Perfluorooctanoic acid impaired glucose homeostasis through affecting adipose AKT pathway

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Abstract Perfluorooctanoic acid (PFOA) is commonly applied in manufactured products, and its potential health risk is concerned greatly. Increasing evidences have indicated PFOA-induced liver dysfunction. However, detailed molecular mechanism has not been completely identified. In this study, we aimed to investigate the mechanical association between PFOA exposure and AKT pathway in white adipose tissue. As results, PFOA-treated mice showed increased blood glucose and insulin levels, and induced insulin resistance. In addition, serum levels of leptin and adiponectin in PFOA-treated mice were elevated. As shown in histological examination, increased cell death counts in PFOA-treated adipose were observed, as well as ultrastructural impairment in adipose cells was found. Further, immunohistochemical stains exhibited GLUT4, p-AKT positive cells were down-regulated in PFOA-treated adipose, while PTEN immune-labeled cells were reduced. In validated data, RT-PCR assay suggested adipose AKT mRNA was down-regulated in PFOA-treated mice, and PTEN mRNA was increased. Western blot data showed that intracellular PTEN protein level in PFOA-treated adipose was up-regulated, while phosphorylation of AKT, GSK3 β levels were lowered

dose-dependently. Taken together, the present findings indicate that PFOA impaired glucose homeostasis via negatively regulating AKT pathway in white adipose tissue.

Keywords PFOA · Adipose · AKT pathway · Metabolism

Introduction

Perfluorooctanoic acid (PFOA), simply termed as C₈, is commonly used in the process of manufacturing fluoropolymer chemicals (Nicole 2013). Notably, PFOA has a potential health risk as it can accumulate in the environment and human body for a long period of time (Post et al. 2012). Multiple studies have found that PFOA is linked to induction of insulin-dependent metabolic disorders in wildlife and humankind (Steenland et al. 2015). Insulin-sensitive adipose tissue is closely associated with insulin sensitivity or resistance in metabolic diseases, characterized with glucose dysmetabolism (Mlinar and Marc 2011). In physiology, adipose tissue functions as a vital endocrine organ that secretes metabolism-regulated hormones including leptin, estrogen, resistin, and other cytokines (Ercin et al. 2015). Adipose-originated cells play a major physiological role in maintaining triglyceride and free fatty acid levels in the body, and may induce insulin resistance in case of adipose impairment (Patel

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et al. 2016; Tam et al. 2010). Increasing epidemiological evidences suggest that PFOA pollutant is detected in human body widely, such as adult blood or cord blood, breast milk and liver tissue (Domingo 2012; Fromme et al. 2009). Due to potential adverse health risks, manufacturing PFOA has been phased out in most developed nations. However, some developing countries are still using PFOA-containing products (Chang et al. 2016). More notably, PFOA-induced liver lipid disturbance is responsible for promotion of nonalcoholic liver disease via pleiotropic actions (Das et al. 2017). However, potential impact of PFOA exposure on hormone-produced adipose cells is less been investigated. Thus, this study was designed to investigate the underlying molecular mechanism that is responsible for PFOA-induced adipose dysfunction and resultant glucose disorder through a group of biochemical tests and immunoassays.

Methods

Reagents

PFOA (96% purity) was purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). Notably, other chemicals and laboratory materials were indicated in respective experimental sections.

Animal design

Male Balb/c mice (aged 6–7 weeks) were obtained from the Experimental Animal Centre of Weifang Medical University (Permit No. 2014-0002). Animal study was performed in accordance with the protocols of *Institutional Ethical Committee* in Weifang Medical University.

All mice were housed under controlled temperature (20 ± 2 °C) and cycled light (12 h/12 h) to acclimatization for 7 days. The mice were randomly assigned as follows, dosed mice were orally given 1 and 5 mg PFOA/kg (dissolved with DMSO and then further mixed with peanut oil for getting a final DMSO concentration of 0.5%) per day for 3 weeks; PFOA-free mice were orally given a similar volume of 0.5% DMSO-containing peanut oil as a control. All mice were given with standard rodent chow (Beijing science and Technology Co., Ltd, China) and had access to distilled water ad libitum. At the end of

experiment, mouse blood was collected from retro-orbital venous plexus, and serum was prepared by centrifugation of 3000 rpm at 4 °C for 10 min, prior to being stored at -20 °C immediately until further test. In addition, abdominal white adipose was removed for respective biochemical assays and histological examinations.

Blood glucose and insulin measurements

The fasting blood glucose contents (24 h) were ($n = 8$ per group) measured by using an ACON-Biotech Glucometer (Hangzhou, China). Furthermore, serum insulin content was measured by using a mouse insulin ELISA kit (Shanghai Elisa Biotech Co., Ltd, China) according to the manufacturer's protocols. The homeostatic model assessment of insulin resistance (HOMA-IR) index was calculated by blood glucose (mmol/L) $\times$ insulin (mU/L)/22.5 (Wan et al. 2014).

Serum hormone analysis

Blood hormonal concentrations of adipose-produced leptin and adiponectin in mice ($n = 8$ per group) were assayed by using a commercially available ELISA kits (Shanghai Elisa Biotech Co., Ltd., China) in according to the manufacturer's instructions (Li et al. 2016a).

Pathophysiological examination

As described previously (Li et al. 2016b, 2017), ventral adipose tissues ($n = 5$) were fixed with 4% paraformaldehyde. Paraffin-embedded adipose was prepared as 5 μ m section for staining with hematoxylin and eosin (HE). Five nonoverlapping fields of view were selected for assaying the counts of death cells in each view, and then the mean value was converted to the number of cells per square millimeter.

In brief, the dewaxed sections were ($n = 3$ per group) subject to blocking by 5% BSA for 1 h at room temperature. After washing for three times, the sections were incubated with anti-glucose transporter 4 (GLUT4) and p-AKT antibodies (1:200; Boster, Wuhan, China) overnight at 4 °C, followed by incubation with horseradish peroxidase (HRP) conjugated anti-rabbit secondary antibody (1:200; Boster, Wuhan, China) for 1 h at room temperature. Correspondingly, chromogenic diaminobenzidine (DAB) served as a HRP developer prior to nucleus being stained with

haematoxylin. Finally, the section was mounted and imaged, and assay data were calculated. Five nonoverlapping fields of view were selected for assaying the numbers of positive cells in each view, and then the mean value was converted to the counts of positive cells per square millimeter.

In transmission electron microscopy (TEM) protocol, adipose samples were ($n = 3$ per group) fixed in 2.5% glutaraldehyde at pH 7.2 for 2 h and post-fixed in 1% osmium tetroxide (OsO_4) in 0.1 M cacodylate buffer for 1 h. Then, samples were dehydrated through an ethanol series and transferred to several changes of a transitional solvent, propylene oxide and embedded in epoxy resin. The samples were sectioned and stained with 2% uranyl acetate/lead citrate. Subsequently, all sections were examined and captured by using a TEM Imager working at 80 kV (Li et al. 2012).

Real time-PCR assay

Mouse adipose RNAs were ($n = 5$ per group) extracted with Trizol solution (Thermo Fisher Scientific, Waltham, MA, USA). Purified RNA was reverse transcribed to cDNA by using a Revert Aid First Strand cDNA Synthesis Kit (TIANGEN Biotech Co., Ltd., Beijing, China) following the manufacturer's instructions. Adipose AKT, PTEN mRNA expressions were measured by using quantitative real-time PCR of ABI PRISM 7500 Sequence Detector System (Applied Biosystems, Carlsbad, CA, USA). The threshold cycle (C_t) reading was recorded and the relative expressions of AKT, PTEN mRNA was calculated with $2^{-\Delta\Delta C_t}$ method. Transcript mRNA levels were normalized to levels of GAPDH in all samples for final result (Chen et al. 2015a, b).

Western blotting test

Fresh adipose proteins were ($n = 5$ per group) extracted by using a lysis buffer (Beyotime, Shanghai, China) containing 1 mM protein inhibitor (Beyotime, China). Adipose protein content was calculated by using a BCA protein assay kit (Beyotime). Each sample, loaded at equal protein amount (20 μg per lane) was subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by blotting to polyvinylidene fluoride membranes (Beyotime). The membrane was blocked with 5% non-fat milk buffer for 1 h at 37 °C, and then incubated with

primary antibodies (1:500, anti-PTEN, anti-GSK3 β , anti-phospho-GSK3 β , anti-AKT, anti-phospho-AKT antibodies; Beyotime) at 4 °C overnight. After being washed with TRIS-buffered saline/0.1% tween 20 for 3 times, membrane was incubated with horseradish peroxidase-coupled secondary antibodies (Beyotime) for 1 h at 37 °C. Membrane-blotted protein bands were developed by using chemiluminescence (ECL) detection kit (Beyotime) and assayed by an Imager (Bio-Rad, Hercules, CA, USA), as described previously (Wang et al. 2014; Zhao et al. 2016).

Statistical analysis

Statistical data were analyzed through statistical product and service solutions (SPSS) 19.0 software. Differences between comparable data were determined by a one-way analysis of variance (ANOVA) with Student's t test. Results were expressed as mean $\pm$ SD, when a P less than 0.05 was considered as statistically significant.

Results

PFOA induced changes of metabolic parameters in blood

Male Balb/c mice were orally dosed every day with 1 and 5 mg/kg/PFOA for 3 weeks. Sera were collected on day-21 and analyzed by commercial biochemical kits. Our results showed that the body weight of PFOA-treated mice was reduced time-dependently ($P < 0.05$), and the blood glucose level in PFOA-treated mice was increased when these comparable data showed statistically significant ($P < 0.05$). In addition, blood insulin concentration was elevated in PFOA-treated mice in a dose-dependent manner, and increased levels were greater than that in control ($P < 0.05$). Further, HOMA-IR (homeostatic model assessment of insulin resistance) was found to be significantly greater in PFOA-treated mice when compared to the control ($P < 0.05$). In serological tests, the hormonal levels of leptin and adiponectin in PFOA-treated mice were reduced dose-dependently, especially in 5 mg/kg PFOA treatment. Notably, these levels were greater than those in control mice ($P < 0.05$) (Fig. 1).

Fig. 1 PFOA induced changes of metabolic parameters in blood. PFOA-treated mice showed body weight loss time-dependently, while the blood glucose level and insulin content

were elevated, characterized by increased HOMA-IR. Moreover, the hormonal levels of leptin and adiponectin in PFOA-treated mice were reduced in a dose-dependent manner

PFOA induced cytoarchitectural changes of adipose tissue

As shown in HE stain, the white adipose tissue of mice treated with a dose of 5 mg PFOA/kg showed visible cell atrophy, nucleolus deformation, and cytoskeletal impairment. In ultrastructural observations, adipose cells in PFOA-free mice exhibited intact nucleolus and plenty of organelles. However, PFOA-exposed adipose cells showed lesioned nucleolus, vacuolation, and reduced organelles. In addition, cell death number in PFOA-exposed adipose tissue was greater than that in control ($P < 0.05$) (Fig. 2).

PFOA induced changes of mRNAs in adipose tissue

In order to assess the impact of PFOA on AKT pathway in adipose cells, some key effectors of mRNAs were determined by using RT-PCR assay. As results, adipose of PFOA treated mice showed reduced AKT mRNA when compared to that in control ($P < 0.05$), while adipose PTEN mRNA level in PFOA-treated mice was up-regulated dose-dependently ($P < 0.05$) (Fig. 3).

PFOA induced changes of proteins in adipose tissue

Based on the results of PFOA-impaired serum hormones, we further assessed possible mechanism of

Fig. 2 PFOA induced cytoarchitectural changes of adipose tissue. PFOA-treated mice showed cell atrophy, nucleolus deformation, and cytoskeletal loosening in white adipose tissue.

In ultrastructural observations, PFOA-exposed adipose cells showed lesioned nucleolus, vacuolation, and reduced organelles

Fig. 3 PFOA induced changes of mRNAs in adipose tissue (RT-PCR assay). As results, AKT mRNA levels in PFOA-exposed adipose cells were decrease, while adipose PTEN mRNA content in PFOA-exposed mice was up-regulated dose-dependently

PFOA-induced glucose disorder, the expression of some vital proteins of AKT signaling pathway was assessed in adipose tissue through immunostainings. In immunohistochemistry and immunofluorescence assays, the number of GLUT4-/p-AKT-positive cells was reduced and that of PTEN-labeled cells was increased in PFOA-treated adipose in a dose-dependent manner. In addition, these positive cell counts showed statistical significance when compared to those in control ($P < 0.05$) (Fig. 4).

PFOA induced changes of AKT pathway in adipose tissue

To further validate the proposed mechanism of PFOA-impaired AKT pathway, western blotting was performed for these key effector proteins. In

Fig. 4 PFOA induced changes of proteins in adipose tissue (immunostaining, scale bar = 100 μ m). In immunohistochemistry and immunofluorescence assays, GLUT4/p-AKT-positive cells were reduced and PTEN-labeled cells were increased in

consequence, significant reductions of phosphorylated AKT (Ser473) and phosphorylated GSK3 β in PFOA-treated adipose cells were observed when compared to those in untreated control ($P < 0.05$), while intracellular PTEN expression was elevated dose-dependently ($P < 0.05$) (Fig. 5).

PFOA-treated adipose were observed in a dose-dependent manner. In addition, these positive cell counts showed statistical significance when compared to those in control. Scale bar: 100 μ m

Discussion

Environmental exposure to perfluoroalkyl acids (PFAAs) is positively related to development of metabolic disease (Lin et al. 2009). Epidemiological evidences have also suggested potential association between PFAAs exposure and glucose disorder (Fisher et al. 2013). Perfluorooctanoic acid (PFOA), one of representative PFAAs, has potential health

Fig. 5 PFOA induced changes of AKT pathway in adipose tissue (western blot assay). In consequence, significant reductions of phosphorylated AKT (Ser473) and phosphorylated GSK3β in PFOA-treated adipose cells were observed when

compared to those in untreated controls, while intracellular PTEN expression was elevated dose-dependently. Abbreviations: *p-AKT* = phosphorylated AKT, *T-AKT* = total AKT, *p-GSK3β* = phosphorylated GSK3β, *T-GSK3β* = total GSK3β

concern in connection with metabolic dysfunction (Hui et al. 2017). However, molecular mechanism of bioeffect of PFOA-impaired adipose on glucose disorder remains less investigated. In this study, PFOA-treated mice were dose-dependently resistant to endogenous glucose and insulin, as shown in increased blood glucose and insulin. Some reports showed that adipose-secreted leptin can regulate insulin-dependent glucose metabolism, and leptin inhibits hyperglycemia in diabetic animals via a molecular mechanism of modulating the neuroendocrine system (German et al. 2011). Adiponectin, a key adipose-produced hormone, functions as an effector in regulation of glucose homeostasis. Notably, increased blood adiponectin is found to be linked to induction of insulin-independent glucose tolerance (Hwang et al. 2012). In the present study, we found that PFOA-treated mice resulted in reduced blood adipose-produced hormones of leptin and adiponectin that might be responsible for inducing insulin-dependent glucose dysmetabolism. These impaired hormonal levels were consistent with elevated blood glucose in PFOA-treated mice. However, the biological effects of PFOA-treated adipose need to be further investigated.

In biology, adipose cells chiefly play role in storing energy in form of lipids, and it has been considered as a key endocrine tissue characterized with hormonal function (Troike et al. 2017). In addition, PFOA can widely accumulate in body tissue, resulting in possible adverse effect on targeted tissue cells (Hines et al.

2009). GLUT4 is an insulin-regulated glucose transporter expressed mainly in adipose and muscle tissues (Smith and Kahn 2016). In order to assess biological mechanism of PFOA-impaired glucose, the expression of adipose GLUT4 that regulates glucose uptake was determined by using immunohistochemistry analysis. As a result, reduced adipose GLUT4 expression in PFOA-treated mice suggested PFOA-induced dysfunction of adipose on glucose uptake. And abnormal GLUT4 expression might be linked to changed cytoarchitecture in adipose, as shown in morphological and structural observations. Further, impaired GLUT4 function might be related to increased blood glucose and insulin resistance induced by PFOA, as shown in increased HOMA-IR.

Biologically, AKT signaling pathway exerts a key role in the metabolic homeostasis in an insulin-dependent manner (Beg et al. 2017). AKT, also termed as PKB (protein kinase B), is activated in phosphorylated way when induced by insulin and insulin-like growth factor (Hernandez et al. 2001). The phosphatase and tensin homologue (PTEN), an inhibitor of the AKT pathway, can be indirectly involved in metabolic functions (Tsai et al. 2017). To characterize adverse effect of PFOA on glucose metabolism, we tested these key effectors of AKT pathway in further experiments. In accordance with the changes of phosphorylated AKT and PTEN expressions in PFOA-treated adipose cells, increased blood glucose and insulin resistance were observed in a dose-dependent manner, indicating insulin-

dependent glucose disorder. Interestingly, decreased mRNA of AKT and unchanged level of AKT in PFOA-treated adipose were observed. These outcomes indicated that PFOA inhibited adipose AKT expression to respond potential stresses. Moreover, glycogen synthase kinase-3 (GSK-3) refers to a serine-threonine kinase that functions as a regulator enzyme in glycogen synthase (Aoki et al. 2004). Our results exhibited that reduced phosphorylation of AKT and GSK3 β levels, and increased PTEN expression, were detected in the PFOA-treated adipose cells when compared to those in controls. These findings indicated that PFOA disrupted glucose metabolism and hormonal homeostasis, as well as impaired AKT pathway in adipose tissue.

Conclusions

Overall, our findings demonstrate that PFOA may impair adipose AKT pathway in association with glucose metabolism. In addition, further study needs to be conducted for better understanding molecular mechanism involved.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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