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Perfluorooctanoic acid (PFOA) Activates the Unfolded Protein Response in Pancreatic Acinar Cells

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Abstract

Perfluoroalkyl substances, such as perfluorooctanoic acid (PFOA), are widely used in consumer and industrial applications. Human epidemiologic and animal studies suggest that PFOA exposure elicits adverse effects on the pancreas; however, little is known about the biological effects of PFOA in this organ. In this study, we show that PFOA treatment of mouse pancreatic acinar cells results in ER stress and activation of the PERK, IRE1 α , and ATF6 arms of the Unfolded Protein Response (UPR) pathway. PFOA-stimulated activation of the UPR was blocked by pretreatment with specific PERK and IRE1 α inhibitors and the chemical chaperone 4-phenyl butyrate, but not the antioxidants N-acetyl cysteine and Tiron. PFOA treatment led to increased cytosolic Ca⁺² levels and induction of the UPR was blocked by an inhibitor of the inositol 1,4,5-trisphosphate receptor (IP₃R). These findings indicate that PFOA-induced ER stress may be the mechanistic trigger leading to oxidative stress in the pancreas.

Keywords

perfluorooctanoic acid; PFOA; ER stress; pancreas; pancreatic acinar cell

1 INTRODUCTION

Perfluorooctanoic acid (PFOA) is a chemical that is widely used in consumer and industrial applications. PFOA does not readily decompose in the environment and due to widespread human exposure¹, detectable levels of PFOA are found in 98% of the American population^{2,3}. Human epidemiological evidence and animal studies demonstrate that PFOA adversely affects the pancreas. PFOA exposure is associated with hyperlipidemia, a cause of both acute and chronic pancreatitis, in occupationally exposed and the general population^{4–6}. Further, mortality due to diabetes was increased in occupationally exposed workers⁷. Exposure to PFOA *in utero* induced obesity in female offspring, concomitant with increased serum leptin and insulin levels^{8,9}. Obesity has been linked to both pancreatitis and diabetic complications, and is also a risk factor for pancreatic cancer development¹⁰. In rodents,

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CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interest.

PFOA induced pancreatic acinar cell tumors (PACTs) through a yet to be determined mechanism¹¹.

Physiologic states that increase protein-folding demand or stimuli that disrupt protein folding create an imbalance resulting in accumulation of unfolded or misfolded proteins in the ER lumen that is referred to as ER stress. Pancreatic acinar cells exhibit a high rate of protein synthesis and possess a highly developed ER^{12, 13} and are highly susceptible to ER stress. To mitigate ER stress, cells activate the unfolded protein response (UPR) that serves to restore ER function by coordinating temporal shutdown of gene transcription while selectively upregulating expression of targets involved in protein folding, ER quality control, and ER-associated degradation (ERAD)¹⁴. The UPR is activated as an adaptive response in several diseases of the pancreas including diabetes and alcohol-induced pancreatitis^{15, 16}, and has been implicated as a cell survival mechanism following chemotherapy in pancreatic cancer¹⁷.

The UPR consists of three main signaling cascades: the double stranded RNA-dependent protein kinase-like endoplasmic reticulum kinase (PERK), the inositol-requiring kinase/endonuclease 1 α (IRE1 α), and activating transcription factor 6 (ATF6)¹⁴. The most immediate response to ER stress is a transient attenuation of cap-dependent protein translation mediated by PERK phosphorylation of eIF2 α . Phosphorylation of eIF2 α selectively enhances the transcription and translation of Atf4, which induces expression of UPR target genes such as C/EBP homologous protein (Chop). PERK has also been shown to phosphorylate Nrf2, promoting dissociation from its cytosolic repressor Keap1, leading to nuclear translocation and upregulation of antioxidant response genes¹⁸. Activation of the RNase function of IRE1 α results in splicing of the X-box binding protein 1 (Xbp1u), generating a potent transcription factor (Xbp1s), which induces expression of itself as well as ER chaperones and foldases, enzymes for lipid synthesis for expansion of the ER membrane and components of the ERAD pathway. ATF6 activation also upregulates Xbp1u, as well as ER chaperones and foldases, and components of ERAD¹⁴. Prolonged ER stress can stimulate Chop gene induction through all three pathways, leading to a switch from the adaptive response of the UPR to induction of apoptosis¹⁹. ER stress has been shown to induce oxidative stress with generation of reactive oxygen species (ROS) involved in both the adaptive and proapoptotic responses of the UPR²⁰.

We have previously shown that exposure of C57Bl/6 mice to PFOA for 7 days elicited dose-related increases in oxidative stress/damage in the pancreas²¹. Here, we investigate whether PFOA induces ER stress and the UPR in pancreatic acinar cells, which may identify a mechanistic link between PFOA exposure and oxidative stress.

2 MATERIALS AND METHODS

2.1 Reagents and antibodies

PFOA (96%), Thapsigargin (TG), Ionomycin (ION), Thiazolyl Blue Tetrazolium Bromide, Ceapin-A7, 4-Phenylbutyric acid (4-PB), N-acetyl-L-cysteine (NAC), 4,5-Dihydroxy-1,3-benzenedisulfonic acid disodium salt monohydrate (Tiron), Hydrogen peroxide (30%), 2-aminoethoxydiphenyl borate (2-APB) and Dantrolene (Dan) were purchased from Sigma-

Aldrich (St. Louis, MO). PERK Inhibitor I (GSK2606414), PERK Inhibitor II (GSK2656157), IRE1 Inhibitor III (4 μ 8c), and IRE1 Inhibitor IV (KIRA6) were purchased from Millipore Sigma (Burlington, MA). Tunicamycin (TM) was purchased from Santa Cruz Biotechnology (Dallas, TX) and Palmitoleic acid (POA) was purchased from Cayman Chemical (Ann Arbor, MI). Cell culture media and antibiotics were purchased from Sigma-Aldrich (St. Louis, MO) and fetal bovine serum (FBS) was purchased from Hyclone Laboratories (Logan, UT). The following primary antibodies were used for Western analysis: phospho-eIF2 α (3597) and Chop (2895) from Cell Signaling Technology (Danvers, MA); eIF2 α (sc-133132) and Gapdh (sc-365062) from Santa Cruz Biotechnology (Dallas, TX); and Atf4 (10835-1-AP) from Proteintech Group (Rosemont, IL). Secondary antibodies against mouse or rabbit species were conjugated to HRP and obtained from Cell Signaling Technology (Danvers, MA).

2.2 Cell culture and treatments

Mouse acinar 266-6 cells were obtained from ATCC (Manassas, VA) and cultured in RPMI 1640 media containing 10% FBS and 1% Antibiotic/Antimycotic (complete media) at 37°C and 5% CO₂. For treatment with inhibitors, PFOA, and other agents, serum content in the media was reduced to 2%. Cells were pretreated with inhibitors for 1 hr prior to addition of PFOA, TG or H₂O₂ for the indicated time periods.

2.3 Measurement of cell viability

266-6 cells (8 $\times$ 10⁴ cells/well) were cultured in 24-well plates overnight in complete media. The following day, media was replaced with RPMI-1640 media containing 2% FBS and DMSO (vehicle) or varying PFOA concentrations for 6 or 24 hrs. Cell viability was assessed following addition of Thiazolyl Blue Tetrazolium Bromide (MTT) (0.5mg/ml) which was added for 1 hr prior to cell harvest. Formazan dye was solubilized with DMSO and absorbance measured at 560nm in a Synergy HT (Biotek) plate reader.

2.4 Measurement of cytosolic calcium levels

Cytosolic Ca²⁺ measurements were conducted utilizing the Fluo-4 No Wash Calcium Assay kit (Thermo Fisher Scientific, Waltham, MA) according to manufacturer's instructions. 266-6 cells were plated at 5 $\times$ 10⁴ cells/well in 96-well plates and cultured overnight in complete media. Media was then removed and replaced with dye loading solution and incubated at 37°C for 30 min before being stimulated with DMSO (vehicle), PFOA, POA or ION and responses measured in a Synergy HT (Biotek) plate reader at 37°C. Fluorescence emission at 516nm was monitored following excitation at 494nm.

2.5 Quantitative RT-PCR analysis

Total RNA was isolated from 266-6 cells (RNeasy, Qiagen) following manufacturer's instructions. 2.5 μ g of total RNA was reverse-transcribed with Superscript II reverse transcriptase (Invitrogen) using random hexamers (Roche) for priming. Real-time PCR was performed using FastStart Universal SYBR Green Master Mix (Roche) and gene-specific primers on an Applied Biosystems QuantStudio3 Real-time PCR System. Primer pairs for specific genes were designed using the Primer Express program (Applied Biosystems), with

β -actin amplification used as the endogenous control. Samples were measured in triplicate and analyzed by the threshold cycle (C_t) comparative method. The 2^{-C_t} value was calculated, where $C_t = C_{t_{\text{target}}} - C_{t_{\beta\text{-actin}}}$, and $C_t = C_{t_{\text{sample}}} - C_{t_{\text{reference}}}$. Relative quantitation for each gene is shown, where control levels are set to 1.0. Sequences of primers used for quantitation are included in Supplemental Table 1.

2.6 Western analysis

Cells were lysed in Buffer D [20mM Tris, pH 7.5; 137 mM NaCl; 2mM EDTA; 1% Triton X-100, 10% glycerol, COMPLETE protease inhibitor, and PhosStop phosphatase inhibitor (Roche)]. Equal amounts of protein were resolved on SDS/PAGE gels, transferred to PVDF membrane (Immobilon, Millipore) and subjected to Western analysis. Immunoblots were visualized utilizing enhanced chemiluminescence (WesternBright ECL, Advansta) and autoradiography film.

2.7 Statistical analysis

The data were analyzed by one-way ANOVA followed by a Tukey's post-hoc test for comparison against controls when the overall model indicated a statistically significant effect. For all studies, treatment groups were considered significantly different from control values when $p < 0.05$.

3 RESULTS

3.1 PFOA triggers ER stress and activation of the UPR

The mouse pancreatic acinar cell line 266-6 was utilized to assess whether PFOA induces ER stress in cell culture, as it has been previously used as a model system to study acinar cell function in vitro ²². Cellular toxicity was assessed following exposure to varying doses of PFOA for either 6 or 24 hrs. As shown in Figure 1A, exposure to only the highest concentration of PFOA, 100 μ g/mL, resulted in a significantly decreased loss of cell viability. As treatment with 50 μ g/mL (~120 μ M) PFOA did not cause significant effects on cell viability at either the 6 or 24 hr timepoint, this concentration was chosen for subsequent cell culture exposures. To determine whether PFOA treatment induces ER stress leading to activation of the UPR, 266-6 cells were treated with vehicle (DMSO) or 50 μ g/mL PFOA. The response to PFOA treatment was compared to other compounds known to induce ER stress and the UPR by various mechanisms, including palmitoleic acid (POA), a non-oxidative metabolite of ethanol ¹⁵ thapsigargin (TG), a non-competitive inhibitor of the sarco/endoplasmic reticulum Ca^{+2} ATPase (SERCA), tunicamycin (TM), which blocks N-linked glycosylation, and Ionomycin (ION), which raises intracellular Ca^{+2} levels by facilitating Ca^{+2} ion flow into cells. As shown in Figure 1B, exposure of 266-6 cells to PFOA for 4 hours triggered phosphorylation of eIF2 α , as well as induction of Atf4 and Chop protein expression to similar levels as TG, TM and ION treatment, indicating activation of the UPR through the PERK pathway.

Activation of one arm of the UPR has been shown to facilitate activation of other arms of the UPR, while significant crosstalk between the pathways has previously been demonstrated ²³. To characterize which arms of the UPR are activated by PFOA, 266-6 cells were treated with

50µg/mL PFOA for various times, followed by gene and protein expression analysis for pathway-specific gene and protein responses. Activation of the PERK pathway, assessed by phosphorylation of eIF2α, occurred within 5 min, which persisted for the entire duration of treatment (4hrs) (Figure 2A). In addition, protein expression of the PERK-dependent targets Atf4 and Chop began to increase starting at the 1 hr time point (Figure 2A). PFOA-mediated mRNA induction of gene targets indicative for activation of the PERK, IRE1α, and ATF6^{23, 24} pathways was also examined following 2, 4, 8 and 24 hrs of exposure (Figure 2B, C, and D). While gene induction of the PERK-dependent targets Atf4, Chop and Trb3 were significantly increased as early as 2 hrs (Figure 2B), significant mRNA induction of genes indicative of IRE1α and ATF6 activation only occurred after 8 and 24 hrs of exposure (Figure 2C, D). All three arms of the UPR pathway are activated following exposure to TG for 6 hrs, which was included as a positive control (Figure 2B, C, D).

To determine which UPR pathway may predominate following PFOA exposure, we utilized specific inhibitors for the individual pathways in conjunction with PFOA treatment. Treatment of 266-6 cells with the PERK inhibitors GSK2606414 and GSK2656157^{25, 26} decreased PFOA-induced protein expression of the PERK target genes Atf4 and Chop (Figure 3A), while treatment with GSK2606414 also led to decreased upregulation of Atf4 and Chop mRNA by PFOA (Figure 4B, C). Interestingly, inhibition of IRE1α using 4µ8c²⁷ and KIRA6²⁸, blocked PFOA-mediated protein and mRNA induction of the PERK-responsive genes Atf4 and Chop more efficiently than PERK-specific inhibitors (Figure 3B, C, D). Pretreatment with the IRE1α inhibitor 4µ8c decreased basal levels of Xbp1s mRNA and also blocked PFOA-mediated upregulation of Xbp1s (Figure 3E). In contrast, PERK-specific inhibitors were more efficient at blocking TG-mediated upregulation of Atf4 and Chop mRNA expression, while IRE1α inhibitors exerted much lesser effects (Figure 3 A–D). Pretreatment with a specific inhibitor of ATF6, Ceapin-A7²⁹, however, had no effect on PFOA-stimulated induction of Atf4 and Chop mRNA and protein expression (data not shown).

As PERK has also been shown to regulate Nrf2 signaling¹⁸ we assessed the effects of inhibitors of PERK and IRE1α on upregulation of antioxidant response genes with Nrf2/Antioxidant response elements (AREs). Pretreatment of 266-6 cells with the specific PERK inhibitor GSK2606414, but not the IRE1α inhibitor 4µ8c, efficiently blocked upregulation of Sod1 and Sod2 (Figure 3F, G). While the IRE1α inhibitor 4µ8c decreased PFOA-mediated upregulation of Nqo1, the PERK inhibitor GSK2606414 was more effective at inhibiting mRNA induction (Figure 3H). Together, these results demonstrate that PFOA exposure can trigger ER stress and activation of all three arms of the UPR and suggests that significant crosstalk exists between the PERK and IRE1α arms of the UPR following PFOA treatment.

3.2 PFOA-mediated ER stress precedes oxidative stress

ER stress has been shown to generate oxidative stress^{20, 30} and we have previously shown that oxidative stress is observed in the pancreas following PFOA exposure²¹. To assess the temporal relationship between PFOA-mediated induction of ER and oxidative stress, 266-6 cells were pretreated with either the antioxidants N-acetyl cysteine (NAC)³¹, 4,5-

Dihydroxy-1,3-benzenedisulfonic acid disodium salt monohydrate (Tiron)³² or the chemical chaperone 4-phenylbutyric acid (4-PB) which has been shown to alleviate ER stress^{33, 34}. As a positive control for oxidative stress induction, cells were treated with hydrogen peroxide (H₂O₂). As shown in Figure 4, pretreatment of 266-6 cells with 4-PB prior to PFOA exposure decreased the expression of Atf4 and Chop protein and mRNA levels, while pretreatment with NAC or Tiron failed to block PFOA-stimulated induction (Figure 4A–C). In contrast, both NAC and 4-PB pretreatment were able to block H₂O₂-mediated induction of Atf4 and Chop protein and mRNA induction, while Tiron failed to do so (Figure 4A–C). To further extend these results, we assessed whether inhibition of ER stress could decrease the induction of the oxidative stress response gene Sod1. While treatment with 4-PB, NAC and Tiron blocked PFOA-mediated induction of Sod1 mRNA levels, NAC and Tiron treatment, but not 4-PB treatment, blocked H₂O₂-mediated induction of Sod1 (Figure 4D). Together, these results suggest that PFOA exposure induces ER stress which subsequently induces oxidative stress.

3.3 PFOA increases intracellular Ca²⁺ Levels

The ER is a major storage site for intracellular calcium that, when released into the cytoplasm, mediates the regulated secretion of stored pancreatic digestive enzymes into the ductal system and can also trigger ER stress. Alcohol has been shown to trigger pancreatic ER stress¹⁵ while non-oxidative metabolites such as POA, result in a toxic increase in cytosolic calcium levels³⁵. As PFOA has a similar structure to POA, we investigated whether PFOA treatment also increases cytosolic calcium levels. To determine this, 266-6 cells were incubated with the Fluo-4 AM fluorescent calcium indicator dye in the presence of PFOA, POA, or vehicle, followed by monitoring of fluorescence levels over a 160 min time course. Treatment of 266-6 cells with 20, 50 or 100 µg/ml PFOA resulted in time- and dose-dependent increase in cytosolic calcium levels which began to deviate from vehicle-treated control levels at 40 min post-stimulation and continued to increase over the 160 minute time course (Figure 5A). Treatment of the cells with POA followed a similar increase in cytosolic calcium levels, while treatment of cells with ION as a positive control, led to a rapid increase in cytosolic calcium levels that plateaued at 80 min post-treatment. Intracellular calcium release can occur through activation of two intracellular receptors, the inositol 1,4,5-triphosphate receptor (IP₃R) and the ryanodine receptor (RyR), which are located primarily in the ER. To probe whether PFOA-mediated calcium intracellular calcium release occurs through activation of the IP₃R or RyR leading to ER stress, cells were treated with specific inhibitors to each prior to addition of PFOA. As shown in Figure 5, pre-treatment of 266-6 cells with a specific inhibitor of the IP₃R, 2-aminoethoxydiphenyl borate (2-APB)³⁶, significantly decreased PFOA-stimulated protein and mRNA induction of Atf4 and Chop, while a specific inhibitor of the RyR, dantrolene (Dan)³⁷, had little effect on protein and mRNA levels (Figure 5B–D). 2-APB treatment significantly decreased Atf4 and Chop mRNA induction following TG stimulation, however, similar to PFOA stimulation, Dan had a minimal effect on protein and mRNA levels of Atf4 and Chop. We also observed that 2-APB pretreatment blocked PFOA-stimulated upregulation of Xbp1s, while Dan treatment exhibited a lesser effect (Figure 5E). In contrast to PFOA, 2-APB pretreatment failed to block TG-mediated Xbp1s mRNA induction (Figure 5E). These results suggest that PFOA increases intracellular Ca²⁺ levels predominantly through activation of the IP₃R. Overall, our

results support the following model for activation of the UPR and generation of oxidative stress in the pancreas triggered by exposure to PFOA (Figure 5F). PFOA increases intracellular Ca^{+2} levels through activation of the IP_3R and primary activation of the IRE1 α and PERK response pathways of the UPR. Continued ER stress generates ROS leading to oxidative stress, the PERK-dependent activation of Nrf2, and induction of antioxidant response genes.

4 DISCUSSION

Widespread exposure to PFOA, coupled with its long half-life in humans³⁸, suggests that PFOA may contribute to development of chronic diseases such as cancer. In a two-year rodent bioassay, PFOA induced PACTs through an undetermined mechanism¹¹. Pancreatic acinar cells are highly susceptible to ER stress, and diseases of the exocrine pancreas, such as pancreatitis and cancer, have been associated with induction of ER stress. Exposure to exogenous agents or inherited gene mutations lead to ER stress and development of pancreatitis^{15, 30, 39}. As chronic pancreatitis is a risk factor for pancreatic cancer⁴⁰, these studies suggest that agents that induce ER stress may increase the development of pancreatic cancer. Here we show that PFOA results in ER stress and activation of the UPR in mouse pancreatic acinar cells, and may be the mechanism through which PFOA stimulates PACT development.

The IRE1 α /Xbp1 arm of the UPR is constitutively active in the exocrine pancreas with Xbp1 required for neonatal development⁴¹ and function of mature acinar cells⁴². Mice with pancreatic exocrine-specific PERK deficiency exhibited pancreatic atrophy; however, they did not exhibit perturbation of the UPR⁴³. No organ defects were observed following germline deletion of ATF6, although the mice were more susceptible to chemically-induced ER stress⁴⁴. Our results indicate that the IRE1 α /Xbp1 pathway is a predominant pathway mediating the response to PFOA exposure, as IRE1 α inhibitors blocked PFOA-stimulated induction of IRE1 α /Xbp1s targets and PERK-stimulated gene targets (Figure 3). However, activation of the PERK pathway appears key for induction of antioxidant response genes following PFOA exposure (Figure 3F–H).

In experimental models of pancreatitis, altered ER calcium levels can cause ER stress. Cholecystokinin (CCK), which stimulates secretion of digestive enzymes from acinar cells, can deplete ER calcium stores, leading to an ER stress signal in acinar cells. Acinar cells treated with CCK concentrations that elicit pancreatitis exhibited phosphorylation of eIF2 α ⁴⁵, linking alteration of calcium levels to ER stress and pancreatitis. Elevation of intracellular calcium by PFOA was observed in mast cells⁴⁶, in cultured neurons⁴⁷ and we show here increased intracellular calcium in pancreatic acinar cells (Figure 5A). Further, blockade of the IP_3R , which mediates intracellular calcium release, attenuated PFOA-stimulated mRNA induction of the ER stress markers Atf4, Chop, and Xbp1s (Figure 5).

PFOA exposure resulted in oxidative DNA damage and increased ROS levels in liver cells^{48, 49}, and an inflammatory response with increased ROS production in mast cells⁵⁰. We previously demonstrated that PFOA triggers focal ductal hyperplasia, and an inflammatory response concomitant with oxidative stress in the pancreas²¹. Here, we show that 4-PB, a

chemical chaperone that alleviates ER stress, but not the antioxidants NAC or Tiron, blocked PFOA-stimulated induction of Atf4 and Chop indicating that PFOA-induced ER stress precedes oxidative stress (Figure 4). Similarly, 4-PB, but not NAC, alleviated PFOA-induced activation of the UPR in HepG2 cells⁵¹. PFOA-stimulated induction of antioxidant genes was shown to be PERK-dependent (Figure 3F–H), suggesting that activation of PERK may be a key cellular response to counteract PFOA-induced oxidative stress.

In summary, we show that PFOA increases intracellular calcium levels through the IP₃R, leading to ER stress, induction of the UPR and oxidative stress. As oxidative stress and ROS are known cancer promoters, activation of ER stress may be a common mechanism through which PFOA and other PFAS influence PACT development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1. PFOA induces ER stress and activation of the UPR in pancreatic acinar cells. **A**, Effect of PFOA exposure on cell viability in 266-6 cells. 266-6 cells were plated in 24 well plates and treated with vehicle (DMSO), 1, 10, 20, 50, or 100 µg/mL PFOA for 6 and 24 hrs. Cell viability was assessed using the MTT assay as detailed in Materials and Methods. Shown is the average $\pm$ std dev of cell viability measurements performed in quadruplicate, expressed as % value of DMSO-treated cells (set to 100%), from a representative experiment ($n = 4$). **B**, Immunoblot analysis showing the levels of phosphorylated eIF2 α , total eIF2 α , Atf4, and Chop following exposure of 266-6 cells to DMSO, 50 µg/mL PFOA, 100µM palmitoleic acid (POA), 1µM thapsigargin (TG), 5µM tunicamycin, (TM) or 3µM ionomycin (ION) for 4 hrs. Levels of Gapdh are included as loading control. Shown is a representative experiment ($n = 4$). * indicates significantly different from DMSO control $p < 0.05$. Noted are the

phosphorylated forms of Atf4 detected by the Atf4 antibody while NS indicates recognition of a non-specific band.

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FIGURE 2.

Time-dependent activation of the PERK, IRE1 α , and ATF6 arms of the UPR by PFOA. A, Immunoblot analysis for protein expression of phosphorylated eIF2 α , total eIF2 α , Atf4, and Chop following treatment of 266-6 cells with DMSO (0) or 50 μ M PFOA for the indicated time points. Gapdh expression is shown for verification of equal protein loading. Shown is a representative experiment (n = 4). Noted are the phosphorylated forms of Atf4 detected by the Atf4 antibody while NS indicates recognition of a non-specific band. B-D, qRT-PCR analysis was performed to determine mRNA expression of gene targets specific for the PERK, IRE1 α , and ATF6 arms of the UPR pathways following treatment of 266-6 cells with DMSO or 50 μ M PFOA for the various time points indicated, or 1 μ M thapsigargin (TG) for 6 hrs. Shown is the average fold-induction $\pm$ std dev of measurements performed in triplicate from a representative experiment with DMSO values set to 1.0 (n = 3). * indicates significantly different from DMSO control p < 0.01; # indicates significantly different from DMSO control p < 0.05.

FIGURE 3.

PFOA-mediated induction of Atf4 and Chop can be abrogated by both PERK and IRE1 α selective chemical inhibitors. A and D, Immunoblot analysis for expression of Atf4 and Chop following 1 hr pretreatment of 266-6 cells with the indicated μ M concentrations of PERK-selective inhibitors (GSK2606414 and GSK 2656157) (A) or IRE1 α -selective inhibitors (4 μ 8c and KIRA6) (D) followed by treatment with DMSO (-), 50 μ g/mL PFOA or 1 μ M thapsigargin (TG) for 6 hrs. Gapdh expression is used to verify equal protein loading. Shown is a representative experiment (n = 4). Noted are the phosphorylated forms of Atf4 detected by the Atf4 antibody while NS indicates recognition of a non-specific band. (B,C,E-H) Gene expression of Atf4, Chop, Xbp1s, Sod1, Sod2 and Nqo1 was determined by qRT-PCR analysis following 1 hr pretreatment of 266-6 cells with 1 μ M GSK2606414 or 25 μ M 4 μ 8c followed by treatment with DMSO (CTL), 50 μ g/mL PFOA or 1 μ M thapsigargin (TG) for 6 hrs. Values are expressed as average fold-induction $\pm$ std dev of assays performed

in triplicate with DMSO (CTL) values set to 1.0. Shown is a representative experiment (n = 3). * indicates significantly different from DMSO (CTL) $p < 0.05$; # indicates significantly different from respective PFOA or TG values $p < 0.05$.

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FIGURE 4.

PFOA-mediated ER stress precedes oxidative stress. A, Immunoblot analysis for Atf4 and Chop expression in 266-6 cells following 1 hr pretreatment with 5 μ M 4-phenylbutyrate (4PB), 1mM N-acetyl-cysteine (NAC), or 0.4mM 4,5-Dihydroxy-1,3-benzenedisulfonic acid disodium salt monohydrate (Tiron) prior to treatment with DMSO (-), 50 μ g/mL PFOA or 10 μ M H₂O₂ for 6 hrs. Gapdh expression is included as a loading control. Shown is a representative experiment (n = 4). Noted are the phosphorylated forms of Atf4 detected by the Atf4 antibody while NS indicates recognition of a non-specific band. B-D, mRNA expression of Atf4, Chop and Sod1 was determined by qRT-PCR analysis following 1 hr pretreatment of 266-6 cells with 5 μ M 4-phenylbutyrate (4PB), 1 mM N-acetyl cysteine (NAC), or 0.4mM 4,5-Dihydroxy-1,3-benzenedisulfonic acid disodium salt monohydrate (Tiron) followed by treatment with DMSO (CTL), 50 μ g/mL PFOA or 10 μ M H₂O₂ for 6 hrs. Quantitation is expressed as fold-induction $\pm$ std dev of assays performed in triplicate with

DMSO (CTL) levels set to 1.0 from a representative experiment ($n = 3$). * indicates significantly different from DMSO (CTL) $p < 0.05$; # indicates significantly different from respective PFOA or TG values $p < 0.05$.

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FIGURE 5.

PFOA treatment increases intracellular Ca^{+2} levels in pancreatic acinar 266-6 cells. A, Intracellular cytosolic Ca^{+2} levels were determined in 266-6 cells treated with DMSO (Control), 20, 50, or 100 $\mu\text{g}/\text{mL}$ PFOA, 100 μM palmitoleic acid (POA), or 3 μM ionomycin (Ion) utilizing the Fluo-4 AM indicator as described in Materials and Methods. Fluorescence levels are expressed as a ratio of fluorescence at specified time (F)/ fluorescence at time 0 (F0). Plotted is the average of values from 6 replicate wells of a representative experiment ($n = 2$). B, Immunoblot analysis for expression of Atf4 and Chop expression following 1 hr pretreatment of 266-6 cells with DMSO (–), or the indicated μM concentration of 2-aminoethoxydiphenyl borate (2-APB) or Dantrolene (Dan) followed by 6 hr treatment with DMSO (–), 50 $\mu\text{g}/\text{mL}$ PFOA or 1 μM thapsigargin (TG). Gapdh expression is included as a loading control. Shown is a representative experiment ($n = 4$). Noted are the phosphorylated forms of Atf4 detected by the Atf4 antibody while NS indicates recognition of a non-specific

band. C-E, 266-6 cells were either pretreated with DMSO, 5 μ M 2-aminoethoxydiphenyl borate (2-APB) or 10 μ M Dantrolene (Dan) followed by 6 hr treatment with DMSO (CTL), 50 μ g/mL PFOA or 1 μ M thapsigargin (TG). qRT-PCR analysis was performed for expression of Atf4, Chop and Xbp1s. Quantitation is expressed as average fold-induction $\pm$ std dev of assays performed in triplicate, with levels in DMSO (CTL) set to 1.0. Shown is a representative experiment (n = 3). * indicates significantly different from DMSO (CTL) p < 0.05; # indicates significantly different from respective PFOA or TG values p < 0.05. F, Model for stimulation of the UPR flowing exposure to PFOA. PFOA increases intracellular Ca⁺² levels through activation of the IP₃R, leading to ER stress and induction of the UPR. Prominent activation of IRE1 α and PERK is observed, as indicated by increased arrow thickness. Continued ER stress generates ROS leading to oxidative stress, the PERK-dependent activation of Nrf2, and induction of antioxidant response genes.