Perfluorooctanoic acid promotes pancreatic β cell dysfunction and apoptosis through ER stress and the ATF4/CHOP/TRIB3 pathway

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Abstract
Perfluorooctanoic acid (PFOA), a widely used chemical substance, causes an increased risk of human type 2 diabetes (T2D), but its underlying mechanism is not well elucidated. The aim of the present study was to investigate whether PFOA regulates the functions of pancreatic β cells, which are specialized for the biosynthesis and secretion of insulin. The treatment of the mouse pancreatic β cell line (MIN6 cells) with PFOA caused a time- and dose-dependent inhibition of cell viability in CCK-8 assays. Annexin V/PI and TUNEL staining results confirmed that exposure to a high PFOA dose (500 μM) promoted apoptosis of β cells, while a low dose (300 μM) had no effects on β cell survival. PFOA treatment, even at a low dose, diminished glucose-stimulated insulin secretion (GSIS) in both primary islet perfusion and MIN6 cell experiments. RNA-sequencing data showed significantly increased expression of endoplasmic reticulum (ER) stress-associated genes, with \( \text{tribbles homolog 3 (Trib3)} \) ranking first among the altered genes. The activation of ER stress pathways was verified by qRT-PCR assays, and the ATF4/CHOP/TRIB3 pathway contributed to PFOA-induced β cell damage. The inhibition of TRIB3 expression significantly protected MIN6 cells from PFOA-induced GSIS defects and apoptosis by ameliorating ER stress. These findings reveal a link between ER stress and PFOA-induced β cell defects, opening up a new set of questions about the pathogenesis of T2D due to environmental chemicals.

Highlights
• PFOA exposure results in pancreatic β cell apoptosis.
• PFOA treatment diminishes glucose-stimulated insulin secretion in β cells.
• PFOA activates endoplasmic reticulum stress and increases TRIB3 expression.
• Inhibition of TRIB3 expression ameliorates PFOA-caused β cell dysfunction and apoptosis.

Keywords Perfluorooctanoic acid · Pancreatic β cell dysfunction · Apoptosis · Endoplasmic reticulum stress · TRIB3

Introduction
The burgeoning epidemic of type 2 diabetes (T2D), a disease associated with significant individual morbidity and mortality, threatens the stability of healthcare systems globally (Shrestha et al. 2018). Understanding the key drivers of this health crisis is vital for relieving the recent dramatic surge in T2D. The clear risk factors include genetic background, age, physical inactivity, and excess caloric consumption (Karnes et al. 2014). However, these factors alone still do not fully explain the prevalence and rapid growth of T2D worldwide (Sargis 2014). Recent studies have implicated that certain chemical pollutants may have endocrine-disrupting properties (Gore et al. 2015), which can cause disturbed insulin secretion, oxidative damage, and impaired signal transduction, thereby contributing to the occurrence and
development of T2D (Domazet et al. 2016; Kaur et al. 2016; Kurita et al. 2009).

One widely used chemical compound that is associated with serious health problems, including metabolic disorders such as T2D, is perfluorooctanoic acid (PFOA or C8) (Bach et al. 2015, 2016). PFOA does not occur naturally but can be introduced into the environment from various industrial and commercial products, such as surfactants, lubricants, emulsifiers, paper and textile coatings, nonstick frypan coatings, fire extinguishing foams, and food packaging (Calafat et al. 2007; Su et al. 2016). The median concentration of PFOA was 3 ng/L in surface wastewater (ranging from 0.011 to 65.7 ng/g) and 24 ng/L in wastewater (ranging from 1 to 1057.1 ng/g) (Zareitalabad et al. 2013). In addition to water samples, PFOA can also be detected in sediment (ranging from 0.018 to 203 ng/g), sewage sludge (ranging from 0.5 to 4780 ng/g), and soils (ranging from 0.1 to 47.5 ng/g) (Zareitalabad et al. 2013). Widespread exposure to PFOA has also been recognized in both occupational and general populations (Fei et al. 2007; Steenland and Woskie 2012), involving biosamples including plasma tissue, umbilical cord blood, and breast milk (Arbuckle et al. 2013; Fromme et al. 2010). PFOA has been banned in many countries and regions, but it persists in the environment because of its high stability and its long half-life in humans (Eriksson et al. 2017; Seo et al. 2018), which may indicate a role of bioaccumulation and would lead to long-term body burdens and health risks (Poet et al. 2016). Epidemiologic studies in the population excessively exposed to PFOA in the community have shown its associations with chronic diseases, including kidney and testicular cancers (Barry et al. 2013), high serum lipids (Steenland et al. 2009), and pregnancy-induced hypertension (Darrow et al. 2013). The negative effects of long-term exposure to PFOA are mainly highlighted in studies of disease risk caused by prenatal exposure to PFOA. Strong relationships between maternal and cord blood PFOA levels imply transfer from maternal circulation to the fetus (Aylward et al. 2014). Birukov et al. reported that PFOA exposure in early pregnancy was associated with maternal blood pressure trajectories in pregnancy, which may have a long-term impact on the health not only of the pregnant woman but also of her offspring (Birukov et al. 2021). PFOA concentrations in mid-pregnancy have been found to be associated with lower offspring birth weight (Starling et al. 2017), whereas some studies reported positive or null associations between prenatal PFOA exposure and body weight in childhood (Andersen et al. 2013; Braun et al. 2016). Additionally, a long-term follow-up study has demonstrated that higher prenatal PFOA exposure was related to delay of menarche (Kristensen et al. 2013).

PFOA is also viewed as a potential diabetogenic factor based on epidemiological data. For example, adverse effects of PFOA (median, 4.5; interquartile range (IQR), 2.1, 5.1 ng/ml) were shown in terms of diabetes risk in a representative National Health and Nutrition Examination Survey (NHANES) population (He et al. 2018). A twofold increase in diabetes mortality was found in workers exposed to PFOA (median, 403 ng/ml) compared with other non-exposed regional workers (Steenland and Woskie 2012). Another study on 258 pregnant women showed a positive association between exposure to PFOA (geometric mean, 3.94; 95% CI, 3.15, 4.93) and gestational DM risk (Zhang et al. 2015). As is well-known, the dysfunction of pancreatic β cells is known to be a key component in the pathogenesis of T2D. However, research elucidating the relationship between PFOA exposure and the function of pancreatic β cells is limited; therefore, further analysis of the exact links between PFOA exposure and β cell function is warranted.

Consistent with epidemiological research, experimental data have provided evidence to support an association between PFOA exposure and metabolome disorders. An in vitro study linked exposure to PFOA to apoptosis of pancreatic β cells, accompanied by increased oxidative stress and mitochondrial dysfunction (Suh et al. 2017). Similarly, mice showed the modulation of the phosphatidylinositol 3-kinase-serine/threonine protein kinase (PI3K-AKT) signaling pathway in their livers after 28 days of PFOA exposure by gavage; the PI3K-Akt pathway is considered a major regulator of cell survival in stressful conditions (Kaur et al. 2019; Yan et al. 2015b). The activation of peroxisome proliferator–activated receptors, which can influence insulin resistance and insulin secretion (Eldor et al. 2013), has also been demonstrated in response to PFOA (Lau et al. 2007; Ma et al. 2018). Taken together, this evidence suggests that multiple mechanisms may be involved in the PFOA-related risk of metabolic disorders. Notably, the direct effects of PFOA on pancreatic β cell function have not been reported. The biochemical processes and underlying molecular mechanisms induced by PFOA exposure need further research.

The aim of the present study was therefore to explore the effects of PFOA at a nontoxic level on a pancreatic β cell line and to reveal the potential molecular mechanism. The information obtained in this study could provide novel insights into the prediction, diagnosis, or treatment of T2D.

Materials and methods

Chemicals and reagents PFOA and β-mercaptoethanol were obtained from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco’s Modified Eagle’s Medium (DMEM) (glucose, 25 mmol/l), RPMI 1640 (glucose, 11.1 mmol/l), TRizol™, and Lipofectamine 2000 transfection reagent were purchased...
from Invitrogen Life Technologies (Grand Island, NY, USA). Fetal bovine serum (FBS), streptomycin sulfate, penicillin G sodium, and phosphate-buffered saline (PBS) were purchased from Gibco (Thermo Fisher Scientific, Waltham, MA, USA). A stock solution of PFOA (10 µM in dimethyl sulfoxide, DMSO, Sigma-Aldrich) was prepared and further diluted in DMEM. The final DMSO concentration was 0.1% in both the PFOA-treated and control groups.

**Cell culture** MIN6 cells, a transformed mouse pancreatic β cell line, are frequently used as in vitro models for insulin secretion (Yamato et al. 2013). MIN6 cells were obtained from Prof. Xiao Han (hanxiao@njmu.edu.cn; Nanjing Medical University) and maintained in DMEM (glucose, 25 mmol/l) supplemented with 15% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 50 µM β-mercaptoethanol (to enhance the antioxidation ability of MIN6 cells) at 37 °C in a humidified atmosphere containing 5% CO₂. The culture medium was changed daily, and the cells were passaged at a ratio of 1:2 every 3 days. The cells were confirmed to be β cells in the laboratory and were not contaminated by Mycoplasma spp.

**CCK-8 assay** Cell viability was determined with the Cell Counting Kit-8 (CCK-8) kit (Vazyme, Nanjing, China) following the manufacturer’s instructions. Briefly, MIN6 cells were plated in 96-well plates for 24 h and then treated with a gradient of PFOA concentrations (0, 0.1, 1, 3, 5, 10, 100, 300, 500, and 800 µM) to determine the concentration of PFOA that suppressed cell proliferation. After 6, 12, 24, 48, and 72 h, 10-µl CCK-8 solution was added to each well in darkness. The cells were incubated at 37 °C in 5% CO₂ for 2 h, and the absorbance at 450 nm was read with a SpectraMax 190 microplate reader (Molecular Devices, California, USA).

**Apoptosis assay** MIN6 cells were plated at a density of 1 x 10⁵ cells in 6-well plates. After a 24-h incubation, the cells were treated with PFOA (0, 300, or 500 µM) for 24 h. The analysis was performed using an Annexin V-FITC Apoptosis Detection Kit (BD Biosciences, Franklin Lakes, NJ) as described by Kaur et al. (Kaur et al. 2021, 2020b). The cells were then washed twice with PBS, trypsinized for collection, and stained with propidium iodide (PI) and annexin V for 30 min in the dark. Apoptosis was determined by flow cytometry in a FACSCalibur flow cytometer (BD Biosciences).

**Islet isolation and culture** Male C57BL/6 J mice were purchased from Nanjing University, China. The animal experiments were approved by the Institutional Animal Care and Use Committee at Nanjing Medical University. Islet isolation and culture were performed as previously described (Han et al. 2001). Briefly, cold type V collagenase (Sigma-Aldrich) solution was injected into the mouse pancreatic duct through a catheter. The expanded pancreas was removed, minced, and digested in a shaking water bath at 37 °C. After centrifugation, discarding sedimentation and extraction by histopaque®-1077 (Sigma-Aldrich), islets were manually picked out under the microscope. After extraction from the mouse pancreas, the islets were transferred to 6-well plates and cultured in RPMI 1640 (glucose, 11.1 mmol/l) supplemented with 10% (v/v) FBS, 10 mmol/l HEPES, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37 °C and 5% CO₂. The islets were equilibrated for 3 h and then counted and replated for further experiments.

**TUNEL staining** After equilibration, the primary islets were treated with PFOA (0 or 500 µM) for 48 h. TUNEL staining was performed using the TUNEL BrightRed Apoptosis Detection Kit (Vazyme, Nanjing, Jiangsu, China) according to the manufacturer’s protocol. Islets were observed with a laser scanning confocal microscope (FV1200, Olympus, Japan).

**Glucose-stimulated insulin secretion (GSIS)** MIN6 cells were seeded in 48-well plates at a density of 2 x 10⁵ cells per well for 24 h and then treated with PFOA (0, 300, or 500 µM) for 24 h. The cells were then incubated in HEPES-balanced Krebs–Ringer bicarbonate buffer (KRBH) for 1 h, followed by KRBH buffer with low glucose (2 mmol/l) for 1 h, and the medium was collected. The cells were then treated for 1 h with KRBH buffer containing a stimulatory glucose concentration (20 mmol/l), and the medium was collected. The collected media from the low glucose and stimulatory glucose conditions were used to determine the basal and stimulated insulin secretions, respectively. Insulin was extracted in an acid–ethanol solution (1.4% HCl:74% ethanol (v/v)).

**Primary islet perfusion assay** Perfusion analysis of insulin secretion from isolated primary islets was conducted after a 24-h treatment with 300 µM PFOA. Identical numbers of islets (120 islets per group) of equal size were placed on a nylon filter in a plastic perfusion chamber (Millipore Millex-GP) and perfused with KRBH or KRBH containing high glucose at a flow rate of 125 µl/min. The insulin levels were measured by radioimmunoassay (BNIBT, China). Data were normalized to the total cellular protein amount.

**qRT-PCR** Cells were plated in 6-well plates at a density of 2 x 10⁵ cells per well and treated with 300 µM or 500 µM PFOA for different times (6, 12, and 24 h). Total RNA was then extracted using TRIzol™ reagent according to the manufacturer’s protocol. After quantitative analysis by spectrophotometry, 1 µg of total RNA was reverse transcribed to complementary DNA (cDNA) using AMV Reverse Transcriptase (Promega, Madison, WI, USA). Quantitative real-time PCR was then performed using the cDNA, forward
and reverse primers, and SYBR Green PCR Master Mix (Applied Biosystems) on a LightCycler 480 II Sequence Detection System (Roche, Basel, Switzerland). The β-actin gene was utilized as an internal standard for quantifying other mRNAs. The sequences of the primers used are provided in Supplemental Table 1.

**Western blot analysis** MIN6 cells (2 × 10⁵ cells per well) were plated in a 6-well plate for 24 h and then treated with 300 or 500 µM PFOA for 24 h. The protocol for western blotting came from Bio-Rad, as described in detail in a previous study (Kaur et al. 2020a). The cells were lysed in ice-cold radioimmunoprecipitation assay buffer (RIPA, 50 mmol/l Tris–HCL, pH 7.4; 1% (v/v) NP-40; 150 mmol/l NaCl; 1 mmol/l EDTA; 1 mmol/l phenylmethylsulfonyl fluoride) mixed with a cocktail of protease inhibitors (Sigma-Aldrich) at a 1:100 dilution. The protein concentrations in the lysates were measured using BCA Protein Assays (Thermo Fisher Scientific), and 30–60 µg protein was separated by electrophoresis on an 8% or 10% polyacrylamide gel. The gel was blotted onto a PVDF membrane (Bio-Rad) and blocked in 5% (w/v) nonfat dry milk in Tris-buffered saline with Tween-20 (TBST buffer). The protein blots were incubated with the primary antibody at 4 °C overnight and then with the secondary antibody at room temperature for 1 h. The primary and secondary antibodies are shown in Supplemental Table 2. Primary antibodies were as follows: PARP 1 (Cell Signaling; 1:1000); cleaved PARP 1 (Cell Signaling; 1:1000); Caspase 3 (Cell Signaling; 1:1000); cleaved Caspase 3 (Cell Signaling; 1:1000); α-Tubulin (Sigma-Aldrich; 1:4000); p-IRE1α (Abcam; 1:1000); ATF4 (Cell Signaling; 1:1000); and TRIB3 (Proteintech; 1:1000). Secondary antibodies were goat anti-rabbit IgG-HRP and anti-mouse IgG-HRP (Cell Signaling; 1:4000). The blots were visualized by chemiluminescence detection (Pierce, Thermo Scientific, Waltham, USA), and the protein bands were analyzed with CLINX Image Analysis software. A visual chart was created to show the average stripe intensity of the study protein/internal reference protein ± the standard error of three experiments, as shown in a previous study (Li et al. 2021).

**RNA sequencing and analysis** The total RNA extracted from MIN6 cells was checked for quality and quantity using a NanoDrop and Agile 2100 bioanalyzer (Thermo Fisher Scientific, MA, USA). The mRNA was then purified, fragmented, end repaired, adapter ligated, and PCR amplified according to standardized protocols. The resulting library was assessed for quality and quantity using the Agilent 2100 bioanalyzer and then amplified on cBot to generate a cluster in the flow cell. The amplified flow cell was single-end sequenced on the HiSeq4000 or HiSeq X-ten platform.

Genes with an FDR value <0.05 were selected as differentially expressed genes (DEGs).

**Transient transfection** Silencer Select Validated siRNA against Trib3 and a scramble control (si-Tirb3, sense, TGC AGGAAAGAACCCTTTGGG, and antisense, CTCGTT TTAGGACTGGAGACTTG; negative control, sense, UUC UCCGAACGUGUCACGUTT, and antisense, ACGUGA CACGUUCGGAGAATT), which were obtained from GenePharma, Shanghai, China, were transfected into MIN6 cells using Lipofectamine 2000 transfection reagent according to the manufacturer’s protocol. The efficiency of TRIB3 knockdown was determined by western blotting after 24 h.

**Statistical analysis** Three independent repetitions were conducted for each experiment. All values, unless otherwise stated, are expressed as the mean ± standard error of the mean (SEM). Statistical significance was determined using the two-tailed Student’s t test or one-way analysis of variance (ANOVA), followed by Duncan’s test. Figures were generated using GraphPad Prism 7. A value of P <0.05 was considered statistically significant.

**Results**

**Effects of PFOA on pancreatic β cell viability** As shown in Fig. 1, PFOA supplied at 0.1–300 µM had no significant inhibitory effect on the cell viability of MIN6 cells during a 72-h exposure. However, 800 µM PFOA significantly reduced cell viability after 6 h or 12 h of treatment (Fig. 1A and B) (P = 0.034 and P = 0.021 vs control), and 500 µM PFOA inhibited cell viability by 76% after 24 h compared with untreated control cells (Fig. 1C) (P = 0.009). Similar decreases were observed after 48 and 72 h of exposure to 500 µM PFOA (Fig. 1D and E) (P = 0.006 and P = 0.004 vs control). PFOA did not show any significant time-dependent inhibition of MIN6 cell viability, except when administered at 800 µM (Fig. 1F).

**Effects of PFOA on pancreatic β cell apoptosis** The effects of PFOA exposure on apoptosis were assessed by flow cytometry analysis and TUNEL staining. Consistent with the CCK8 assay results, Annexin V/PI staining indicated no significant apoptosis in MIN6 cells treated with 300 µM PFOA for 24 h compared with untreated control cells (Fig. 2A and B). Conversely, appreciable apoptosis was evident in cells treated with 500 µM PFOA (Fig. 2A and B) (P = 0.007 vs control for apoptosis). Furthermore, the treatment of primary islets with 500 µM PFOA for 48 h increased the number of TUNEL-positive β cells (Fig. 2C and D) (P = 0.002 vs control). Treatment with 500 µM PFOA for 24 h increased the expression and cleavage of poly(ADP-ribose) polymerase 1.
PARP 1 and caspase-3 (Fig. 2E) \( (P = 0.005 \) vs control for cleaved PARP1 and \( P = 0.004 \) vs control for cleaved caspase 3), which are both molecular biomarkers of apoptosis (Jiao et al. 2020). Taken together, these results indicate that PFOA induces β cell apoptosis in both MIN6 cells and mouse islets.

Effects of PFOA on insulin synthesis and secretion

Since GSIS and insulin biosynthesis are the primary roles of β cells, we examined whether PFOA supplied at a nontoxic dose might affect insulin secretion. Radioimmunoassay results demonstrated that exposure to PFOA at 300 µM significantly inhibited high GSIS from MIN6 cells \( (P = 0.005 \) vs control), suggesting that PFOA induces β cell dysfunction at concentrations of 300 µM that do not affect cell survival (Fig. 3A and B). Exposure to 500 µM PFOA further inhibited GSIS function and reduced insulin biosynthesis (Fig. 3A to C) \( (all \ P < 0.01 \) vs control).

We validated and clarified these results by examining the effect of PFOA at 300 µM on insulin secretion from primary isolated islets. Notably, both the first and second phases of GSIS, as well as KCl-stimulated insulin secretion, were significantly reduced in perfused islets (Fig. 3D and E) \( (all \ P < 0.01 \) vs control). Thus, PFOA at nontoxic levels impaired both insulin secretion and biosynthesis.

Transcriptomic analysis following PFOA exposure

We sought to identify the potential mediator of PFOA effects on insulin biosynthesis and secretion by comparing differentially expressed genes (DEGs) in control and PFOA-treated MIN6 cells using bulk RNA sequencing. A total of 292 genes (136 upregulated and 156 downregulated) were differentially expressed in cells exposed to a low dose of PFOA (Supplemental Fig. 1). The top 54 genes with expression levels exceeding 0.02 were clustered in a heatmap, and Tribbles homolog 3 \( (\text{Trib3}) \) ranked first among all the altered genes (Fig. 4A). The selection of 10 potential genes for validation by qRT-PCR again identified Trib3 as the most affected by PFOA treatment of MIN6 cells at 300 µM (Fig. 4B), and this was also verified by western blotting (Fig. 4C and D).

PFOA-induced ER stress in pancreatic β cells

Trib3 is an ER stress-inducible gene that is induced by the ATF4-C/EBP
homologous protein (CHOP) pathway and participates in cell death (Ohoka et al. 2005). TRIB3 can interact with activating transcription factor 4 (ATF4), and this complex can inhibit insulin secretion by competitive inhibition of cAMP response element-binding (CREB) transcriptional activity (Liew et al. 2010). In our study, PFOA treatment of MIN6 cells activated TRIB3 and induced apoptosis, suggesting that PFOA treatment may have induced endoplasmic reticulum stress. Treatment with 300 and 500 µM PFOA for 12 h significantly increased the mRNA levels of Chop, Trib3, and Atf4 (Fig. 5A and B) (all P < 0.05 vs control). PFOA treatment for 12 and 24 h also increased the protein expression of p-inositol requiring enzyme (IRE)-1α, ATF4, and TRIB3 (Fig. 5C) (all P < 0.05 vs control), thereby confirming an enhancement of ER stress by PFOA treatment.

**TRIB3 depletion ameliorates PFOA-induced ER stress and inhibition of insulin synthesis and secretion** We examined the possibility that TRIB3 might mediate ER stress induced by PFOA by knockdown of TRIB3 with siRNAs. As shown in Fig. 6A, the first two columns of protein bands show the expression results of TRIB3 in cells without siRNA transfection, and the third column shows the expression results of TRIB3 in cells transfected with control siRNA. The expression of PARP1, cleaved PARP1, caspase 3, and cleaved caspase 3 in MIN6 cells treated with 300 µM or 500 µM PFOA for the indicated times was analyzed by western blotting. α-Tubulin was used as the internal control. The value results are expressed as the mean ± standard error of the mean (SEM) for three separate experiments. *P < 0.05, **P < 0.01 vs. control.

**Fig. 2** Effects of PFOA on apoptosis of MIN6 cells. A MIN6 cells were untreated or treated with PFOA at the indicated dose for 24 h, followed by annexin V/PI staining and flow cytometry analysis. B The percentage of apoptotic MIN6 cells expressed as a histogram. Upper left quadrant, dead cells; upper right quadrant, late stage of apoptosis; lower left quadrant, living cells; and lower right quadrant, early stage of apoptosis. C Primary mouse islets treated with 300 µM or 500 µM PFOA for 48 h were triple stained with Hoechst (blue), TUNEL (red), and insulin (green). Laser scanning confocal microcopy images were obtained for every islet (scale bar=50 µm). D TUNEL-positive β cells were counted. E The expression of PARP1, cleaved PARP1, caspase 3, and cleaved caspase 3 in MIN6 cells treated with 300 µM or 500 µM PFOA for the indicated times was analyzed by western blotting. α-Tubulin was used as the internal control. The value results are expressed as the mean ± standard error of the mean (SEM) for three separate experiments. *P < 0.05, **P < 0.01 vs. control.
TRIB3 mediates PFOA-induced cell apoptosis

We also determined whether TRIB3 was a mediator of PFOA-induced cell apoptosis by flow cytometry analysis of TRIB3 knockdown MIN6 cells exposed to PFOA. Treatment with 500 μM PFOA induced significant apoptosis of control cells, but no apoptosis was observed in TRIB3 knockout cells (Fig. 7A and B). Notably, PFOA was significantly less effective at activating apoptosis in TRIB3-depleted cells than in vehicle-treated cells transfected with control siRNA (Fig. 7A and B) (P < 0.01). TRIB3 knockdown also attenuated the increase in apoptosis-related proteins induced by PFOA treatment at 500 μM (Fig. 7C). Collectively, these data indicated a relationship between TRIB3 expression and PFOA-induced β cell apoptosis.

Discussion

In the present study, we aimed to investigate the potential role of PFOA in pancreatic β cell function and survival, and we demonstrated that PFOA at 300 μM significantly impaired the ability of MIN6 cells to perform GSIS, while PFOA at 500 μM induced apoptosis and decreased the viability of MIN6 cells. In addition, Trib3 was proven to be a PFOA-responsive gene that could mediate function and survival in pancreatic β cells.

To explain why the effect of PFOA on the functions of pancreatic β cells is not due to cell death, it is necessary to observe the effect of PFOA without decreasing cell viability,
which is a routine principle in toxicological experiments (Xu et al. 2015; Yuan et al. 2016). Hence, cell viability and cell apoptosis assays were performed in succession to look for a nontoxic dosage for pancreatic β cells. As shown in the results of the cell viability experiment, PFOA treatments after both 24 h and 48 h treatment did not affect cell viability at 300 μM, while PFOA treatment at 500 μM caused decreased cell viability; similar results were found in the apoptosis experiment. Therefore, PFOA at 300 μM and 500 μM was applied to pancreatic β cells in subsequent experiments.

Next, we used pancreatic β cells to observe the ability of insulin secretion stimulated by high glucose, which was the main purpose of our study. A primary islet perfusion assay was also applied to observe the differences in insulin secretion phases. When feeling high blood glucose, pancreatic β cells secrete insulin in a biphasic pattern (Cheng et al. 2013). Therefore, relative to the GSIS experiment conducted in Min6 cells, the GSIS perfusion assay with primary islets can show the response of different stages of insulin secretion to PFOA treatment, as well as the evaluation of basal, glucose stimulation, and total insulin secretion, to accurately evaluate β cell function ex vivo (Zhu et al. 2021). In our results, both the first and second phases were affected by PFOA treatment at 300 μM, indicating impaired insulin secretion and biosynthesis. Notably, the process of GSIS in a single β cell includes multiple procedures, such as glucose internalization through the transmembrane, energy metabolism, protein synthesis, the activation of different ionic channels, and the transport of insulin granules (Hiriart et al. 2014). For this reason, further experiments need to be performed to uncover which process is influenced by the effect of PFOA precisely.

There is a discrepancy in reports pertaining to the relationship between PFOA exposure and β cell function. For example, a prospective association analysis in a multicenter cohort study demonstrated that exposure to PFOA (median, 9.7; IQR, 7.7; 12.1 ng/ml) in childhood was associated with
impaired β cell function at 15 years of age (Domazet et al. 2016). However, strong positive associations between PFOA concentrations (geometric means, 2.20; SEM, 1.02; log PFOA, 1.48; SEM, 0.04, respectively) and β cell function were found in adults from the NHANES study 1999–2004 and 2013–2014 (Lin et al. 2009; Liu et al. 2018). The reasons for the contradictory results between studies are unclear but probably come from multiple confounding factors and studies with cross-sectional attributes.

As emphasized in a previous study, the extrapolation and interpretation of data between experimental animal studies (in a high range) and the general population (in a low range) should be carefully considered (Lin et al. 2009). Actually, the concentration of PFOA used in this study (300–500 µM) was 2 orders of magnitude higher than that measured in the occupational population (400 ng/ml). However, it must be noted that exposure to low-dose perfluoroalkyl chemicals may have an impact on glucose metabolism in the general population. Fundamentally, our results highlight the fact that PFOA is predisposed to impair β cell function at nontoxic levels for cells.

PFOA has been reported to induce apoptosis in different tissues and cells (Bassler et al. 2019; Zhou et al. 2020). For example, Suh et al. reported that 100 µM PFOA could induce apoptosis of RINm5F cells, a rat pancreatic β cell line (Suh et al. 2017). The effectiveness of this much lower concentration compared to the higher concentrations required in the present study may reflect a difference in the tolerance of different cell types.

![Fig. 5](image-url) PFOA treatment can induce ER stress in pancreatic β cells. MIN6 cells were treated with 300 or 500 µM PFOA for 3, 6, 12, or 24 h, and RT–PCR was performed to measure the mRNA levels of Trib3, Chop, and Atf4 (A and B). The protein expression levels of p-IRE1α, ATF4, and TRIB3 in MIN6 cells treated with 300 or 500 µM PFOA for 12 or 24 h were analyzed by western blotting. α-Tubulin was used as an internal standard. The results are presented as the mean ± standard error of the mean (SEM) for three separate experiments. *P < 0.05, **P < 0.01 vs. control.
types to PFOA. Suh et al. implicated mitochondrial-mediated apoptosis as a mechanism, whereas our results suggest that activation of the ER stress pathway may mediate the apoptosis induced by PFOA in MIN6 cells. The ER stress response reflects a change in the homeostatic response to cellular stress (Kaufman 1999). It prevents protein folding and leads to the accumulation of misfolded proteins in the ER; ultimately, the excessive or long-term accumulation of unfolded proteins induces apoptosis. A previous study showed that PFOA can induce ER stress, and this stress plays an important role in the liver lipid metabolism disorder induced by PFOA (Yan et al. 2015a).

In our experiments, RNA-sequencing analysis was performed to seek the underlying mechanism involved in pancreatic β cell dysfunction induced by PFOA treatment, which identified Trib3 as ranking first among all responsive genes. Consequently, a Trib3 depletion experiment was performed to observe its role in endoplasmic reticulum stress and apoptosis induced by PFOA. TRIB3 (also named SIKP3, NIPK, and TRB3) was originally identified as a mitotic blocker in Drosophila (Grosshans and Wieschaus 2000), but it was subsequently shown to regulate a number of cellular functions ranging from cell differentiation to cell growth, survival, and apoptosis (Ord et al. 2015). Under physiological conditions, the expression levels of TRIB3 mRNA and protein are very low and are induced by ER stress in some cells (Ohoka et al. 2005). However, in our study, we found that TRIB3 can mediate the induction of ER stress by PFOA. This may reflect the action of TRIB3 as a sensor of ER stress-induced cell death (Prudente et al. 2012). If ER stress is mild and transient, TRIB3 is induced to bind to and inhibit ATF4/Chop, thereby promoting cell survival. However, when long-term and intense ER stress occurs, significant and long-term overexpression of TRIB3 leads to apoptosis.

In recent years, the overexpression of TRIB3 has been reported in the liver, adipose tissue, skeletal muscle, and pancreatic β cells of patients with insulin resistance and/or T2D (Oberkofler et al. 2010). Research has indicated that
TRIB3 mediates glucose-induced insulin resistance through the hexosamine biosynthesis pathway (Zhang et al. 2013) and that TRIB3 binds to a phosphorylation site of AKT, thereby blocking AKT phosphorylation and activation in response to insulin (Du et al. 2003). In our study, insulin synthesis in MIN6 cells was also decreased by exposure to 300 µM PFOA, which may reflect an inhibition of AKT phosphorylation by TRIB3.

Compared with the effect of TRIB3 on insulin resistance and insulin synthesis, its effect on insulin secretion has seldom been reported. One study found that vesicular exocytosis was reduced after overexpression of TRIB3 in islet β cells, resulting in decreased numbers of insulin granules, whereas knockout of TRIB3 in islet β cells restored insulin secretion (Liew et al. 2010). Qian et al. demonstrated that induction of TRIB3 impaired GSIS in INS-1 cells (Qian et al. 2008), in agreement with the inhibitory effects on insulin secretion observed in our study. Interference with Trib3 expression partially restored GSIS function in MIN6 cells treated with PFOA, providing molecular evidence for damage to GSIS in pancreatic β cells exposed to PFOA.

In general, the current study demonstrated the involvement of TRIB3 in ER stress as a mechanistic link between PFOA exposure and β cell dysfunction and apoptosis. These findings provide strong support for a PFOA effect on glucose metabolism and dysfunction of pancreatic β cells. Taken as a whole, these findings mandate efforts to understand the mechanism of PFOA exposure, as well as to formulate policies to limit emissions of PFOA to the environment.

**Conclusion**

Our study characterized a novel PFOA-induced mouse pancreatic β cell dysfunction and subsequent apoptosis. The activation of ER stress signals and TRIB3-involved metabolism appears to be important components of the mechanism that leads to the perturbation of cellular functions by PFOA at nontoxic levels.
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Data availability The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval The animal experiments were approved by the Institutional Animal Care and Use Committee at Nanjing Medical University.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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