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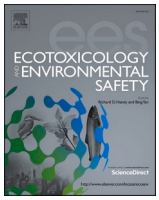

Perfluorooctanoate (PFOA) cell-autonomously promotes thermogenic and adipogenic differentiation of brown and white adipocytes

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ABSTRACT

Perfluorooctanoic acid (PFOA) is a synthetic organofluoride surfactant associated with several toxic effects in humans and animals. Particularly, it has been observed that PFOA treatment of mice results in weight loss associated with recruited brown adipose tissue (BAT), including an increased amount of uncoupling protein 1 (UCP1). The molecular mechanism behind this BAT recruitment is presently unknown. To investigate the existence of possible cell-autonomous effects of PFOA, we treated primary cultures of brown and white (inguinal) adipocytes with PFOA, or with the non-fluorinated equivalent octanoate, or with vehicle, for 48 h (from day 5 to day 7 of differentiation). PFOA in itself increased the gene expression (mRNA levels) of UCP1 and carnitine palmitoyltransferase 1A (CPT1α) (thermogenesis-related genes) in both brown and white adipocytes. In addition, PFOA increased the expression of fatty acid binding protein 4 (FABP4) and peroxisome proliferator-activated receptor α (PPARα) (adipogenesis-related genes). Also the protein levels of UCP1 were increased in brown adipocytes exposed to PFOA. This increase was more due to an increase in the fraction of cells that expressed UCP1 than to an increase in UCP1 levels per cell. The PFOA-induced changes were even more pronounced under simultaneous adrenergic stimulation. Octanoate induced less pronounced effects on adipocytes than did PFOA. Thus, PFOA in itself increased the levels of thermogenic markers in brown and white adipocytes. This could enhance the energy metabolism of animals (and humans) exposed to the compound, resulting in a negative energy balance, leading to diminished fitness.

1. Introduction

Perfluorooctanoic acid (PFOA) has been used in numerous commercial products since the 1950'ies. Although its manufacture has been stopped in the US, this surfactant is almost non-biodegradable, and it is one of the most abundant perfluorinated compounds in the environment (Goosey and Harrad, 2011). PFOA is also found in significant levels in bodily fluids of humans and animals (Kannan et al., 2006; Calafat et al., 2007; Theobald et al., 2012; Beesoon et al., 2012; Zhang et al., 2013; ATSDR 2021), where it can potentially cause toxic effects (see review (Li et al., 2017; ATSDR 2021)).

Among the toxic effects observed are marked negative effects on body weight (Xie et al., 2002; Xie et al., 2003; Shabalina et al., 2015); these effects are thus in marked contrast to the obesogenic effects of certain other pollutants described by Gi Gregori et al. (2019). The negative effects on body weight can partially be understood as being due to a decreased food intake in the PFOA-treated animals, but there are also clear indications that brown adipose tissue is recruited in the PFOA-treated mice (Shabalina et al., 2015). This recruited brown adipose tissue could mediate an "extra" thermogenesis that may aggravate the negative energy balance caused by a lowered food intake. A chronic negative energy balance will evidently be fatal with time. It is therefore of importance to understand the effects of PFOA on brown adipose tissue.

The molecular background for the recruitment of brown adipose tissue – and especially for the increased expression of the thermogenesis-mediating uncoupling protein UCP1 – in the PFOA-treated mice has not as yet been established. The increased UCP1 gene expression could occur indirectly via a stimulation of the sympathetic nervous system or it could be a cell-autonomous effect, i.e. a direct effect on the brown adipocytes. There is also the possibility that PFOA may induce "browning" (i.e. UCP1 expression) in certain white adipocytes (Nedergaard and Cannon,

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2014).

To establish as to whether PFOA possesses the ability to advance thermogenic and adipogenic adipocyte differentiation cell-autonomously, we have here exposed brown and white adipocytes to PFOA during their differentiation process and examined gene expression and corresponding protein levels for genes associated with thermogenesis and adipogenesis, both structural proteins and selected transcription factors. The results indicate that exposure to PFOA could potentially result in an unnecessary increased utilization of limited energy resources and thus could lead to decreased fitness. Our findings thus establish a risk for animals exposed to PFOA and living in conditions with limited energy sources.

2. Materials and Methods

2.1. Animals, cell isolation and cell culture

Procedures involving animals that were performed in this study were approved by the Animal Ethics Committee of the North Stockholm region. Three-week-old male NMRI (Naval Medical Research Institute) mice (purchased from the local supplier Scanbur, Sweden) were used. The mice were kept in our facility for at least 48 h at room temperature (around 21 °C) on a 12:12-h light dark cycle with free access to chow food and water. The mice were euthanized with CO_2, followed by cervical dislocation. The interscapular, cervical and axillary brown adipose tissue (BAT) depots were pooled and used for the preparation of brown adipocyte cultures. Inguinal white adipose tissue (WAT) was used for the preparation of white adipocyte cultures. The preparation of primary cultures of brown and white adipocytes was performed as described in Petrovic et al. (2008), principally through collagenase treatment of the tissues and seeding the stromal-vascular fraction in culture plates.

The primary cultures of brown and white adipocytes were grown and differentiated in twelve-well cell culture plates (Corning Costar, CLS3513). The culture medium was composed of high-glucose DMEM (Sigma-Aldrich, D6429) with 10% (vol/vol) newborn calf serum (Sigma-Aldrich, N4637), 3 nM insulin (Sigma-Aldrich, I9278), 25 μg/ml sodium L-ascorbate (Sigma-Aldrich, A4034), 10 mM HEPES (Sigma-Aldrich, H0887), 4 mM glutamine (Sigma-Aldrich, G7513), 50 U/ml penicillin and 50 μg/ml streptomycin (Sigma-Aldrich, P0781). The cells were grown at 37 °C in an atmosphere of 8% CO_2 in air with 80% humidity.

Cells were grown for 7 days (seeding is considered day 0). Medium was changed on day 1 and then every other day. From day 5 to 7, the culture medium was supplemented with solutions of PFOA (Sigma-Aldrich, 171468) or octanoate (OA, Sigma-Aldrich, C5038) to final concentrations of 200 or 400 μM per well. These concentrations were based on a previous in vitro study (Shabalina et al., 2016)). Octanoate was used due to its similar molecular structure compared to PFOA; it also has an eight-carbon chain with a carboxyl group at the end but no fluorides. PFOA and octanoate solutions were prepared by dissolving the powders in 50% ethanol. Control cells were exposed to 50% ethanol (vehicle) to a final concentration of 0.5% per well (the same concentration as the PFOA- and octanoate-exposed cells). Where indicated, adipocytes were exposed to 1 μM norepinephrine (NE, Sigma-Aldrich, A9512) or ultrapure water (control) for 2 or 48 h. Adipocytes exposed to NE or water for 2 h were used for mRNA analysis, and adipocytes exposed to NE or water for 48 h were used for protein, morphology and immunocytochemical analysis.

2.2. Analysis of mRNA levels

Gene expression was analyzed by quantitative reverse transcription-PCR. The experiments were performed with brown and white adipocyte cultures in 6 independent experiments, each run in duplicate. Cells were exposed to PFOA, octanoate or vehicle for 48 h *plus* NE or water for 2 h. After the treatments, the cells were harvested in TRI Reagent (Sigma-Aldrich, T9424) and kept at − 80 °C until further processing. RNA extraction and quantitative real-time PCR were performed as described in Petrovic et al. (2010). Primers used for qPCR are listed in Supplementary Table S1. The ΔC_t ($2^{-\Delta Ct}$) method was used to calculate relative changes in gene expression. The general transcription factor IIB (TFIIB) was used as a reference gene to adjust for variations in cDNA synthesis; its *C*t value was subtracted from the *C*t value of the target genes in each sample. To normalize between cell cultures, the Δ*C*t value of the NE control group in each experiment was used; the gene expression levels of all other experimental groups in each experiment were normalized to this value; the resulting mean Δ*C*t value is shown. The treatments used did not markedly or systematically alter the total RNA recovery from the cell culture wells (not shown); the results have therefore been given as indicated here (per TFIIB mRNA, i.e. in reality ≈per RNA) and not per total well yield.

2.3. Analysis of protein levels

Brown and white adipocytes were exposed to PFOA, octanoate or vehicle *plus* NE or water for 48 h. Samples from 6 independent experiments were used to analyze UCP1 and fatty acid binding protein 4 (FABP4) levels. Western blotting was performed as described previously (Petrovic et al., 2008). 15 μg protein from each sample (of brown and white adipocytes) was loaded per well in 12% polyacrylamide gel. One standard brown sample was also run on the gels of white samples which were used for UCP1 expression analysis. Primary antibodies against UCP1 (rabbit polyclonal, raised against the C-terminal decapeptide), diluted 1:3000, and against FABP4 (Cell Signaling, D25B3, XP rabbit monoclonal antibody, #3544), diluted 1:2000. The chemiluminescence signal was detected with a CCD camera (FujiFilm, Tokyo, Japan). Exposure time for chemiluminescence detection was 2–5 s for membranes used for UCP1, and 5 s for FABP4. Quantifications were performed with the Image Gauge 3 software. The chemiluminescence signal level of all experimental groups was set in each experiment in relation to the NE control group, which was set as 1 in all independent experiments. The treatments used did not markedly or systematically alter the total protein recovery from the cell culture wells (not shown); the results have therefore been given per mg protein recovered and not per total well yield.

2.4. Morphological and immunocytochemical analysis

For microscopy purposes, cells were grown on glass coverslips (Thermo Fisher Scientific, 1014355118NR1) placed on the bottom of the plate before seeding the cells. At the end of the treatment, cells were examined under phase-contrast microscopy (Olympus) and photographed with an Olympus digital camera. Cells were then washed with PBS, fixed with 4% paraformaldehyde solution and exposed to 5% glycine in PBS to quench unspecific fluorescence. Cells were subsequently permeabilized with cold methanol and blocked with 3% bovine serum albumin (BSA). Cells were incubated overnight with an anti-UCP1 antibody (RD Systems, purified mouse monoclonal antibody, MAB6158, 1:500), and for two h at room temperature with secondary antibodies (Life Technology, Alexa Fluor 594 goat anti-rabbit, A11037, 1:500). The nuclei were stained with 1 μg/ml Hoechst 33258 for 15 min. Cells were washed with PBS before and after the staining steps and then mounted in ProLong Gold antifade reagent (Invitrogen, P36934) onto microscopy slides (Solveco, 1212). Images were taken in a Zeiss LSM 780 confocal microscope.

2.5. Statistical analysis

Data are presented as means ± standard error of the means (SEM). Statistical analyses were performed by paired pre-hoc Student's t-test against control, NE group, between groups exposed and not exposed to NE, and between groups exposed to PFOA or octanoate at the same concentrations. Data were considered significant when $P < 0.05$. The

statistical software GraphPad Prism version 6 was used.

3. Results

In this study, we have investigated whether PFOA has the ability to increase the expression of genes related to thermogenesis and to adipogenesis, in primary cultures of brown adipocytes and white adipocytes, with the implication that such effects may potentially affect energy balance in intact animals and humans. We investigated both the effects of PFOA in itself and how this may affect the response to the physiological gene expression activator norepinephrine: i.e. the gene expression level after norepinephrine and the magnitude of the response to norepinephrine. We primarily followed gene expression at the mRNA level but also investigated to what extent the PFOA-induced changes in mRNA levels were reflected in corresponding protein levels and how the cellular expression pattern was affected.

3.1. PFOA in itself increases expression levels of thermogenesis- and adipogenesis-related genes in brown and white adipocytes

The compound under analysis here is the artificial compound PFOA, but we chose to compare the effects of this agent with that of the most structurally similar endogenous compound: octanoate. This was to establish as to whether the effects of PFOA were principally different from those of an aliphatic fatty acid.

As detailed below, we first analyzed whether PFOA (or octanoate) per se could affect the expression of genes related to thermogenesis and adipogenesis in brown and white adipocytes. The expression of genes generally associated with thermogenesis and genes associated with adipogenesis were here evaluated. Data for brown adipocytes are shown in Fig. 1 and for white adipocytes in Fig. 2.

3.1.1. Thermogenesis-related markers

With respect to thermogenesis-related markers, *uncoupling protein 1* (UCP1, *Ucp1*) is the mediator of adaptative non-shivering thermogenesis (Nedergaard et al., 2001). It uncouples respiration from ATP synthesis, dissipating the energy released from substrate oxidation as heat. As seen, PFOA per se increased UCP1 mRNA levels nearly 5-fold in brown adipocytes; octanoate also increased UCP1 mRNA levels, although the effect was less pronounced (Fig. 1*a*).

In white adipocytes, UCP1 mRNA levels were approximately 100 times lower than in brown adipocytes (Supplementary Fig. S1a). Also in these cells, PFOA, at the lower concentration, was able to increase UCP1 mRNA levels (Fig. 2*a*) but octanoate could not influence UCP1 mRNA levels. These data therefore indicate a pronounced ability of PFOA in itself to enhance the thermogenic capacity of brown and possibly of white adipocytes (but in white adipocytes at a much, much lower level).

Carnitine palmitoyltransferase 1A (CPT1A, *Cpt1a*) mediates the transport of fatty acids from the cytosol to mitochondria, where they can undergo β-oxidation. PFOA increased carnitine palmitoyltransferase 1A mRNA levels in both brown and white adipocytes (Figs. 1*b* and 2*b*), suggesting an ability to increase the capacity for lipid catabolism. The basal mRNA levels of carnitine palmitoyltransferase 1A were about 10 times higher in brown than in white adipocytes (Supplementary Fig. S1b). Octanoate also increased carnitine palmitoyltransferase 1A mRNA levels in brown and white adipocytes, but the effect was again lower than that of PFOA.

In addition to examining the levels of enzymes directly involved in thermogenesis, we also examined whether PFOA would affect the expression level of transcription factors involved in enhancing the thermogenic competence of the cells. The mRNA levels of *peroxisome proliferator-activated receptor gamma coactivator* (PGC-1 alpha, PGC1α, *Ppargc1a*), a transcriptional coactivator that promotes mitochondrial biogenesis, were unaffected by PFOA in brown adipocytes but the level was increased in white adipocytes at the higher PFOA concentration (Figs. 1*c* and 2*c*). The PGC1α mRNA levels were similar in brown and white adipocytes (Supplementary Fig. S1c). No changes in PGC1α mRNA levels in adipocytes exposed to octanoate were found.

Peroxisome proliferator-activated receptor α (PPARα, *Ppara*) is a nuclear receptor involved in lipid metabolism. PFOA markedly increased PPARα levels in both brown and white adipocytes (Figs. 1*d* and 2*d*), while octanoate led to more modest increases. The mRNA levels were some 10-fold lower in white than in brown adipocytes (Supplementary Fig. S1d).

Taken together, these results indicate an ability of PFOA in itself to increase thermogenic capacity in adipocytes. The increases were seen in both brown and white adipocytes, but the actual thermogenic gene expression levels were much lower in white than in brown adipocytes.

3.1.2. Adipogenesis-related markers

Concerning adipogenesis-related marker genes, *fatty acid binding protein 4* (FABP4, also termed aP2, *Fabp4*) is a fatty acid transporter related to lipid accumulation. Its mRNA levels were increased by PFOA exposure in both brown and white adipocytes (Figs. 1*e* and 2*e*). Octanoate caused a modest increase only in brown adipocytes.

We also examined two transcription factors associated with promotion of adipogenesis in the adipocytes. *Peroxisome proliferator-activated receptor γ* (PPARγ, *Pparg*) is a regulator of lipid storage and glucose metabolism and is widely expressed. No differences were observed between the different experimental groups in the mRNA levels of this nuclear receptor (Figs. 1*f* and 2*f*). The PPARγ mRNA measured here consists of both PPARγ1 and PPARγ2; PPARγ2 is considered more adipose tissue-specific. Although the treatments did not affect total PPARγ amounts, the relationship between the two forms could have been altered. However, the level of PPARγ2 did not change due to the treatments either (Figs. 1*g* and 2*g*); clearly, in these measurements about half of the total PPARγ mRNA consisted of PPARγ2 and the other half thus presumably of PPARγ1; this ratio is different from what we have earlier seen in these cells with a somewhat different method (Lindgren et al., 2004). PFOA treatment did not alter this relationship in either tissue investigated. The levels of the PPARγs were similar in brown and white adipocytes (Supplementary Fig. S1f *g*).

Thus, PFOA seems to be able to promote adipogenesis, probably mainly by interacting with existing transcription factors.

3.1.3. Stability of reference gene

No differences were found in the expression levels of the general transcription factor IIB (*TFIIB*) between the experimental groups of brown and white adipocytes exposed or not to PFOA or octanoate (Figs. 1*h* and 2*h*; Supplementary Fig. S1h); this “housekeeping” gene thus fulfils the criteria to serve as a reference gene for mRNA normalization between cell types and between treatments, and the gene-of-interest/TFIIB mRNA levels can be directly compared under the different conditions here investigated.

3.2. The effect of PFOA pretreatment on gene expression levels after NE stimulation

Since sympathetic signalling physiologically is the most important factor influencing the recruitment state of brown and white adipose tissues (Cannon and Nedergaard, 2004), we explored whether pretreatment with PFOA (or octanoate) would affect the differentiation state of the adipocytes such that the outcome of stimulation of brown and white adipocytes with the sympathetic neurotransmitter norepinephrine (NE) would be altered. For this, adipocytes that had been exposed or not to PFOA or octanoate for 2 days were acutely exposed to 1 μM NE, 2 h before harvest. Data for brown adipocytes are presented in Fig. 3 and for white adipocytes on Fig. 4. To visualize the magnitude of the NE effects, the basal gene expression data from Figs. 1 and 2 are indicated in these figures by dashed lines.

Remarkably, as shown in Fig. 3, for all genes studied in brown adipocytes (except fittingly the reference gene TFIIB), pre-exposure to

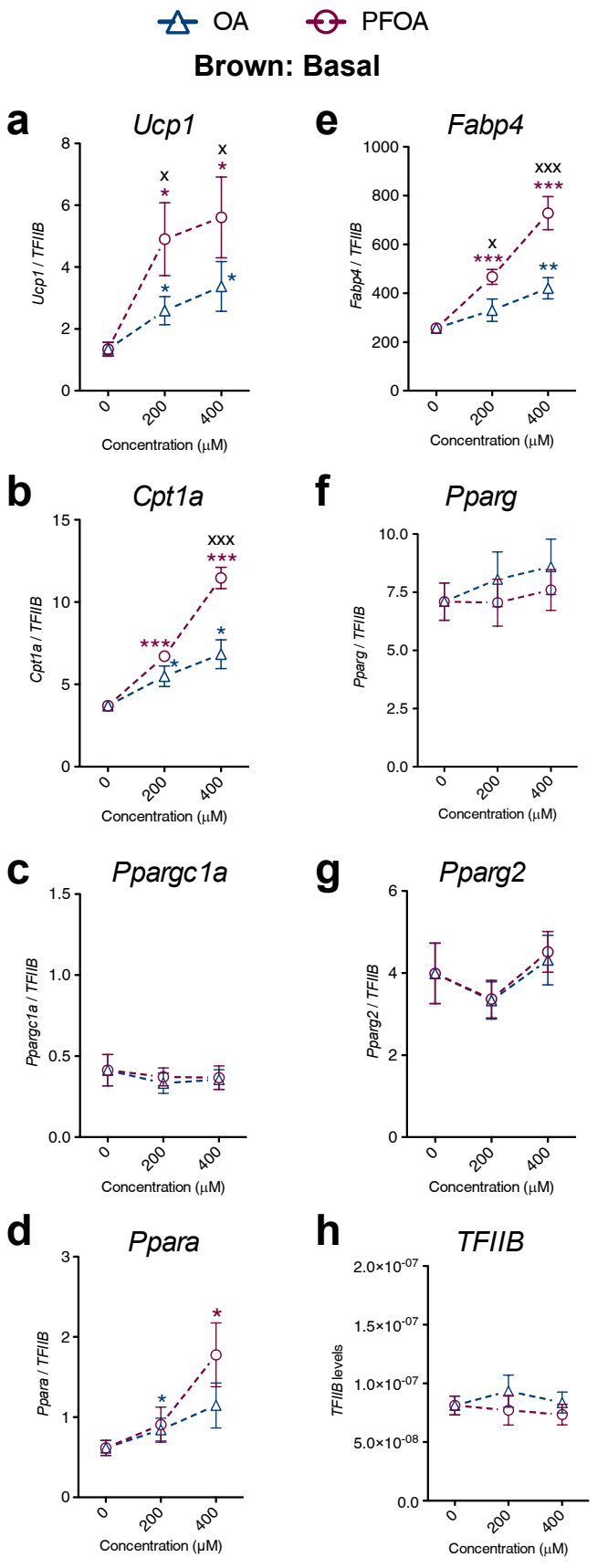

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Fig. 1. Effects of PFOA and octanoate (OA) on basal levels of thermogenesis- and adipogenesis-related genes in brown adipocytes. Graphs show the gene expression levels of uncoupling protein 1 (*Ucp1*) **(a)**, carnitine palmitoyltransferase 1alpha (*Cpt1a*) **(b)**, peroxisome proliferative activated receptor gamma coactivator 1 alpha (*Ppargc1a*) **(c)**, peroxisome proliferator-activated receptor alpha (*Ppara*) **(d)**, fatty acid binding protein 4 (*Fabp4*) **(e)**, peroxisome proliferator activated receptor gamma (*Pparg*) **(f)**, peroxisome proliferator activated receptor gamma 2 (*Pparg2*) **(g)**, and general transcription factor IIB (*TFIIB*) **(h)** in cultured brown adipocytes exposed to PFOA or octanoate for 2 days. Data were normalized between experiments as described in Methods. The points are means ± SEM from 6 independent experiments. Statistical analyses were performed by Student's paired t-test. * shows difference between PFOA or octanoate groups and control group (* $P < 0.05$, ** $P < 0.01$ or *** $P < 0.001$). x shows difference between PFOA and octanoate groups at the same concentrations (x $P < 0.05$, xx $P < 0.01$ or xxx $P < 0.001$).

PFOA clearly augmented the mRNA level observed after NE stimulation.

Thus, although the acute stimulation with NE in itself markedly increased UCP1 mRNA levels (as would be expected (Rehnmark et al., 1990), the level was even higher in the PFOA-pretreated cells (Fig. 3*a*). The same was the case for PGC1α (Fig. 3*c*), whereas concerning carnitine-palmitoyl transferase, PPARα and Fabp4 mRNA levels, NE in itself had no effect – but positive effects of PFOA pretreatment were still evident (Fig. 3*b d e*). For both total PPARγ, as well as for PPARγ2, the expected decreases in mRNA levels (Lindgren et al., 2004) were observed, but the resulting levels were nonetheless higher in the PFOA-pretreated cells than in the untreated cells.

Qualitatively, in the white adipocytes, the effects of PFOA pretreatment on the NE-induced gene expression levels were similar to those observed in the brown adipocytes (Fig. 4). The absolute levels of PGC1α were notably higher in the white adipocytes after NE than in the brown.

Again, no differences were found in the expression of the housekeeping gene *TFIIB* in brown and white adipocytes exposed to PFOA or octanoate and/or NE and controls (Figs. 3*h* and 4*h*).

3.3. The effect of PFOA pre-treatment on the magnitude of the effect of NE on the expression of thermogenesis-related genes

The results above showed the outcome when the sympathetic transmitter NE was added to cells pretreated with PFOA. To evaluate whether PFOA influenced the true magnitude of the response to the adrenergic stimulus, the gene expression levels of untreated, PFOA- or octanoate-exposed cells were subtracted from the respective gene expression levels of the NE-exposed groups in each independent experiment. Data are presented as delta gene expression in Supplementary Fig. S2.

PFOA or octanoate did not influence the magnitude of the NE response on the adipogenesis-related markers FABP4, PPARγ, PPARγ2 and PPARα; these data are therefore not presented. However, pretreatment with PFOA tended to increase the magnitude of the NE-induced UCP1 expression in brown adipocytes, and significantly did so in white adipocytes. Octanoate increased the NE-induced UCP1 expression in both brown and white adipocytes (Supplementary Figs. S2a *d*). PFOA and octanoate also enhanced the NE effect on PGC1α expression in brown adipocytes (Supplementary Fig. S2c), but not significantly in white adipocytes (Supplementary Fig. S2f). Additionally, the presence of PFOA or octanoate tended to indicate a decrease in the magnitude of the CPT1α expression induced by NE in white adipocytes (Supplementary Fig. S2e). These data indicate that PFOA treatment may not only in itself affect the expression levels of important genes but may also influence the response to adrenergic stimulation.

3.4. PFOA per se increases UCP1 and FABP4 protein levels and may affect the protein levels attained after NE stimulation

To examine whether the PFOA-induced changes in the mRNA levels

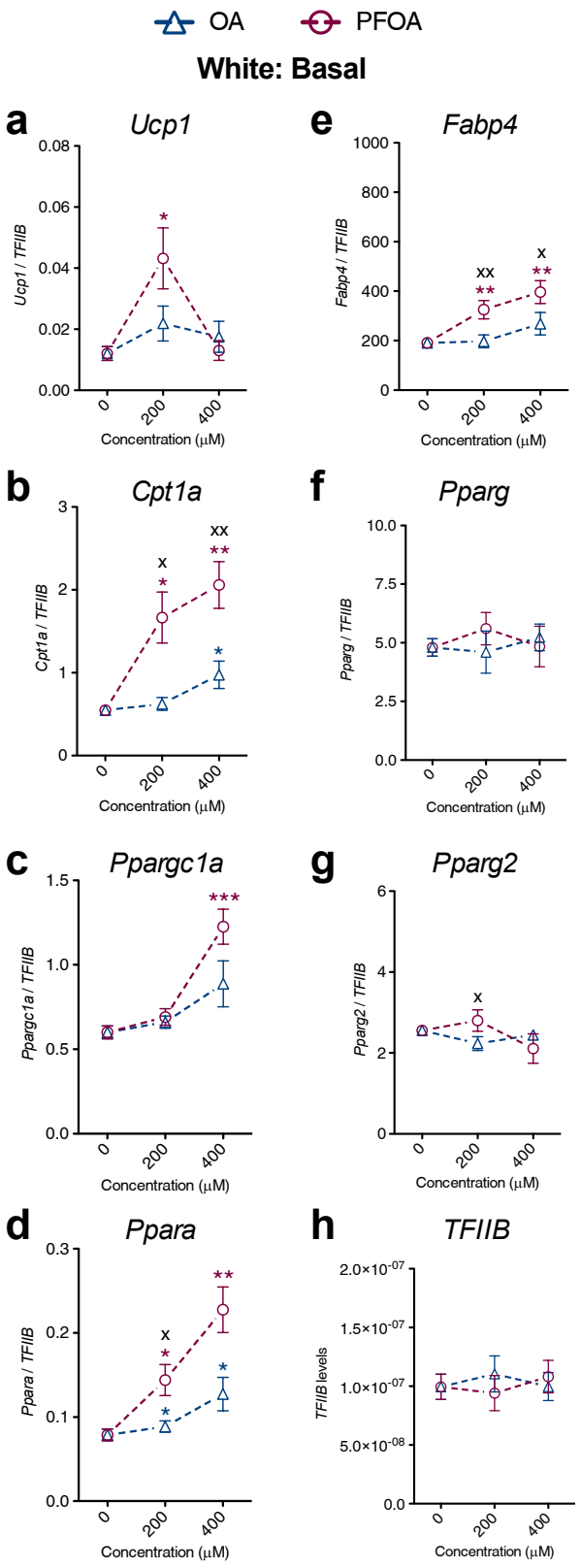

Fig. 2. Effect of PFOA and octanoate (OA) on basal levels of thermogenesis- and adipogenesis-related genes in white adipocytes. Graphs are organized as described in Fig. 1, and show the expression of the indicated genes in white adipocytes exposed to PFOA or octanoate for 2 days. The points are means ± SEM from 6 independent experiments. Statistical analyses were performed as described in Fig. 1.

of genes representative for thermogenesis and adipogenesis, UCP1 and FABP4, were reflected in changes in the corresponding protein levels, western blot analyses were performed with brown and white adipocytes exposed to PFOA, octanoate or vehicle *plus* NE or water. UCP1 was chosen as a marker related to thermogenesis and FABP4 to adipogenesis (Petrovic et al., 2008). To ensure that measurable changes in protein levels could be determined, NE exposure was prolonged to two days, in parallel with the PFOA or octanoate exposures.

A marked increase was observed in UCP1 protein levels in brown adipocytes only exposed to PFOA or octanoate (Fig. 5*a b*). As expected (Puigserver et al., 1992), chronic NE treatment per se markedly increased UCP1 protein levels in brown adipocytes (Fig. 5*a c*). Exposure of brown adipocytes to NE plus PFOA showed a further increase in UCP1 protein levels with 200 μM PFOA but a decrease with 400 μM PFOA (Fig. 5*a c*). – In white adipocytes, UCP1 protein was not detected under any of these conditions (Fig. 5*a*).

PFOA per se increased FABP4 protein levels in both brown and white adipocytes; octanoate did not significantly change FABP4 protein levels (Fig. 5*e g*). Chronic NE treatment led to a consistent increase in FABP4 protein levels (Fig. 5*f h*). The co-exposure of brown and white adipocytes to PFOA *plus* NE further increased FABP4 protein. Octanoate barely affected the FABP4 protein levels seen with NE alone.

Western blot membranes stained for UCP1 and FABP4 on brown and white adipocytes exposed to PFOA or octanoate (OA) plus norepinephrine (NE) or water for 48 h are showed in Supplementary Fig. S3.

Thus, PFOA modified the protein levels of these thermogenesis- and adipogenesis-related genes in adipocytes both per se and in addition to the chronic NE effect.

3.5. FABP4 mRNA and protein levels are positively correlated

The alterations in the levels of UCP1 and FABP4 protein levels induced by treatment with PFOA (or octanoate) could either fully be explainable by alterations in the corresponding mRNA levels, or PFOA could affect the translation processes or e.g. the halflife of the proteins, in which case the relationship between mRNA and protein would be different in the presence or absence of PFOA. We therefore examined the relation between the mRNA levels and the protein levels. Linear regression analyses were performed between the mean mRNA values and the mean protein values for UCP1 and FABP4 in each experimental group. The data are presented in Supplementary Fig. S4. Only non-NE exposed groups were included, since the NE exposure data was for 2 h for mRNA analyses and 48 h for protein analyses. Since UCP1 protein was not observed in white adipocytes, data for UCP1 in these cells could not be analysed.

Formally, no significant correlation was found between UCP1 mRNA and protein levels in brown adipocytes. However, visual examination of the data points indicates that at low UCP1 mRNA levels, a linear correlation exists between the mRNA amounts and the protein amounts (Supplementary Fig. S4a). Apparently, some process becomes saturated at higher UCP1 mRNA levels.

Clear positive correlations were found between FABP4 mRNA and protein levels in both brown and white adipocytes; the numeric ratio protein/mRNA was very similar in the brown and the white adipocytes (Supplementary Fig. S4b *c*). The cell translational machinery must thus be remarkably similar in the two cell types. Notably, the data points for untreated and for PFOA- and octanoate-treated cells all lie on the same line. This indicates that the presence of PFOA or octanoate does not alter the translation processes and that any alterations in mRNA levels will be directly reflected in protein levels. The alterations in protein levels may be expected to affect other processes in the cells, such as their morphology.

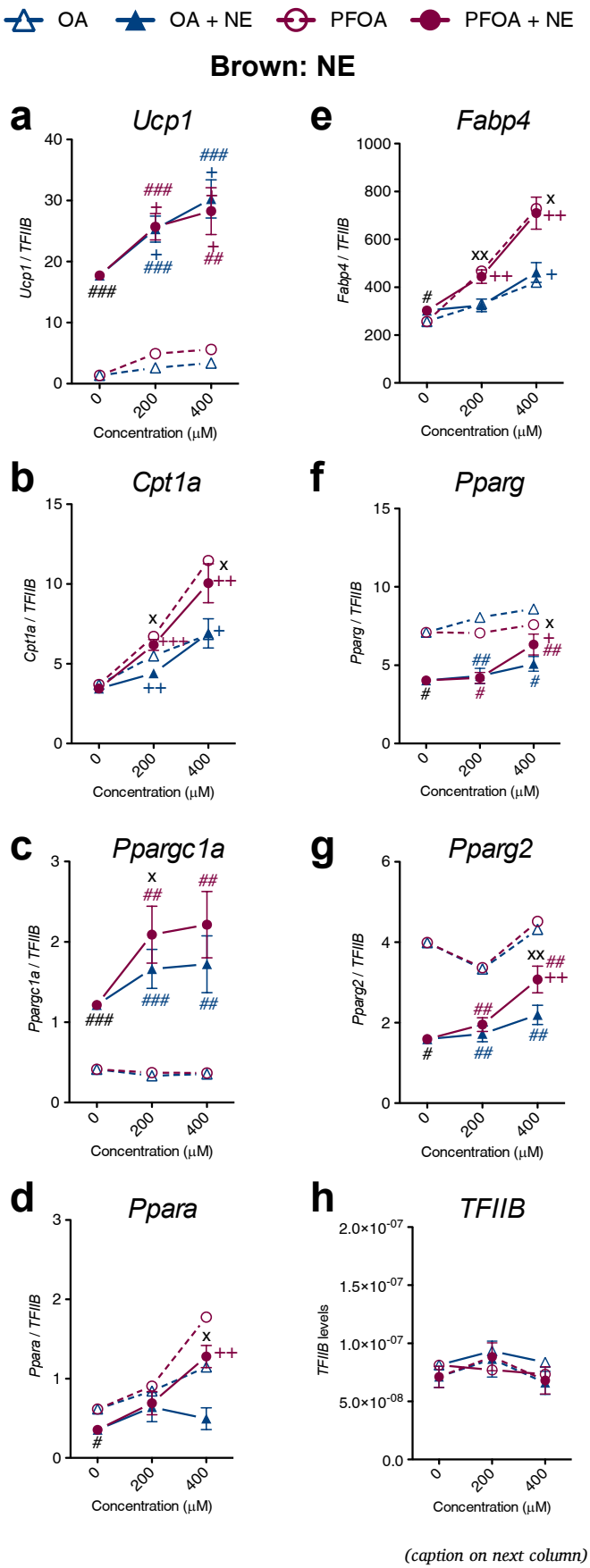

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Fig. 3. Effects of PFOA and octanoate (OA) on basal and NE-stimulated levels of thermogenesis and adipogenesis-related genes in brown adipocytes. Graphs are organized as described in Fig. 1, and show the expression of the indicated genes in brown adipocytes exposed to PFOA or octanoate for 2 days. Filled symbols and solid lines indicate the results from cells also exposed to 1 μM norepinephrine (NE) for 2 h before harvesting. For comparisons, the corresponding mean data from Fig. 1 are shown in open symbols and dashed lines. In each experiment, the data were normalized to the value of the NE control group and replotted as the absolute mean of the values. The points are means ± SEM from 6 independent experiments. Statistical analyses were performed as described in Fig. 1. + shows difference between groups exposed to PFOA or octanoate *plus* NE and NE group (+ $P < 0.05$, ++ $P < 0.01$ or +++ $P < 0.001$). # shows difference between PFOA or octanoate *plus* NE groups and respective PFOA or octanoate groups not exposed to NE (# $P < 0.05$, ## $P < 0.01$ or ### $P < 0.001$). x shows difference between PFOA and octanoate groups at the same concentrations (x $P < 0.05$, xx $P < 0.01$ or xxx $P < 0.001$).

3.6. *Effect of PFOA treatment on cell morphology and the cellular distribution of UCP1*

3.6.1. *No marked alterations of cell structure due to PFOA treatment*

To visualize the effects of PFOA on the cell cultures, cells were examined by digital phase-contrast microscopy. No marked structural effects were observed. However, it would seem from the pictures that in brown adipocytes (Supplementary Fig. S5), PFOA (200 μM) promoted the accumulation of lipid droplets, and the presence of NE led to a decrease in lipid accumulation (due to stimulated lipolysis (Kussela et al., 1986)). These effects were less marked in the white adipocytes (Supplementary Fig. S5).

3.6.2. *Marked effects of PFOA on UCP1 protein presence in certain cells*

To examine to what degree the increase in UCP1 protein amount was caused by a general increase in UCP1 protein in all cells or a large increase only in certain cells, we examined the cellular distribution of UCP1. In accordance with the western blot data above – showing that UCP1 is very weakly expressed in the white adipocytes – the UCP1 signal was so low in the immunohistochemistry studies that these data are not shown. As expected, even in the brown adipocytes, very few cells expressed visualisable amounts of UCP1 protein in the absence of any treatment (Fig. 6*a*). Norepinephrine, again as expected, markedly increased the number of cells that stained positively for UCP1 protein expression (Fig. 6*b*). No notable effect of octanoate (200 μM) on the density of UCP1 positive cells was observed (Fig. 6*c*), nor was there any obvious influence of octanoate in the presence of NE (Fig. 6*d*). PFOA (200 μM), however, in itself markedly increased the number of cells staining positively for UCP1 (Fig. 6*e*). The striking response to PFOA alone demonstrates that the increased mRNA levels indeed resulted in increased UCP1 protein, converting some of the non-thermogenic brown adipocytes into potentially thermogenic and energy-consuming adipocytes. – In this qualitative analysis, it is not possible to strictly determine whether the fraction of cells that express visualisable levels of UCP1 is higher in the cell cultures that have been exposed to both PFOA and NE than in those only exposed to NE. If anything, this would seem to be the case.

4. Discussion

We report here that the environmental pollutant PFOA has the potential in itself to induce the expression of genes related both to thermogenesis and to adipogenesis in both brown and white adipocytes. These findings relate to the potential metabolic toxicity of PFOA, through the impact of this and similar compounds on the environment. Particularly, our results uncover and identify molecular mechanisms through which these effects are mediated and underscore the ability of these compounds to affect the metabolism of animals and thus

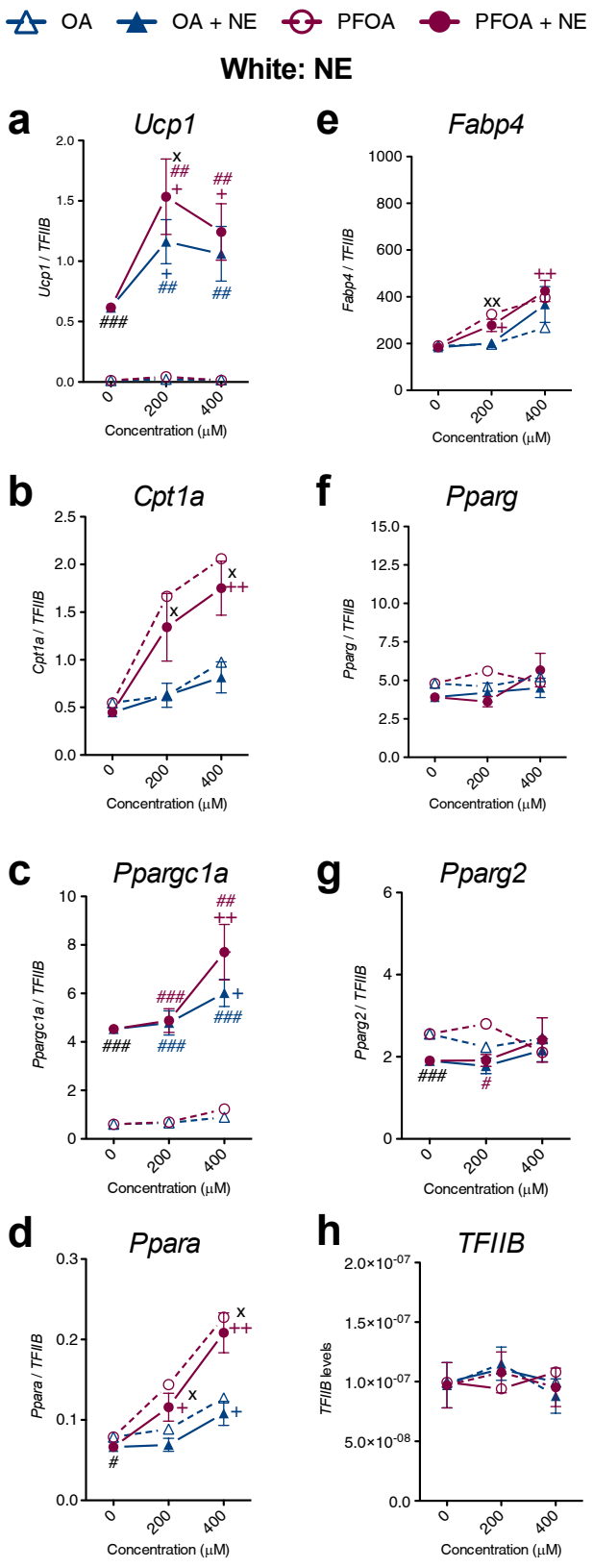

Fig. 4. Effects of PFOA and octanoate (OA) on basal and NE-stimulated levels of thermogenesis and adipogenesis-related genes in white adipocytes. Graphs are organized as described in Fig. 1, and show the expression of different genes in white adipocytes exposed to PFOA or octanoate for 2 days *plus* NE for 2 h before harvesting. Details and statistical analyses are as detailed in legend to Fig. 3.

potentially of humans.

4.1. A direct effect on gene expression

Earlier studies of the effect of PFOA on the expression of thermogenic genes (particularly UCP1) were performed in intact animals (Shabalina et al., 2015). It has therefore not been possible to distinguish as to whether the effects seen are direct effect on gene expression or due to other regulatory mechanisms in the animals, involving e.g. the major regulatory pathway for brown adipose tissue recruitment: the sympathetic nervous system.

Here we show that in the isolated system of primary cultures of brown and white adipocytes, PFOA is fully able in itself to induce the expression of important thermogenesis-related genes: UCP1 and carnitine-palmitoyl transferase (CPT1A). Additionally, it may be said to promote adipogenesis, as demonstrated through its effects on the expression of the adipogenesis marker fatty-acid-binding protein 4 (FABP4, also called aP2). Thus, PFOA not only has the ability to directly activate the controlling enzyme for thermogenesis: UCP1 (Shabalina et al., 2016); it can also in itself promote this thermogenic effect by enhancing the thermogenic machinery of the cell. This endows PFOA with the dual ability to control thermogenic pathways in a pathological manner.

4.2. Mediation of the PFOA effects

The present experiments were not designed to elucidate in detail the molecular mechanisms underlying the effects seen. There are several possibilities. PFOA may act through activation of known and recognized already well-expressed transcription factors affecting thermogenic and adipogenic gene expression, or it may act by enhancing the expression of transcription factors and thus promoting endogenous stimulation of thermogenic and adipogenic gene expression.

4.2.1. Direct activation of the PPARs

PFOA has been demonstrated to function as a ligand for peroxisome-proliferator activated receptors (PPARs) (Takacs and Abbott, 2007; Yamamoto et al., 2015). This ligand action can lead to increased expression of responsive genes involved in adipogenesis and thermogenesis. The liganded receptors dimerize with RXR and the complex then interacts with PPREs to enhance gene expression (see e.g. (Yi et al., 2020) for UCP1). While the nature of the physiological ligands for the PPARs is still discussed, fatty acids and derivatives, as well as thiazolidinediones, act by interaction with these ligand binding sites (Nedergaard et al., 2005; Petrovic et al., 2008, 2010; Shin and Ajuwon, 2018).

Brown adipocytes express all three PPAR isoforms: α, β/δ and γ (Braissant and Wahli, 1998). Lipid catabolism is primarily regulated by the PPARα isoform (Desvergne and Wahli, 1999) through upregulation of fatty acid oxidation-related genes, including carnitine palmitoyltransferase *Cpt1a* determined here, as well as acyl-CoA synthase and acyl/CoA oxidase (Mandard et al., 2004). PFOA has been described as a PPARα agonist (Sohlenius et al., 1992; Yang et al., 2002; Perkins et al., 2004; Martin et al., 2007; Hung et al., 2010).

The process of adipogenesis in adipose tissue is regulated by another PPAR-family member, PPARγ (Spiegelman and Flier, 1996). PPARγ activation regulates induction of genes controlling lipid accumulation, such as fatty acid binding protein FABP4 measured here, as well as fatty acid synthase and lipoprotein lipase (Takahashi et al., 2009).

Thus, the effects reported here of PFOA on UCP1, carnitine palmitoyltransferase and FABP4 may adequately be understood as being mediated through PFOA directly activating these PPARs.

4.2.2. Enhanced expression of the transcription factors themselves

Alternatively, or in parallel with, activating PPARs, PFOA could increase the thermogenic potential by enhancing the expression of transcription factors that would then be able to further promote

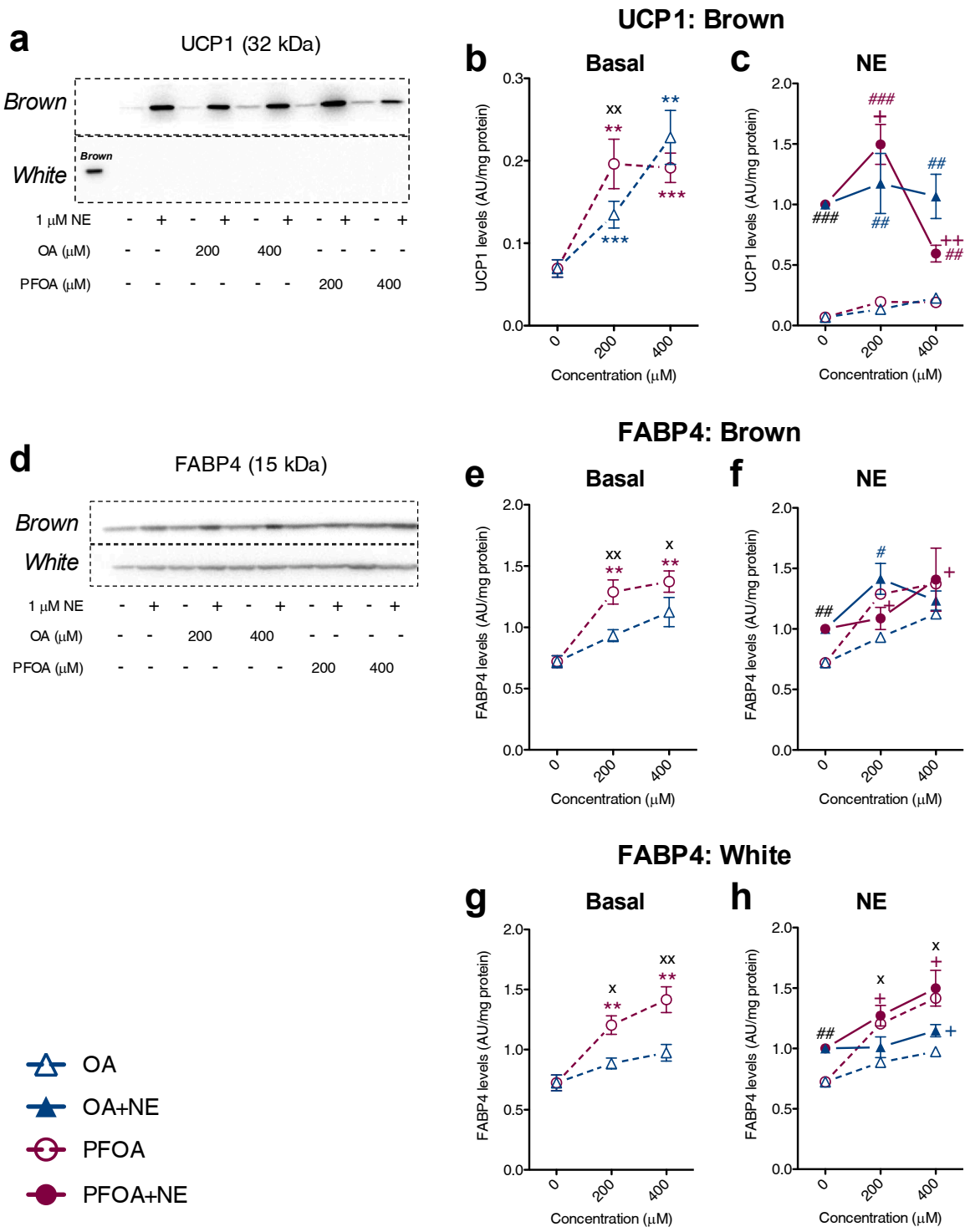

Fig. 5. UCP1 and FABP4 protein levels of brown and white adipocytes exposed to PFOA or octanoate (OA) *plus* norepinephrine (NE) or water for 48 h. (a) shows representative Western blots of UCP1 from brown and white adipocytes exposed to PFOA, octanoate or vehicle *plus* NE or water. Top shows a representative membrane with brown adipocyte samples. Bottom shows a representative membrane with white adipocyte samples; this membrane only shows one band corresponding to a positive control (brown adipocytes exposed to NE for 48 h). **(b-c)** show UCP1 levels of brown adipocytes exposed to PFOA, octanoate or vehicle *plus* water or NE. **(d)** shows representative Western blots of FABP4 from brown and white adipocytes exposed to PFOA, octanoate or vehicle *plus* NE or water. **(e, f)** and **(g, h)** show FABP4 levels of white and brown adipocytes, respectively, exposed to PFOA, octanoate or vehicle *plus* NE or water. The points are means ± SEM from the 6 independent experiments (or only mean for groups not exposed to NE in **c**, **f** and **h**). Statistical analyses were performed by paired Student's t-test. * shows difference between PFOA or octanoate groups and control group (* $P < 0.05$, ** $P < 0.01$ or *** $P < 0.001$); + shows difference between groups exposed to PFOA or octanoate *plus* NE and NE group (+ $P < 0.05$, ++ $P < 0.01$ or +++ $P < 0.001$); # shows difference between PFOA or octanoate *plus* NE groups and respective PFOA or octanoate groups not exposed to NE (# $P < 0.05$, ## $P < 0.01$ or ### $P < 0.001$). x shows difference between PFOA and octanoate groups at the same concentrations (x $P < 0.05$, xx $P < 0.01$ or xxx $P < 0.001$).

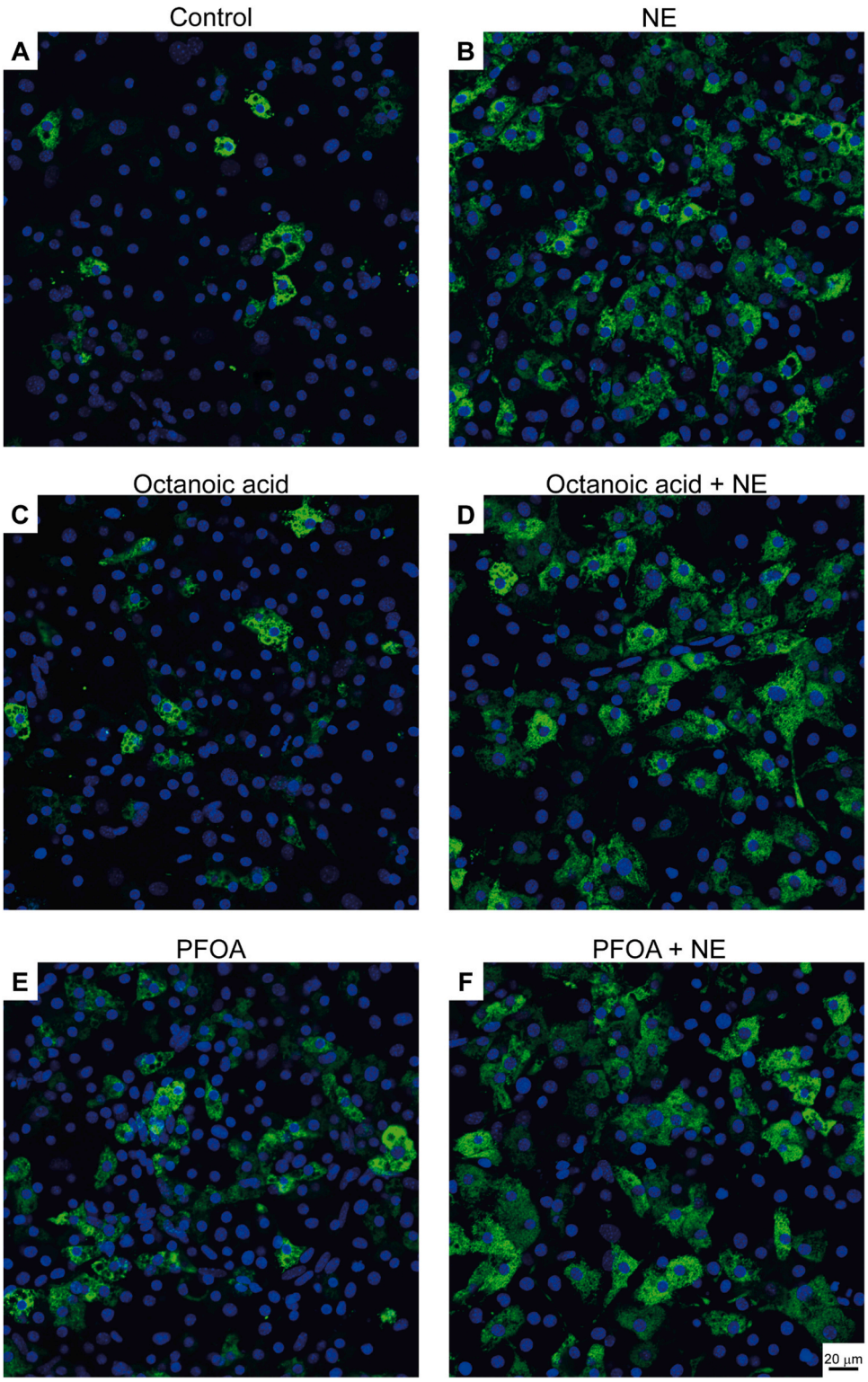

Fig. 6. Brown adipocytes exposed to 200 µM PFOA or octanoate (OA) *plus* 1 µM norepinephrine (NE) or water for 48 h and stained for UCP1 (green) and nuclei (blue). Photos were taken in a Zeiss LSM 780 confocal microscope with a 20x objective. Scale bar indicate 50 µm.

thermogenesis and adipogenesis, by being activated by endogenous or exogenous ligands.

PGC1α is a transcriptional co-activator in the adrenergic recruitment of thermogenic adipocytes (Handschin and Spiegelman, 2006). In response to sympathetic stimulation, adenylyl cyclase is activated, increasing the intracellular cyclic adenosine monophosphate (cAMP) concentration and protein kinase A (PKA) activity, and subsequently PGC1α. Through its action as a co-activator, PGC1α coordinates the expression of genes related to mitochondrial biogenesis and oxidative metabolism. Here we observed that NE, as expected, increased PGC1α mRNA (*Ppargc1a*) levels in both brown and white adipocytes; however, notably, PFOA did not influence PGC1α mRNA levels. Thus, PFOA would appear not to affect the expression of thermogenesis-related genes through a pathway involving enhanced action of cAMP/PGC1α. PGC1α

gene expression may also be increased through activation of PPARα (Hondares et al., 2011) but in our system this did not happen, although the data indeed indicate that PFOA activated PPARα.

The mRNA levels of PPARα in brown and white adipocytes exposed to PFOA demonstrated a potent increase, in agreement with reports that PPARα agonists enhance PPARα mRNA levels (a feed-forward process) (Kim et al., 2019). This may thus make the cells even more susceptible to the PFOA effects.

No alterations in the levels of PPARγ or PPARγ2 mRNA were seen in the PFOA-treated adipocytes.

Thus, in relation to transcription factors, PFOA initiates a feed-forward pathway that would make the cells even more susceptible to the effects of PFOA; the expression of other transcription factors examined here is not directly affected.

4.3. Augmenting effect of PFOA pretreatment on norepinephrine-induced expression of UCP1

A significant finding from the present results is also the observation that the presence of PFOA not only in itself led to a stimulation of the expression of UCP1, but that its presence also enhanced the response of the adipocytes to norepinephrine. Thus, the magnitude of the NE response was notably greater than the sum of the actions of the two agents. Although not studied here, it may be suggested that the enhanced expression of PPARα caused by PFOA may be involved in the mediation of this augmenting effect of PFOA. Stimulation of the cells by norepinephrine leads to activated lipolysis, and some of the induction of the norepinephrine-induced expression of UCP1 may be mediated through the released fatty acids binding to PPARs and the activated PPARs then binding to the PPRE in the enhancer region of the UCP1 gene.

4.4. Comparison between PFOA and octanoate

To elucidate to which degree the responses of the adipocytes to PFOA are understandable as resulting from PFOA being a modified aliphatic fatty acid, octanoate was used as a naturally occurring fatty acid, with the same number of carbons as PFOA but without fluoride substitutions. We observed that octanoate influenced gene expression when PFOA influenced gene expression, although the effects were in general significantly less pronounced that those of PFOA. Thus, octanoate was less potent than PFOA in inducing thermogenic capacity and adipogenesis in adipocytes. The present results also suggest that the response to PFOA in certain cases appeared to have peaked at the lower dose of PFOA, whereas this was not the case for octanoate. Thus, the present data as such do not indicate that the effects of PFOA are principally different from those of fatty acids. However, the large difference is that the effects of natural fatty acids such as octanoate are temporary in that the fatty acids will ultimately be combusted or stored, whereas the PFOA will always be present at a physiologically unregulated level.

4.5. Consequences of the increased FABP4 levels

We find that PFOA increases FABP4 mRNA and protein levels in the adipocytes, indicating stimulation of adipogenesis. Indeed, in our cell culture system, treatment with PFOA seemingly led to an increase in lipid accumulation in the cells. Thus, based on these observations alone, PFOA would be predicted to promote obesity in mice, similarly to what has been reported for other pollutants (Gi Gregori et al., 2019). However, what has been observed is the opposite: that PFOA-treated mice lose weight (Xie et al., 2002, 2003). This is due to decreased food intake and possibly also to stimulated thermogenesis (Shabalina et al., 2015). Probably, these effects would exceed the PFOA-induced effects on adipogenesis. It may be noted that, in this way, there are some similarities between the in-vivo effects of the thiazolidinediones (plitazones), such as pioglitazone (Valdivia et al., 2023), and PFOA. The thiazolidinediones promote obesity and promote thermogenic gene expression. However, they do not decrease appetite; thus, their obesity-promoting effects are those that become manifest. PFOA may affect lipid storage as such – and in this way withhold energy from the circulation in a pathophysiological imbalance – but the effects will not be visible due to the decreased food intake.

4.6. Consequences of the increased UCP1 levels

We show here that PFOA increases the expression level of the UCP1 gene, both in itself and when UCP1 gene expression is also stimulated by norepinephrine. This will result in higher UCP1 protein levels, at least if a saturation level is not encountered. It may be anticipated that an increased UCP1 amount will necessarily result in a higher metabolic rate, leading to uncontrolled loss of energy. However, this is not necessarily the case. UCP1 is not leaky (Shabalina et al., 2010), and the presence of high amounts of UCP1 does not necessarily lead to decreased metabolic efficiency (Valdivia et al., 2023; von Essen et al., 2023); i.e. the UCP1 has also to be stimulated in order to be active. However, PFOA is of a special nature in that it can, in itself, activate UCP1 (Shabalina et al., 2016). Thus, whereas we have earlier shown that PFOA is able to directly and acutely activate already existing UCP1 in brown-fat mitochondria, we additionally show here that chronic PFOA can increase the number of cells in brown adipose tissue that express UCP1, and increase the total amount of UCP1 in the cells, through the increased expression of UCP1. This means that the action of PFOA on brown-fat thermogenesis is multiplied in that it both increases the thermogenic capacity and the thermogenic activity. Whereas there may be conditions under which such increases could be helpful for survival, they would be pathophysiologically governed and may thus be generally detrimental.

4.6.1. Environmental effects

If animals that are competent to express UCP1 absorb PFOA found in the environment, this would enhance expression of UCP1 and genes related to thermogenesis and adipogenesis under conditions that would not normally require expression of UCP1. In a cold environment, animals could use PFOA-mediated thermogenesis to help maintain body temperature and the effects would be minor, but in a warmer environment, the extra heat would be a burden and lead to unnecessary and unwanted energy consumption. Since PFOA has a very long half-life in humans and animals, being almost non-degradable and not eliminated, its effects will persist. As a consequence of increased thermogenesis, PFOA-exposed animals could present with decreased fitness because of the excessive combustion of limited energetic resources. Furthermore, to aggravate this scenario, PFOA also induces a decrease in food intake and body weight in mice in a partly UCP1-dependent manner (Xie et al., 2002, 2003; Shabalina et al., 2015). The immediate environmental implication of the present study is that PFOA exposure has a direct negative effect on the energy metabolism of animals, and this can be particularly relevant for predators that experience biomagnification.

While the concentrations of PFOA used in this study were much higher than those currently found in samples from humans and animals (although a wide range of PFOA concentrations have been reported (Death et al., 2021)), this study should be seen as an indication of what may occur, especially as PFOA contamination may be more widespread than is normally realized (including being present in paper straws (Boisacq et al., 2023)).

4.6.2. Possible human anti-obesity effects

It is now recognized that most younger adult humans possess significant amounts of active brown adipose tissue (Nedergaard et al., 2007; van Marken Lichtenbelt et al., 2009; Nedergaard and Cannon, 2010; Yoneshiro et al., 2011). It has been proposed that the activation of BAT, as well as the browning and activation of WAT, may be helpful in ameliorating obesity and its co-morbidities (e.g. (Nedergaard and Cannon, 2010)). Since PFOA and similar compounds decrease food intake by

pathways in part involving UCP1 (Xie et al., 2002, 2003; Shabalina et al., 2015), increase UCP1 expression as shown here, and can directly activate UCP1 (Shabalina et al., 2016), our results provide insights for the potential discovery of new pathways that, when stimulated, could be utilized to counteract obesity.

Ethical standards

All procedures performed in this study involving animals were in accordance with the ethical standards of the institution at which the studies were conducted.

CRediT authorship contribution statement

Nedergaard Jan: Conceptualization, Writing – original draft, Writing – review & editing. **Petrovic Natasa:** Conceptualization, Investigation, Writing – review & editing. **Cannon Barbara:** Conceptualization, Writing – original draft, Writing – review & editing. **Reckziegel Patrícia:** Conceptualization, Investigation, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Jan Nedergaard reports financial support and equipment, drugs, or supplies were provided by Swedish Research Council for Sustainable Development (FORMAS). Patricia Reckziegel reports a relationship with São Paulo Research Foundation (FAPESP) that includes: funding grants. The authors declare that they have no conflict of interest associated with this manuscript.

Data Availability

Primary data will be provided by the authors on request.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2024.115955.

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