for peroxidized cardiolipin; therefore, a decrease in NAO fluorescence reflects the peroxidation of intracellular cardiolipin (29). Cells were labeled with NAO (5 μM, at 37˚C for 20 min), washed twicewithDulbecco's PBS, and the fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

Statistical analysis. The results were expressed as the mean ± standard error of the mean, for triplicate experiments. One-way analysis of variance was followed by Dunnett's t-test. $P<0.05$ was considered to indicate a statistically significant difference. Data was analyzed using SAS statistical software (version 9.1.3; SAS Institute, Inc., Cary, NC, USA).

Results

Cytotoxicity of PFOA in RIN-m5F cells. To determine whether PFOA induced cytotoxicity and apoptosis in RIN-m5F cells, cells were treated with 0-500 μM PFOA for 48 h. PFOA significantly decreased RIN-m5F cell viability in a concentration-dependent manner (≥100 μM, Fig. 2A), with cells treated with 100 μM PFOA exhibiting 76% cell viability, compared with control. Furthermore, apoptosis was significantly increased in PFOA-treated cells (100-500 μM; Fig. 2B); following treatment with 500 μM PFOA apoptosis was increased 2.4-fold. These results indicated that PFOA may reduce cell viability by inducing apoptosis.

PFOA induces oxidative/nitrosative stress in RIN-m5F cells. To investigate whether PFOA-induced apoptosis in RIN-m5F cells was associated with the accumulation of ROS, RIN-m5F cells were treated with DCFH-DA, a fluorescent probe for ROS. PFOA (≥200 μM) significantly increased DCF fluorescence, which was 3.2-fold greater following treatment with 500 mM PFOA (Fig. 3A).

Mitochondria are a major source of ROS (25). To determine whether PFOA regulated mitochondrial ROS accumulation in RIN-m5F cells, cells were incubated with MitoSOX Red, a fluorogenic dye that specifically detects superoxide in the mitochondria of live cells (30). Cells treated with PFOA (≥200 μM) demonstrated significantly higher levels of MitoSOX™ Red

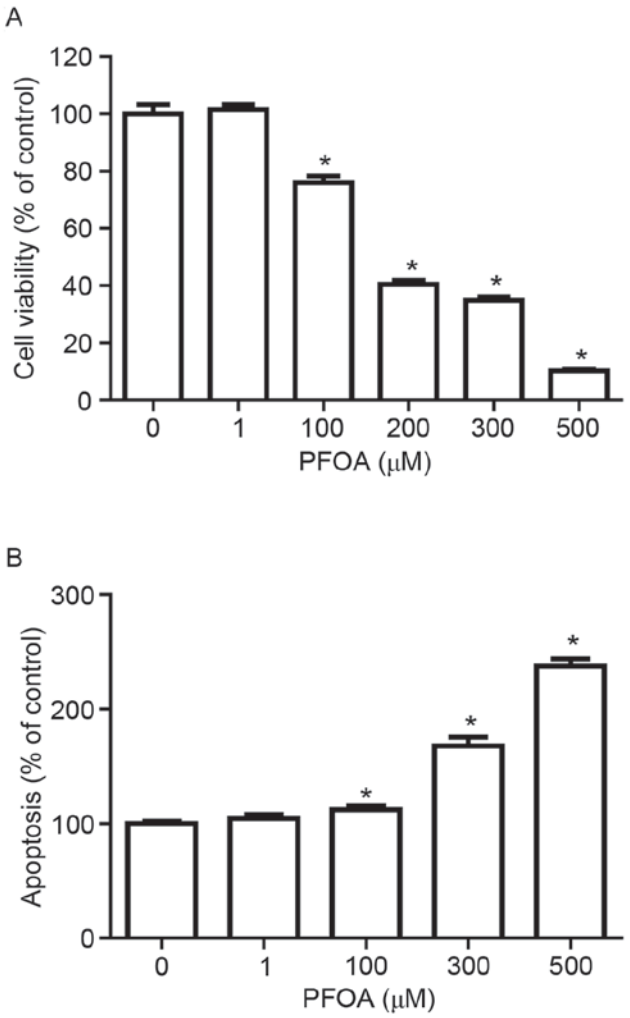

Figure 2. Effects of PFOA on cell viability and apoptosis in RIN-m5F cells. (A) Cell viability and (B) apoptosis in RIN-m5F cells treated with PFOA for 48 h. *P<0.05, vs. control. PFOA, perfluorooctanoic acid.

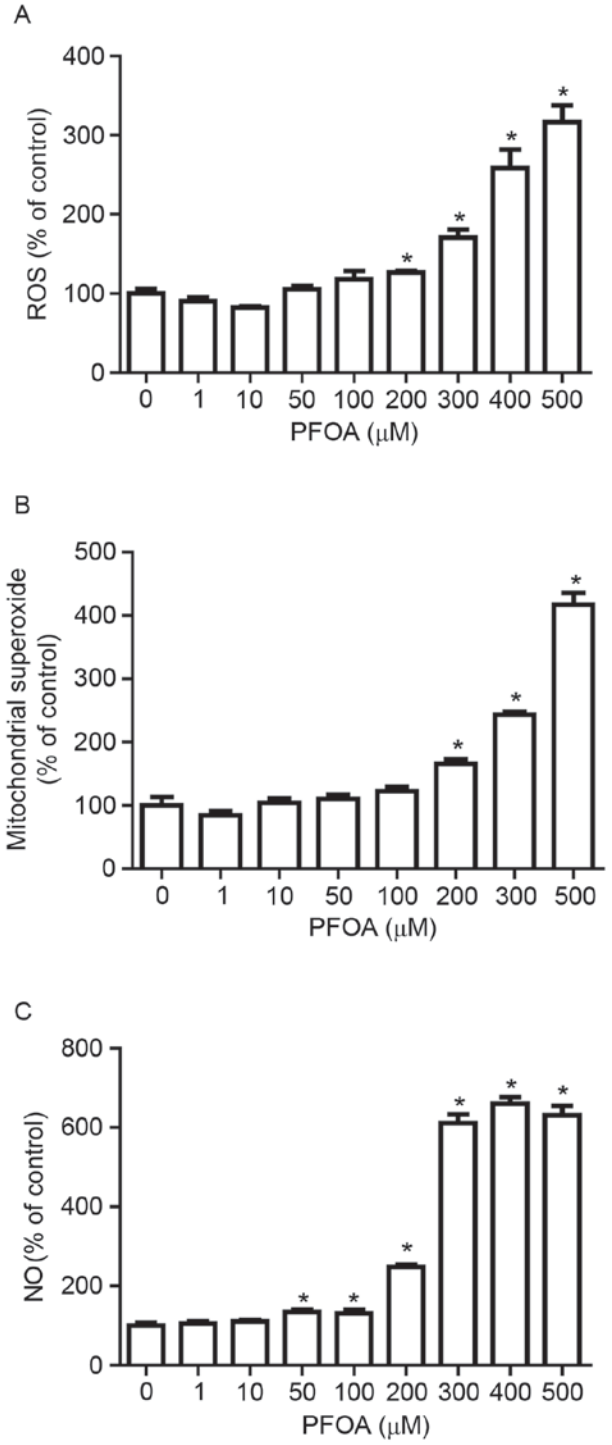

Figure 3. Effects of PFOA on oxidative stress in RIN-m5F cells. RIN-m5F cells were incubated with PFOA for 48 h. (A) Treatment with PFOA significantly increased ROS levels as measured by dichlorofluorescin fluorescence. (B) Mitochondrial superoxide levels were determined using MitoSOX™ Red mitochondrial superoxide indicator. (C) NO levels were measured using 2',7'-difluorofluorescein diacetate. Data are expressed as relative percentages of the control fluorescence (n=6). *P<0.05, vs. control. PFOA, perfluorooctanoic acid; ROS, reactive oxygen species; NO, nitric oxide.

fluorescence, compared with control cells, which was 4-fold greater following treatment with 500 μM PFOA. This result indicated that high concentrations of PFOA may increase superoxide accumulation in the mitochondria of RIN-m5F cells (Fig. 3B).

NO overproduction induces oxidative/nitrosative stress, which results in cell apoptosis or necrosis. The NO-specific DAF-FM probe was employed to investigate PFOA-induced NO production. NO production was greater in RIN-m5F cells incubated with PFOA for 48 h (≥50 μM) compared with control cells, up to 6.6-fold following treatment with 400 μM PFOA (Fig. 3C). This result suggested that PFOA may induce oxidative/nitrosative stress in pancreatic β-cells, via stimulation of NO overproduction.

PFOA increases cytosolic TNF-α and IL-1β levels in RIN-m5F cells. Proinflammatory cytokines activate various metabolic pathways in pancreatic β-cells that result in cell death (31). Therefore, the present study investigated whether PFOA modulates the production of the cytokines TNF-α and IL-1β. The production of TNF-α and IL-1β was significantly increased at PFOA concentrations ≥100 and ≥200 μM respectively; TNF-α was increased by 2.7-fold and IL-1β was increased by 4.6-fold following treatment with 500 μM PFOA (Fig. 4).

Effects of PFOA on MMP and ATP production in RIN-m5F cells. The irreversible loss of mitochondrial function is a

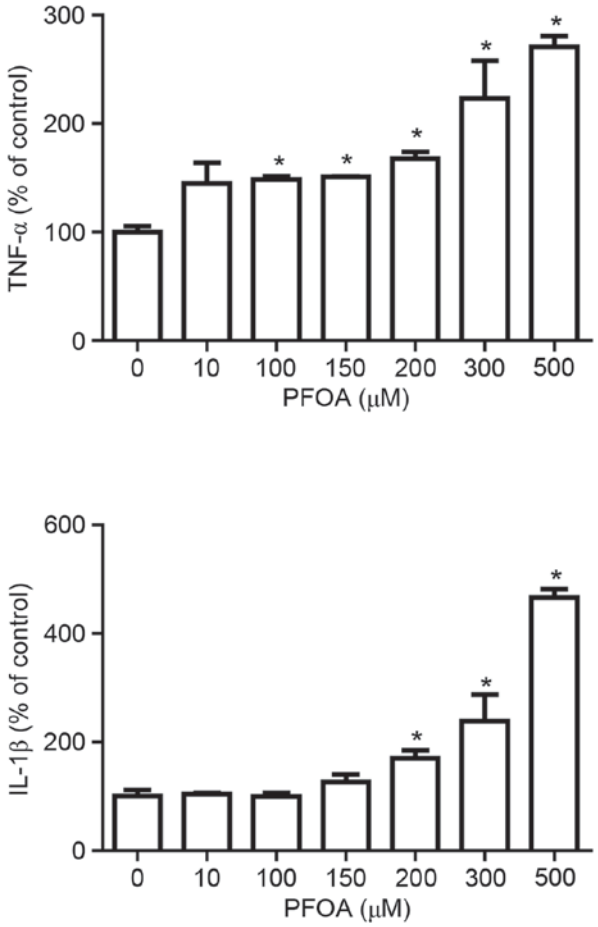

Figure 4. Effects of PFOA on cytosolic TNF-α and IL-1β levels in RIN-m5F cells. RIN-m5F cells were incubated with PFOA for 48 h. (A) TNF-α and (B) IL-1β control values were 1.28±0.071 and 2.56±0.29 ng/mg, respectively. Data are expressed as percentages of the control (n=6). *P<0.05, vs. control. PFOA, perfluorooctanoic acid; TNF-α, tumor necrosis factor α; IL-1β, interleukin-1β.

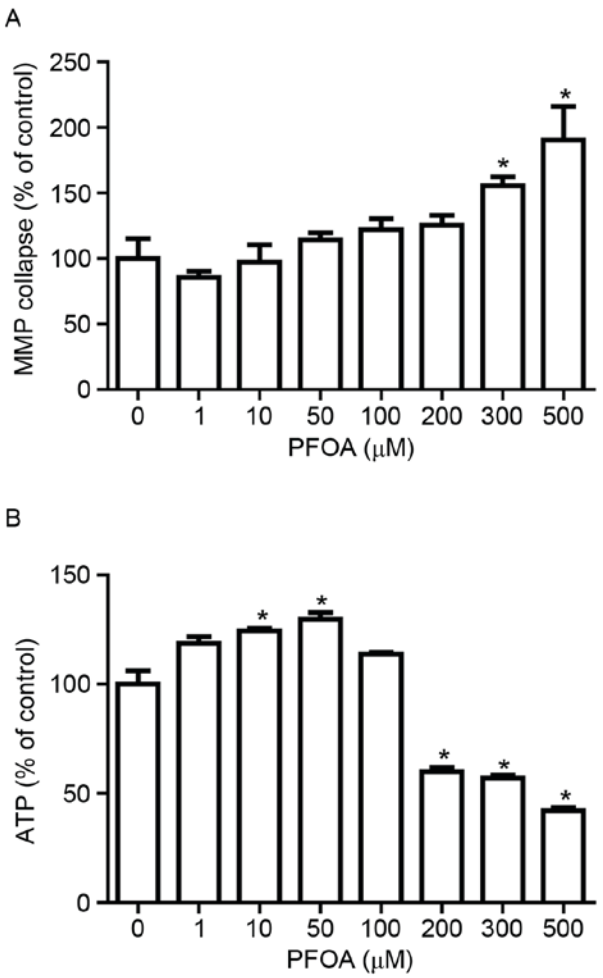

Figure 5. Effects of PFOA on MMP stability and ATP levels in RIN-m5F cells. RIN-m5F cells were incubated with PFOA for 48 h. (A) Rhodamine 123 was used to measure MMP collapse. Data are expressed as relative percentages of the fluorescence emitted by rhodamine 123 with respect to non-treated control cells (n=6). (B) Cellular ATP levels in control and PFOA-treated cells. The ATP control concentration was 0.326±0.019 nmol/mg. Data are expressed as percentages of the control (n=6). *P<0.05, vs. control. PFOA, perfluorooctanoic acid; MMP, mitochondrial membrane potential; ATP, adenosine triphosphate.

prerequisite for apoptosis (32). To determine whether PFOA treatment disrupts the MMP in RIN-m5F cells, MMP was evaluated using the fluorescent dye rhodamine 123, which accumulates in the mitochondrial compartment in an MMP-dependent manner. PFOA treatment (≥300 μM) for 48 h significantly disrupted the MMP in RIN-m5F cells, up to 1.9-fold following treatment with 500 μM PFOA (Fig. 5A). Living cells require a continuous supply of ATP to support the complex biological functions that are essential for survival, and ATP concentration is an important indicator of mitochondrial function. Intracellular ATP was measured in cells exposed to increasing concentrations of PFOA. ATP levels were significantly increased in response to low PFOA concentrations (10-50 μM), up to 1.3-fold following treatment with 50 μM PFOA (Fig. 5B). However, intracellular ATP decreased following exposure to high concentrations (≥200 μM) of PFOA, and had decreased to 42%, compared with the control, in cells treated with 500 μM PFOA.

Effects of PFOA on cytochrome c release and cardiolipin peroxidation in RIN-m5F cells. Mitochondrial cytochrome *c* release is a key event in apoptotic initiation. PFOA treatment (≥200 μM) stimulated a significant increase in cytochrome *c* release into the medium, and was increased 4-fold following treatment with 500 μM PFOA (Fig. 6A). Cardiolipin, which is a phospholipid found almost exclusively at the inner mitochondrial membrane, is an early target of oxygen-free radical attack due to its high content of unsaturated fatty acids. An oxidation-induced decrease in cardiolipin may facilitate the release of cytochrome *c* into the cytosol. The fluorescent probe NAO binds to cardiolipin, and this was used to determine whether PFOA induced cardiolipin peroxidation in RIN-m5F cells. NAO binding was reduced in cells treated with PFOA (≥200 μM), and was reduced to 27%, compared with the control, in cells treated with 500 μM PFOA, thus indicating the occurrence of mitochondrial PFOA-induced cardiolipin peroxidation (Fig. 6B).

Discussion

The present study demonstrated that PFOA induces apoptosis and impairs the viability of the rat pancreatic β-cell line RIN-m5F. Furthermore, PFOA damages β-cells by mediating

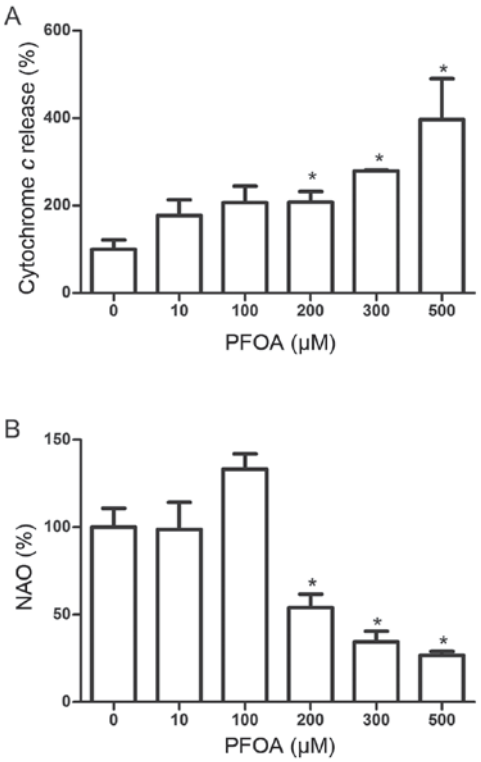

Figure 6. Effects of PFOA on cytochrome *c* release and cardiolipin peroxidation in RIN-m5F cells. RIN-m5F cells were incubated with PFOA for 48 h. (A) Cytochrome *c* release into the culture media was determined. The control value for cytochrome *c* was 1.31±0.286. (B) Cardiolipin oxidation was measured using 5 μM NAO. Data are expressed as relative percentages of the fluorescence emitted by bound NAO with respect to non-treated control cells; a decrease in NAO binding was related to cardiolipin peroxidation. Data are expressed as percentages of control (n=6). *P<0.05, vs. control. PFOA, perfluorooctanoic acid; NAO, 10-N-nonyl-Acridine Orange.

the accumulation of reactive nitrogen species (RNS) and ROS, and through mitochondrial dysfunction. To the best of our knowledge, the present study is the first to demonstrate a direct cytotoxic effect of PFOA on pancreatic β-cells. Treatment with PFOA independently disrupted MMP and reduced intracellular ATP levels in β-cells, thus suggesting that inhibition of mitochondrial metabolism may mediate this cytotoxic effect. Mitochondria are the main source of intracellular ROS; superoxide is produced at complexes I and III in the mitochondrial matrix and is converted to hydrogen peroxide (H_2O_2) by manganese superoxide dismutase (1). Pancreatic β-cells are particularly sensitive to destruction by mitochondrial ROS because the expression of antioxidant enzymes is relatively low in these cells (33). PFOA-induced alterations in mitochondrial membrane integrity result in the subsequent leakage of ions, which may affect the proton gradient and, subsequently, the oxidative phosphorylation required to produce ATP. Therefore, it may be hypothesized that in β-cells exposed to PFOA, RNS/ROS-induced oxidative stress damages the cell membrane, and the subsequent oxidative stimulus induces the cytosolic signaling pathway of the cell, disrupts mitochondrial function and initiates apoptosis.

The mitochondrial-mediated apoptotic pathway serves a major role in pancreatic β-cell death. Previous research has demonstrated that a common effector phase of mitochondrial permeability transition (MPT) may be shared by several types of apoptosis and necrosis (34). MPT is caused by the opening of a multiprotein complex pore positioned at a connecting point between the inner and outer mitochondrial membranes leading to MMP disruption, uncoupling of the respiratory chain and termination of ATP production, hyperproduction of ROS, Ca^{2+} release and depletion of reduced-glutathione, some of which may provoke necrosis (34). Furthermore, MPT is associated with the mitochondrial release of apoptosis-inducing factor and cytochrome *c*, which may activate caspases involved in the degradation phase of apoptosis (35). A decrease in cardiolipin levels induces cytochrome *c* detachment from the inner mitochondrial membrane, and facilitates the subsequent release into the cytosol (36). Once released into the cytoplasm, cytochrome *c* promotes assembly of the apoptosome in response to the cell-death stimulus, and activated caspase-9 subsequently induces the processing and activation of effector caspases, ultimately culminating in apoptosis (37). The discovery that PFOA treatment results in the release of mitochondrial cytochrome *c* indicates that this mechanism may serve an important role in the promotion of oxidative damage and pancreatic β-cell damage.

Mitochondrial ROS stimulate lipid peroxidation and reactive aldehyde formation in pancreatic β-cells, leading to the development of type 2 diabetes (38). The present study demonstrated that PFOA-induced apoptosis is associated with increased levels of mitochondrial ROS production and cardiolipin peroxidation. Cardiolipin is a unique dimeric phospholipid found almost exclusively in the inner mitochondrial membrane. This lipid contains a high percentage of unsaturated fatty acids and is readily oxidized by ROS, a step that is considered essential for the release of cytochrome *c* (39). Following mitochondrial damage, cardiolipin is repositioned to the outer membrane of the mitochondria where it functions as a recognition signal for dysfunctional mitochondria. Cardiolipin and cytochrome *c* interact at two sites on cytochrome *c* (40); cytochrome *c* can accept a hydrogen proton and it can oxidize cardiolipin with an extra oxygen molecule (41). Korytowski *et al* (42) demonstrated that oxidized cardiolipin species were significantly increased in the mitochondria, following exposure to apoptotic stress.

The outcomes of the present study are consistent with previous investigations into the toxicity of fluorochemicals. Panaretakis *et al* (43) observed MMP dissipation in HepG2 cells following incubation with PFOA. Starkov and Wallace (44) reported that treatment with perfluorinated derivatives induced collapse of the MMP and subsequent swelling of rat liver mitochondria. The study by Starkov and Wallace (44) indicated that PFOA may induce peroxisome proliferation and interfere with mitochondrial metabolic pathways. Free acid PFOA has previously been reported to induce a small increase in the intrinsic proton leak of the mitochondrial inner membrane, and the resulting alteration in membrane fluidity was similar to that induced by a surfactant (44). Peroxisome proliferators can slightly increase the steady-state level of H_2O_2 in rodents, perhaps due to an upregulation of acyl-CoA oxidase combined with a small increase in catalase activity (45), and it is well known that long-chain fatty acids are capable of inducing MPT *in vitro* (46).

The present study indicated that PFOA induces the production of high levels of NO. Increased generation of NO during insulitis may contribute to pancreatic β-cell destruction (47). NO can diffuse through the mitochondrial membrane and react with H_2O_2 in an environment rich in free-iron, which promotes the formation of hydroxyl radicals. The mitochondria are therefore the predominant site of hydroxyl radical formation in pancreatic β-cells, and the primary site of ROS toxicity, suggesting that mitochondrial damage may be responsible for PFOA-induced cell death. Catalase enzyme activity is reduced by the induction of iNOS and the accompanying production of NO, which binds to the iron moiety in the catalase heme groups (48). Furthermore, a reaction of NO with superoxide results in the production of peroxynitrite (49), a highly reactive oxidant species that is associated the development of autoimmune diabetes (50). Although the precise role of NO in the development of diabetes is not fully understood, synthesis of the NO radical contributes significantly to β-cell dysfunction and apoptosis. Previous research demonstrated that iNOS serves an important role in reduced mitochondrial function in β-cells and islets (51). Hirst and Robson (52) reported that NO initiated alterations in the MMP, and the subsequent release of cytochrome *c* induced apoptosis. Notably, cytokine-induced production of NO inhibits the mitochondrial enzyme aconitase, thus resulting in a subsequent reduction in ATP production, which may contribute to the promotion of necrotic cell death (53). The reaction of NO with superoxide mediates physiological processes, another method through which this may occur involves NO interaction with a metal at enzymatic active sites, particularly in the Krebs cycle, which ultimately results in significantly decreased glucose metabolism and ATP production (54). Therefore, it is possible that PFOA may exert its toxic effect through the damaging effects of NO on mitochondrial function.

Pancreatic β-cell damage-initiated diabetes is a complex process that is mediated, at least in part, by interactions among cytokines, NO and oxygen-free radicals with the target β-cells (55). The present study revealed that PFOA increased the release of TNF-α and IL-1β. These inflammatory cytokines are cytotoxic to β-cells *in vitro*, and they have demonstrated an ability to induce apoptosis in primary human (56) and mouse (57) pancreatic β-cells, possibly by stimulating NO synthesis (58). These cytokines increase mitochondrial ROS production in several cell types (59). Therefore, mitochondrial ROS serves an important role in cytokine toxicity. The mechanism that underlies mitochondrial ROS signaling in cytokine-induced apoptosis remains unknown, however it has been suggested that the destructive effects include cardiolipin peroxidation, MPT facilitation and inhibition of mitochondrial metabolism (60). Previous research has indicated that various inhibitors of NO generation may protect insulin-secreting cells against cytokine-mediated toxicity, however this occurs with variable efficiency depending on the chemical inhibitor involved and the cytokine combination (61).

In conclusion, the present study demonstrated that PFOA induced an increase in the production of RNS, ROS and inflammatory cytokines, and reduced ATP levels. Furthermore, PFOA induced MMP collapse and the release of cytochrome *c* from RIN-m5F rat pancreatic β-cell mitochondria. Therefore, these results indicated that PFOA may exert its cytotoxic effect on RIN-m5F cells through the increased oxidative stress and mitochondrial dysfunction associated with the induction of apoptosis.

Acknowledgements

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