trans-Activation of PPARα and Induction of PPARα Target Genes by Perfluorooctane-Based Chemicals

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Peroxisome proliferator-activated receptors (PPARs) are ligand-dependent transcription factors that activate target genes involved in lipid metabolism, energy homeostasis, and cell differentiation in response to diverse compounds, including environmental chemicals. The liver-expressed receptor PPARα mediates peroxisome proliferative responses associated with rodent hepatocarcinogenesis. Previous studies have established that certain perfluorooctanesulfonamide-based chemicals (PFOSAs) alter lipid metabolism, are hepatic peroxisome proliferators, and induce hepatocellular adenoma formation in rodents, suggesting that they activate PPARα. The present study investigates this question and characterizes the activation of mouse and human PPARα by PFOSAs. Perfluorooctanesulfonate (PFOS), an end-stage metabolite common to several PFOSAs, was found to activate both mouse and human PPARα in a COS-1 cell-based luciferase reporter *trans*-activation assay. Half-maximal activation (EC_{50}) occurred at 13–15 µM PFOS, with no significant difference in the responsiveness of mouse and human PPARα. Mouse and human PPARα were activated by perfluorooctanesulfonamide (FOSA) over a similar concentration range; however, cellular toxicity precluded an accurate determination of EC_{50} values. Studies of 2-*N*-ethylperfluorooctanesulfonamido ethanol were less informative due to its insolubility. These findings were verified in an FAO rat hepatoma cell line that stably expresses PPARα, where the endogenous PPARα target genes peroxisomal bifunctional enzyme and peroxisomal 3-ketoacyl-CoA thiolase were activated up to ∼10–20-fold by PFOS and FOSA. The interactions of PPARα with PFOS and FOSA, and the potential of these chemicals for activation of unique sets of downstream target genes, may help explain the diverse biological effects exhibited by PFOSAs and may aid in the evaluation of human and environmental risks associated with exposure to this important class of fluorochemicals.

Key Words: **perfluorooctanesulfonamide; perfluorooctanesulfonate; *N*-ethyl perfluorooctanesulfonamidoethanol; rodent hepatocarcinogenesis; ppar; peroxisome proliferation.**

Perfluorooctanesulfonate (PFOS; $C_8F_{17}SO_3$-) and perfluorooctanesulfonamide derivatives (PFOSAs) constitute a class of fluorochemicals used in many industrial and consumer applications as powerful surfactants or components of products which provide oil and water-resistant properties to paper and fabrics (MacKay, 1991). In May 2000, 3M Company announced that it would voluntarily cease production of perfluorooctanyl-based chemicals due to widespread presence of PFOS in the environment (Giesy and Kannan, 2001; Hansen *et al.*, 2001). Epidemiological studies have documented human occupational exposure to PFOS but revealed no associations of PFOS with hepatic enzyme and lipid clinical chemistry parameters (Olsen *et al.*, 1999). A substantial body of research has been undertaken to characterize the metabolic actions and potential toxicities associated with these fluorochemicals, with the goal of evaluating the risk associated with exposures at both occupational and environmental levels. These and other studies have shown that PFOS and related chemicals can have a variety of metabolic and other effects. PFOS and *N*-ethyl perfluorooctanesulfonamidoethanol (*N*-EtFOSE) stimulate hepatic peroxisome proliferation when administered to rodents. PFOS, the ultimate metabolite of the perfluorooctanesulfonamido derivatives in mammalian systems, accumulates in liver tissue (Butenhoff and Seacat, 2001; Gibson *et al.*, 1983). PFOS and perfluorooctanoate (PFOA), a chemically distinct fluorochemical, in addition to certain other perfluorinated carboxylic acids, have also been shown to increase liver triglyceride and free cholesterol levels, lower serum cholesterol, cause hypolipidemia, increase liver weight, and cause aphagia (Berthiaume and Wallace, 2002; Haughom and Spydevold, 1992; Ikeda *et al.*, 1987; Seacat *et al.*, 2002). Perfluorooctanesulfonamide (FOSA), which is a minor metabolite of *N*-EtFOSE (Butenhoff and Seacat, 2001) is a potent uncoupler of mitochondrial oxidative phosphorylation (Schnellmann and Manning, 1990). Mitochondrial uncoupling has also been reported following PFOS and PFOA treatment (Starkov and Wallace, 2002).

Peroxisome proliferation and certain other metabolic effects of PFOSAs may be a consequence of the activation of the nuclear receptor PPARα. PPARα is a member of the nuclear receptor superfamily and mediates a broad range of biological responses

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to fatty acids, their synthetic analogs and structurally diverse lipophilic chemicals containing an acidic group (Gonzalez *et al.*, 1998). Peroxisomes are organelles involved in fatty acid oxidation, cholesterol metabolism, and hydrogen peroxide-linked respiration (Lock *et al.*, 1989). Activation of PPARα leads to altered regulation of distinct sets of genes involved in lipid metabolism and homeostasis, peroxisome proliferation, and cell growth (Corton *et al.*, 2000). Long-term exposure of rodents to peroxisome proliferator chemicals causes a PPARα-dependent increase in liver tumors (Peters *et al.*, 1997), raising concerns about the safety of these compounds for humans and other species (Cattley *et al.*, 1998). In contrast to rats and mice, guinea pigs and other species, including cynomolgus monkeys (Seacat *et al.*, 2002), show little or no evidence of peroxisome proliferation when treated with fluorochemicals or other peroxisome proliferating compounds. Studies with human hepatocytes suggest that humans are also likely to be poorly responsive to hepatic peroxisome proliferators. This species-dependence reflects several factors, one of which is the expression of PPARα in humans and other unresponsive species at a substantially lower level than in rat and mouse liver (Palmer *et al.*, 1998).

A second important factor contributing to the species-specificity of peroxisome proliferative responses is the lower intrinsic responsiveness of human PPARα compared to rodent PPARα to some, but not all, peroxisome proliferator chemicals (Keller *et al.*, 1997; Maloney and Waxman, 1999). Human PPARα requires a 5-fold higher concentration of the classical peroxisome proliferator Wy-14,643 for transcriptional activation when compared to mouse PPARα activation (Keller *et al.*, 1997; Maloney and Waxman, 1999). A similar species difference in PPARα responsiveness is seen with the phthalate monoester mono(2-ethylhexyl)-phthalate (MEHP), a plasticizer metabolite and widespread environmental pollutant (Hurst and Waxman, 2003). Human PPARα also requires a higher concentration of perfluorooctanoate to achieve maximal activation than does mouse PPARα (Maloney and Waxman, 1999). Important differences in PPAR ligand specificity are also apparent in the comparison of PPARα and PPARγ (Forman *et al.*, 1997). For example, PPARα but not PPARγ is strongly activated by classic rodent peroxisome proliferators, such as Wy-14,643 and fibrate drugs (e.g., clofibrate, nafenopin), whereas PPARγ is preferentially activated by a distinct set of chemicals, including anti-diabetes type II drugs of the thiazolidinedione class, such as troglitazone. PPARγ can, however, be efficiently activated by certain activators of PPARα, as shown in the case of MEHP (Hurst and Waxman, 2003; Maloney and Waxman, 1999). It is suspected that PPARα can be activated by PFOSAs; however, it is uncertain whether species-dependent differences in receptor responsiveness, which could be important for human risk assessment, characterize this class of fluorochemicals.

The specific goals of the present study were (1) to ascertain whether, and with what potency, PFOSAs activate PPARα, the major mediator of hepatic peroxisome proliferation responses, as determined using *trans*-activation reporter assays and by evaluation of the responsiveness of endogenous PPARα target genes in a liver cell model; and (2) to characterize the sensitivity of human PPARα to PFOSAs, which may aid in the extrapolation to human toxicology and human risk assessment findings based on rodent model studies.

MATERIALS AND METHODS

Chemicals. Wy-14,643 and DMSO were purchased from Sigma Chemical Co. (St. Louis, MO). Fluorochemicals were obtained from 3M Corporation (St. Paul, MN). Ammonium acetate (99.999%) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Acetonitrile was purchased from EM Science (Gibbstown, NJ). Gas chromatography (GC) and high performance liquid chromatography (HPLC) columns were purchased from Phenomenex (Torrance, CA).

Fluorochemical analysis. Working solutions of each fluorochemical were prepared in DMEM as described below at concentrations of 8, 16, 32, 64, 125, 250, 500, and 1000 μM, with a final DMSO concentration of 0.1%. HPLC/electrospray mass spectrometry (ESI-MS) was used to quantify the concentrations of PFOS and FOSA in spiked DMEM samples using a Thermo-Finnigan LCQ HPLC/MS system (LCQ) and a Thermo-Finnigan aQa HPLC/MS system (aQa). Both HPLC/MS instruments included electrospray ionization sources that were operated in the negative ionization mode. The ESI probes were maintained at ~4 kV and 285°C. LCQ mass spectral data were acquired by full scan with the selected-ion-current area of m/z 499 and m/z 498 being used for quantitation. aQa mass spectral data were acquired in the selected-ion-monitoring mode using the same masses for quantification. PFOS and FOSA were separated from other sample components using a 4 mm × 125 mm Phenosphere ODS (2), 5 μm column and a 30 to 95% acetonitrile gradient in water (over 4 min) at a flow rate of 0.5 ml/min. Both solvents contained 6 mM ammonium acetate as a modifier.

The responses of the HPLC/MS systems were calibrated by analyzing a series of PFOS and FOSA reference standards. The areas of the m/z 499 ions (PFOS) and the m/z 498 ions (FOSA) were used to generate calibration curves that were used to quantify the concentrations of PFOS and FOSA in the spiked MEM samples. The m/z 499 ion is the anion of PFOS acid and the m/z 498 ion is the $(M-H)^-$ ion of FOSA. Prior to the analyses, each of the samples was diluted with acetonitrile such that the resulting solutions had PFOS and FOSA concentrations that produced responses within the linear response range of the HPLC/MS. Portions of these diluted solutions were then transferred to glass autosampler vials for HPLC/ESI-MS analysis. The areas of the m/z 499 and m/z 498 responses were compared to the calibration curves to calculate the PFOS and FOSA concentrations. The reported concentrations included the initial dilution factor in the concentration calculations.

N-EtFOSE concentrations in the spiked MEM samples were quantified using a Hewlett Packard Model 5890 Series II GC equipped with a flame ionization detector, a Hewlett Packard Model 7673 autosampler, and ChemStation data system. *N*-EtFOSE was separated from other compounds present in the samples using a Zebron ZB-5 30 meter fused silica capillary column having an inside diameter of 0.32 mm and a ZB-5 film thickness of 0.25 μm. Helium was used as the carrier gas at a flow rate of 40 cm/s. The column temperature was programmed from an initial temperature of 80°C (1-min hold time) to 190°C at a linear rate of 15°C/min, then to a final temperature of 340°C at a linear rate of 35°C/min (3-min hold time). A 2-μl portion of each diluted sample was injected using a 10:1 split ratio. Prior to sample analyses, the GC was calibrated with a series of *N*-EtFOSE reference standards.

Plasmids. The firefly luciferase reporter pHD(x3)Luc, obtained from Dr. J. Capone (McMaster University, Toronto, ON, Canada), contains three tandem copies of a PPAR-activated DNA response element derived from the rat enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase gene linked

directly to a minimal promoter (Zhang *et al.*, 1993). Expression plasmids for mouse PPARα and human PPARα, respectively provided by Dr. E. Johnson (Scripps Research Institute, La Jolla, CA) and Dr. F. Gonzalez (National Cancer Institute, Bethesda, MD), were described previously (Hurst and Waxman, 2003). *Renilla* luciferase reporter plasmid, pRL-CMV, was purchased from Promega (Madison WI). pBabe-puro expression vector containing the full-length cDNA encoding mouse PPARα, which was used to generate FAO-PPARα cells (see below), was obtained from Dr. B. Spiegelman (Dana-Farber Cancer Institute, Boston, MA).

Transient transfection assays. COS-1 cells were grown in Dulbecco's modified Eagles medium (DMEM) containing 10% fetal bovine serum. COS-1 cells were plated at a density of 3×10^4 cells/well of a 48-well tissue culture plate containing 500 µl of culture medium per well. The medium was replaced with 0.25 ml of fresh DMEM + FBS 24 h after plating, at which time transfection was carried out using 0.3 µl FuGENE 6 transfection reagent (Roche Diagnostics Corp., Indianapolis, IN) per well. Twenty-four h after addition of the DNA-FuGENE mixture to the cells, the medium was changed to serum-free DMEM containing each test compound at concentrations specified in each experiment. Cells were lysed 24 h later and Firefly luciferase and *Renilla* luciferase activities were measured using a dual luciferase assay kit (Promega). Transfections were performed using the following amounts of plasmid DNA/well unless indicated otherwise: 90 ng of pHD(x3)Luc, 5 ng of mPPARα or hPPARα expression plasmid and 1 ng of pRL-CMV. Salmon sperm DNA was added to the plasmid DNA mix to give a total of 200 ng.

Cell treatment and data analysis. Fluorochemicals were dissolved in DMSO to give a 250 mM stock solution. Serial dilutions were prepared in DMSO, followed by a final 1000-fold dilution into serum-free DMEM. Stock solutions of fluorochemicals in DMSO were prepared in triplicate, followed by dilution of each sample into DMEM in duplicate, to give six replicates (tested in six parallel wells) for each fluorochemical at each concentration. Data presented are mean values $\pm$ SD based on $n = 3$ independent dilutions, with each value being an average of duplicate determinations, except as noted. Luciferase activity values were normalized for transfection efficiency by dividing the measured Firefly luciferase activity values by the *Renilla* luciferase activity obtained for the same cell extract, i.e., (Firefly/*Renilla*) $\times$ 1000. Statistical analysis was performed using SAS Jump software, Version 3. Individual pairwise comparison of means were performed using Student's *t*-test, with $p < 0.05$ deemed significant.

Construction of FAO cells that overexpress mPPARα. Rat hepatoma FAO cells (Moore and Weiss, 1982) were obtained from Dr. J. Vanden Heuvel (Penn State University) and were grown in DMEM containing 5% fetal bovine serum. Transfection of the packaging cell line Bosc 23 with rat PPARα-encoding retroviral plasmid DNA, harvesting of the retroviral supernatant, and infection of FAO cells were carried out as described previously (Jounaidi *et al.*, 1998). Pools of puromycin-resistant cells were selected using 2 µg/ml puromycin for 2 weeks. Drug-resistant clones (FAO-PPARα cells) were selected and analyzed for nafenopin responsiveness by Western blot analysis of the PPARα target gene peroxisomal 3-ketoacyl-CoA thiolase (PTL).

Western blotting. Cell extracts (40 µg) were electrophoresed on 10% Laemmli SDS gels, electrotransferred onto nitrocellulose membranes, and then probed with anti-acyl-CoA oxidase (ACOX), anti-PTL antibody, or anti-catalase, generously provided by Drs. T. Hashimoto and J. K. Reddy (Northwestern University, Chicago IL), as described elsewhere (Zhou *et al.*, 2002). Antibody binding was visualized on x-ray film by enhanced chemiluminescence using the ECL kit from Amersham Pharmacia Biotech. Scans of Western blots were obtained using a Canon IX-4015 scanner and Ofoto software. Protein band intensities were quantitated using ImageQuant, v1.2 software (Molecular Dynamics, Sunnyvale, CA).

Quantitation of mRNA levels by real-time PCR. Relative cellular levels of rat 18S rRNA and PTL, ACOX, peroxisomal bifunctional enzyme (PBE), and urate oxidase mRNAs were quantified by real-time PCR analysis using the ABI 7900 Prism Sequence Detection System (Applied Biosystems). Total RNA was extracted using TRIZOL reagent (Gibco BRL) from FAO-PPARα cells that were seeded in six-well plates at 7×10^5 cells/well and treated for 48 h with nafenopin (100 µM) or the indicated fluorochemical beginning 4 h after cell seeding. The RNA obtained was treated with DNase I to remove contaminating DNA. SYBR Green real time PCR assays were used to quantify the following rat mRNAs: PTL (forward primer 5′-GGC-ACA-AGG-GCA-TCC-AAT-C-3′, reverse primer 5′-GTG-CGC-TGT-CTT-TGG-TTC-AA-3′); ACOX (forward primer 5′-CCT-CTG-TCG-ACC-TTG-TTC-GG-3′, reverse primer 5′-ACG-ACC-ACG-TAG-TGC-CAA-TG-3′); PBE (forward primer 5′-GCC-TTG-GGC-TGT-CAC-TAT-CG-3′, reverse primer 5′-CAA-GCC-GAC-ACG-AGC-CTT-T-3′); urate oxidase (forward primer 5′-ACT-GCA-AGT-GGC-GCT-ACC-A-3′, reverse primer 5′-CCC-AGG-TAG-CCT-CGA-AAT-CC-3′); and 18S rRNA (forward primer 5′-CGC-CGC-TAG-AGG-TGA-AAT-TC-3′, reverse primer 5′-CCA-GTC-GGC-ATC-GTT-TAT-GG-3′). For reverse transcription reactions, 0.4 µg RNA was transcribed into cDNA using random hexamer primers and MuLV reverse transcriptase (Applied Biosystems). Each real-time PCR reaction contained SYBR Green PCR Master Mix and 300 nM of each primer in a volume of 4 µl and was carried out in triplicate. The PCR program was 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min and 95°C for 15 s. No PCR amplification was observed in control reactions that omitted reverse transcriptase or the cDNA template. Relative levels of PTL, ACOX, PBE, and urate oxidase mRNA were calculated for each cDNA sample after subtracting the threshold cycle (C_T) for 18S RNA (determined in triplicate for each cDNA) from the C_T values (determined in triplicate) for PTL, ACOX, PBE, and urate oxidase to adjust for small differences in the amount of cDNA template present in each sample (ΔC_T). The average ΔC_T for untreated FAO-PPARα cells was then subtracted from the corresponding ΔC_T for fluorochemical-treated cells ($\Delta\Delta C_T$) and the values were back-transformed ($2^{-\Delta\Delta C_T}$) to calculate the relative abundance of each RNA in the treated cells compared to untreated controls.

RESULTS

Solubility of Fluorochemicals

Initial experiments indicated that some of the fluorochemicals under investigation were not fully soluble when diluted into DMEM media, used for culturing COS-1 cells. The solubilities of each fluorochemical diluted 1000-fold into DMEM from a DMSO stock solution were examined more closely using HPLC coupled to ESI-MS, for PFOS and FOSA, and capillary column GC with flame ionization detection for *N*-EtFOSE. PFOS was found to be partially soluble in DMEM: ∼40–50% recovery of PFOS was achieved at concentrations up to 64 µM, with progressively lower recoveries observed at higher PFOS concentrations, e.g., 19% recovery at 1 mM PFOS (Fig. 1). FOSA was fully soluble at concentrations up to 64 µM. However, the solubility of FOSA decreased at higher concentrations, e.g., 12% solubility observed at 1 mM FOSA. By contrast, *N*-EtFOSE was essentially insoluble when diluted 1000-fold into DMEM from a DMSO stock solution. Several experiments were performed to 'determine why the quantifications of PFOS differed so much from theoretical. DMEM at concentrations ranging from 0 to 100% were analyzed and the recoveries of spikes of PFOS were determined to range from 100% with 0% DMEM to 49% with 100% DMEM, with a relatively linear change in recoveries as the DMEM concentration changed (data not shown). This corresponds to the well known ''salting out'' effect

that is often used for improving extraction efficiency. Severe solubility problems were encountered with two amido acetate derivatives of PFOS, *N*-ethyl-perfluorooctanesulfonamido acetate and perfluorooctanesulfonamido acetate, precluding an evaluation of the effects of these PFOSAs on PPARα.

trans-*Activation of PPARα by PFOS*

To investigate the potential of PFOS to activate PPARα, COS-1 cells were transiently transfected with either mouse or human PPARα expression plasmid, together with the reporter plasmid pHD(x3)luc, which contains three PPAR binding sites linked to a minimal promoter controlling the gene for Firefly luciferase. Cells were cotransfected with an expression plasmid encoding *Renilla* luciferase, which serves as an internal control
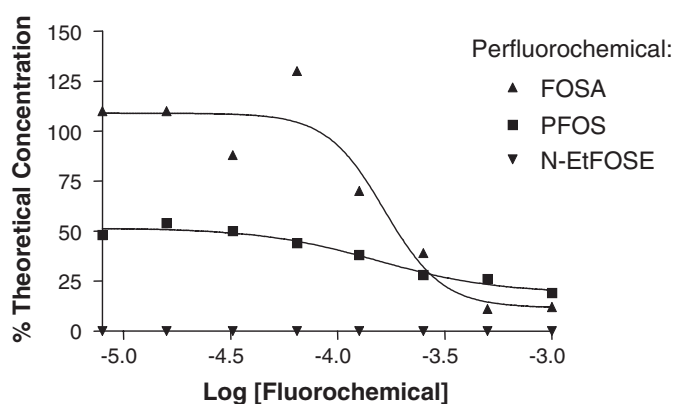

FIG. 1. Solubility of PFOS, FOSA, and *N*-EtFOSE. HPLC/ESI-MS was used to determine the solubility of the indicated fluorochemicals in DMEM culture medium. Concentrations of each fluorochemical were measured for each of eight diluted samples, ranging from 8 μM to 1000 μM nominal concentration. Shown are the actual concentrations, as a percentage of the nominal concentration. The x-axis presents the nominal fluorochemical concentration in units of molarity, on a logarithmic scale.

to normalize individual samples for variation in transfection efficiency and as a monitor of fluorochemical-induced cell toxicity. The potent PPARα activator Wy-14,643 was included as a positive control. Table 1 summarizes the results of 3–4 independent experiments, each of which demonstrates that PFOS can activate both mouse PPARα and human PPARα. The maximal activation achieved (4–6-fold) was similar to that seen with a saturating concentration of Wy-14,643. The PFOS-stimulated reporter activity was mediated by PPARα, as demonstrated by the absence of a significant response in cells lacking transfected PPARα (data not shown). Representative experiments showing dose-dependent activation of mPPARα and hPPARα by PFOS are presented in Figure 2. Average EC_{50} (apparent) values based on 3–4 independent sets of experiments were 13 μM (mPPARα) and 15 μM (hPPARα) and were not significantly different. Inter-experiment differences in PFOS dose-response curves (Table 1) may, in part, result from difficulties in achieving a consistent degree of PFOS solubility in the cell culture medium. PPARα activation was near-maximal ($\geq$90%) at PFOS concentrations of 32 μM (mPPARα) and 64–128 μM (hPPARα). Some cellular toxicity was seen at a PFOS concentration of 250 μM, as indicated by the significant drop in the activity of Firefly luciferase and the *Renilla* luciferase internal control. No such effect was seen at PFOS concentrations $\leq$125 μM (data not shown). The concentrations of PFOS reported in these experiments are nominal values, in view of the partial solubility data shown in Figure 1. Attempts to enhance the solubility of PFOS and increase the *trans*-activation of mPPARα, e.g., by the addition of 10% fetal calf serum to the culture medium during fluoro-chemical treatment, were unsuccessful (data not shown).

trans-*Activation of PPARα by FOSA*

FOSA activated mouse and human PPARα in transiently transfected COS-1 cells (Fig. 3), albeit with some variability

TABLE 1
Activation of PPARα by Fluorochemicals

	mPPARα					hPPARα			
	1	2	3	4	Mean $\pm$ SD	1	2	3	Mean $\pm$ SD
PFOS									
EC_{50} (μM)	10.4	6.9	10.8	22.5	12.7 $\pm$ 5.9	9	15.8	19.9	14.9 $\pm$ 5.5
EC_{90} (μM)	32	32	32	32	32 $\pm$ 0	125	64	64	84.3 $\pm$ 35.2
Activation by PFOS at EC_{90} (fold)	4[*]	6.2[*]	3.3[*]	4.9[*]	4.6 $\pm$ 1.1[*]	3.5[*]	4.2[*]	2.6[*]	3.4 $\pm$ 0.8[*]
FOSA									
EC_{90} (μM)	45	8	34		29 $\pm$ 19	45	45	34	41 $\pm$ 6.4
Activation by FOSA at EC_{90} (fold)	3.3	1.6[*]	6.7[*]		3.9 $\pm$ 2.6	2.1[*]	4.3[*]	2.7[*]	3 $\pm$ 1.1[*]

Note. COS-1 cells were transfected with the PPAR reporter plasmid pHD(x3)Luc together with an expression plasmid encoding either mouse or human PPARα and an expression plasmid for *Renilla* luciferase, pRL-CMV, as an internal control. Transfected cells were treated for 24 h with DMSO (vehicle control), Wy-14,643 (5 μM), or PFOS. Typical fold-activation values for Wy-14,643 (positive control; data not shown) ranged from 3 to 5-fold in individual experiments, and were comparable to the maximal fold-activation values for PFOS and FOSA. Data is presented in the form of EC_{50} and EC_{90} values for 3–4 independent experiments (designated 1–4) for mPPARα and hPPARα treated with PFOS or FOSA, as indicated. Statistical analysis was carried out as described under Materials and Methods.

*PPAR reporter activity determined in the experiment indicated was significantly different from that of untreated cells by Student's *t*-test ($p < 0.05$).
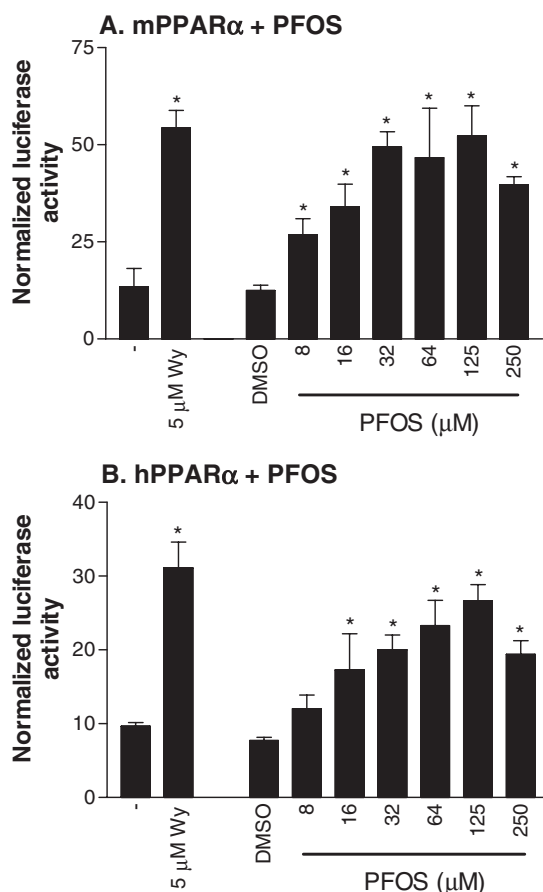
FIG. 2. PFOS activation of mPPARα (panel A) and hPPARα (panel B). COS-1 cells were transfected with the PPAR reporter plasmid pHD(x3)Luc and an expression plasmid encoding mPPARα or hPPARα as described under Materials and Methods. Transfected cells were treated for 24 h with DMSO (vehicle control), 5 μM Wy-14,643, or PFOS at the concentrations indicated. Data shown are mean ± SD Firefly luciferase activity values normalized to the *Renilla* internal control for *n* = 3 data points, where each of the three values is the mean of duplicate wells. *PPAR reporter activity was significantly different from that of untreated cells by Student's *t*-test (*p* < 0.05).

FIG. 3. FOSA activation of mPPARα (panel A) and hPPARα (panel B). COS-1 cells were transfected as described under Figure 1 and then treated for 24 h with DMSO (vehicle control), 5 μM Wy-14,643, or FOSA at the concentrations indicated. Data shown are mean ± SD Firefly luciferase activity values normalized to the *Renilla* internal control for *n* = 3 data points, where each of the three values is the mean of duplicate wells. Firefly luciferase activities were unusually high for the experiment shown in panel A; however, the overall pattern of response to FOSA was representative of several other experiments (Table 1). Symbol (∇) indicates luciferase activity undetectable, due to cell toxicity of FOSA at 60 μM. *PPAR reporter activity was significantly different from that of untreated cells by Student's *t*-test (*p* < 0.05).

in terms of the fold-activation achieved and the consistency of the dose-response curves between experiments (Table 1). Also notable was the shape of the FOSA dose-response curves: no activation was seen until the concentration of FOSA was increased to the point where maximal or near-maximal PPARα activation was obtained. At higher concentrations, FOSA exhibited substantial toxicity to COS-1 cells, such that the Firefly and *Renilla* luciferase activity values were undetectable. For example, in the experiment shown in Figure 3B, FOSA had no effect on hPPARα activity up to 19 μM, whereas FOSA induced a 3–4-fold increase in receptor activity at 25, 34, and 45 μM and was toxic to the cells at 60 μM. Significant intersample variation was sometimes observed, as is apparent from the large error bars seen in Figure 3A, where one series of FOSA dilutions strongly stimulated mPPARα activity, while a second series of dilutions prepared in parallel induced a more
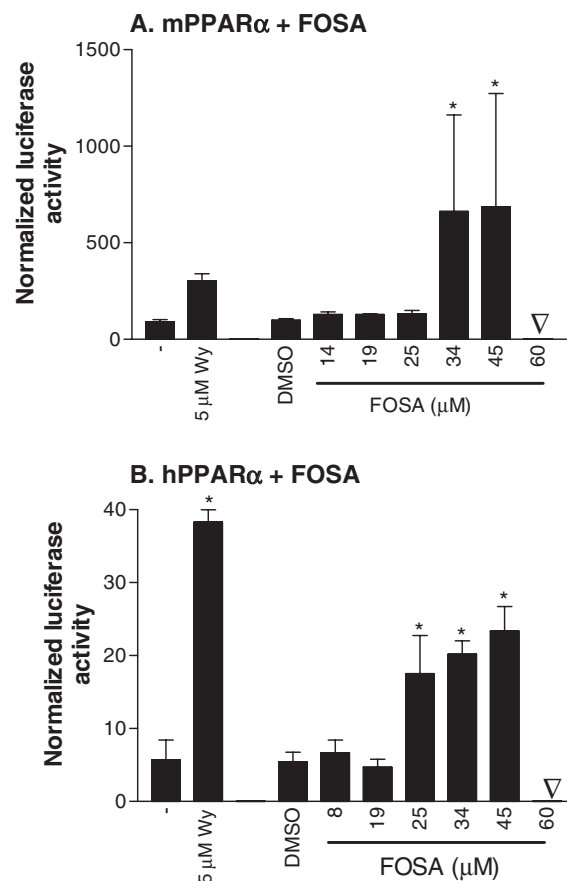
modest PPARα response. These general trends were observed in all experiments with FOSA, making it difficult to determine a reliable EC_{50} value for this fluorochemical. Nevertheless, it is evident from these studies that FOSA can activate both mouse and human PPARα in a manner similar to Wy-14,643, albeit at substantially higher concentrations (c.f., EC_{50} (Wy-14,643) = 0.3–1 μM; Hsu *et al.*, 1995).

Effects of N-EtFOSE on PPAR Activity

N-EtFOSE did not activate mouse PPARα or human PPARα in a consistent manner in the COS-1 cell *trans*-activation assay. An apparent 1.5-fold activation seen at several concentrations of *N*-EtFOSE in the experiment shown (Fig. 4) reflects a decrease
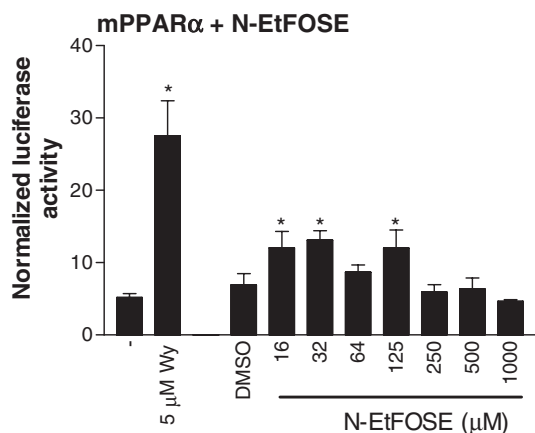
mPPARα + N-EtFOSE

FIG. 4. *N*-EtFOSE fails to activate mPPARα. COS-1 cells transfected as described in Figure 1 were treated with Wy-14,643 (5 μM) or *N*-EtFOSE at the concentrations indicated. Cell lysates from triplicate samples were assayed for luciferase activity 24 h later. Activities are expressed as Firefly luciferase activity normalized to the reporter activity of the *Renilla* luciferase internal standard. Activation of mPPARα was shown to be statistically significant at 16, 32, and 125 μM; however, the low-level activation by *N*-EtFOSE was variable between samples as shown by the lack of response at 64 μM and at ≥250 μM. *PPAR reporter activity was significantly different from that of untreated cells by Student's *t*-test ($p < 0.05$).

in the activity of the *Renilla* luciferase internal control and was not reproducible in other experiments. The apparent inactivity of *N*-EtFOSE is consistent with its insolubility in DMEM culture medium (Fig. 1).

PFOS and FOSA Induce Peroxisomal Enzymes in FAO Rat Hepatoma Cells

To further establish the ability of PFOS and FOSA to stimulate PPARα-regulated transcription, we examined the effect of these fluorochemicals on specific PPARα target genes using the rat hepatoma cell line FAO. FAO cells are responsive to PPARα activators (Bayly *et al*., 1993) but in our studies the cells displayed very weak responses compared to rat liver (data not shown). In an effort to improve the cell's responsiveness, FAO-PPARα cells were prepared by infection of wild-type FAO cells with retrovirus encoding mouse PPARα (see Methods). The clonal FAO-PPARα cell line obtained expressed a four-fold higher level of PPARα mRNA than wild-type cells, as revealed by quantitative real-time PCR analysis. Treatment of FAO-PPARα cells with the PPARα activator nafenopin stimulated a substantially stronger increase in expression of the PPARα target genes ACOX and PTL compared to wild-type FAO cells, as revealed by Western blot analysis (data not shown). The increased PPARα target gene expression seen in FAO-PPARα cells compared to wild-type FAO cells provides good evidence that these increases are bona-fide, PPARα-dependent transcriptional responses.

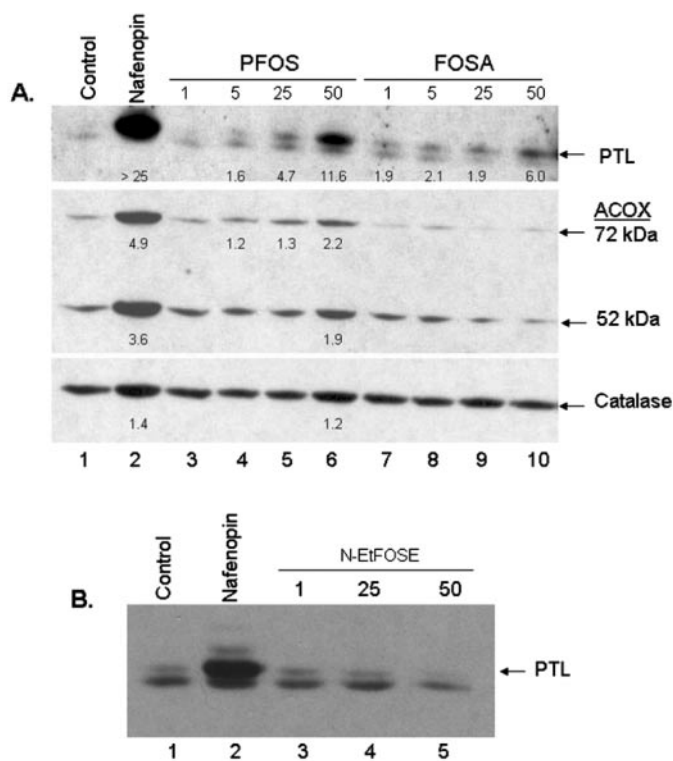FAO-PPARα cells were treated with PFOS, FOSA, or *N*-EtFOSE (1–50 μM) for 48 h. Western blot analysis revealed

FIG. 5. Induction of liver cell peroxisomal enzymes by PFOS, FOSA, and *N*-EtFOSE. (Panel A) FAO-PPARα cells were treated for 48 h with DMSO (control; lane 1), PFOS (lanes 3–6), or FOSA (lanes 7–10) at the indicated concentrations (1–50 μM). Whole cell extracts were assayed by Western blotting for PTL protein (upper band of doublet), ACOX, and catalase. Nafenopin (25 μM) was included as a positive control (lane 2). ACOX is expressed in rodent liver cells as a primary 72 kD protein product, a portion of which is cleaved to give ∼52 kD and ∼20 kD polypeptides. Both the 72 kD and the 52 kD forms are shown on the gel where they were induced by nafenopin. Fold increases compared to the DMSO control in lane 1 are indicated below those proteins bands whose intensity was measurably different (≥1.1) from the DMSO control. Catalase was minimally responsive to PPARα ligands, as seen by a 1.4-fold increase in protein expression in response to treatment with nafenopin. Catalase was relatively unchanged in the PFOS- and FOSA-treated cells and served as a loading control between protein samples. (Panel B) FAO-PPARα cells were treated for 48 h with DMSO as a control (lane 1) or with *N*-EtFOSE at the micromolar concentrations indicated. Whole cell extracts were assayed by Western blotting for PTL protein (upper band of doublet). Nafenopin (25 μM) was included as a positive control (lane 2).

a substantial, dose-dependent induction of PTL protein (upper band of doublet; Fig. 5A) in FAO-PPARα cells treated with either PFOS (panel A, lanes 3–6) or FOSA (panel A, lanes 7–10) compared to the DMSO control (panel A, lane 1). The fact that PFOS and FOSA both induced PTL expression indicates that these are true PPARα-dependent responses. The clear, positive response of PTL to PFOS and FOSA contrasts with the weak response of ACOX (∼2-fold) in FAO-PPARα cell extracts after PFOS treatment (Fig. 5A). ACOX was induced 4–5-fold in FAO-PPARα cells treated with nafenopin, which serves as a positive control for PPARα activation (panel A, lane 2 vs. lane 1). *N*-EtFOSE had no reproducible effect on PTL protein levels in

TABLE 2
Fatty-Acid β-Oxidation Genes Induced by PFOS Treatment of FAO-PPARα Cells: Real-Time PCR Analysis

	Relative mRNA level					
		PFOS (μM)				
	DMSO	5	25	50	75	Nafenopin (100 μM)
PTL	1.00 ± 0.01	1.4 ± 0.6	1.6 ± 0.4	$8.7 \pm 4.4^*$	$8.8 \pm 1.9^*$	$47 \pm 18^*$
PBE	1.00 ± 0.01	$1.5 \pm 0.01^*$	$1.7 \pm 0.4^*$	$9.5 \pm 0.1^*$	$23.3 \pm 0.2^*$	$63 \pm 3^*$
ACOX	1.00 ± 0.02	1.4 ± 0.6	0.9 ± 0.08	$3.4 \pm 1.9^*$	1.9 ± 0.04	$11 \pm 0.5^*$
UOX	1.00 ± 0.02	1.2 ± 0.4	1.2 ± 0.4	$2.7 \pm 0.6^*$	1.9 ± 0.6	$2.4 \pm 0.6^*$

Note. Real-time PCR analysis was carried out as described in Materials and Methods. Values shown are relative mRNA levels (mean $\pm$ SD) in comparison to DMSO-treated controls. PTL, PBE, ACOX, and urate oxidase mRNA values were normalized to the 18S RNA levels from the same cDNA sample to control for differences in the amount of cDNA template. Data shown are based on triplicate PCR analyses of $n = 2$ independent cell culture RNA samples.

*$p < 0.05$ from DMSO control values by Student's *t*-test.

TABLE 3
Fatty-Acid β-Oxidation Genes Induced by FOSA Treatment of FAO-PPARα Cells: Real-Time PCR Analysis

	Relative mRNA level					
		FOSA (μM)				
	DMSO	5	25	50	75	Nafenopin (100 μM)
PTL	1.00 ± 0.01	$3.3 \pm 1.3^*$	$9.4 \pm 2.0^*$	$11.9 \pm 1.5^*$	$12.0 \pm 4.3^*$	$167 \pm 31^*$
PBE	1.00 ± 0.01	2.8 ± 0.6	7.7 ± 3.9	$86 \pm 59^*$	6.6 ± 2.9	$73 \pm 59^*$
ACOX	1.00 ± 0.02	1.5 ± 0.1	0.8 ± 0.03	2.2 ± 1.3	0.7 ± 0.07	$7.8 \pm 2.0^*$

Note. Real-time PCR analysis was carried out as described in Table 2. Values shown are mean $\pm$ SD based on triplicate PCR analyses of $n = 2$ independent cell culture RNA samples.

*$p < 0.05$ from DMSO control values by Student's *t*-test.

FAO-PPARα cells (Fig. 5B), in agreement with the reporter gene assay presented in Figure 4. Weak induction of catalase (1.4-fold) was apparent in FAO-PPARα cells after nafenopin treatment (Fig. 5A). Since catalase was nearly unchanged after PFOS and FOSA treatment (panel A, lanes 3–10), the catalase band intensities serve as a loading control.

The induction of PTL gene expression in FAO-PPARα cells following treatment with PFOS or FOSA was verified by real-time PCR analysis. PTL mRNA was induced ~9-fold at 50 μM PFOS (Table 2). Similarly, FOSA induced PTL mRNA ⩾10-fold at concentrations ⩾25 μM (Table 3). PBE mRNA was also strongly induced following treatment of the FAO-PPARα cells with PFOS or FOSA. These mRNA inductions were substantially lower than those achieved in FAO-PPARα cells treated with nafenopin (Table 2 and Table 3, last column), in agreement with the Western blot results shown in Figure 5. ACOX mRNA was induced in nafenopin-treated cells (8- to 11-fold increase), albeit to a much lower extent than PTL and PBE mRNA (Table 2 and Table 3). By contrast, little or no consistent increase in ACOX mRNA was seen with PFOS or FOSA, in agreement with the modest increase in ACOX protein shown in Figure 5.

Urate oxidase mRNA, which encodes a peroxisomal enzyme that is not responsive to peroxisome proliferator chemicals, was not induced by PFOS (Table 2) or by FOSA (data not shown).

DISCUSSION

PFOS is an end-stage metabolite of *N*-EtFOSE and related perfluorooctanesulfonamido chemicals that accumulate in liver tissue when administered to rats (Butenhoff and Seacat, 2001). In the present study, PFOS and FOSA were shown to activate PPARα when assayed in a COS-1 cell-based *trans*-activation assay and in a rat liver cell model where the induction of endogenous PPARα target genes was monitored. Dose-response curves for PPAR activation indicated that mPPARα and hPPARα both respond similarly to PFOS and to FOSA. Moreover, PPARα activation occurred at similar concentrations of both perfluorooctane chemicals, indicating that PPARα is intrinsically responsive to both compounds. PFOS activated mPPARα and hPPARα to the same maximum extent as the potent peroxisome proliferator Wy-14,643, but substantially

higher concentrations were required to elicit a PPAR transcriptional response. The effective concentration of PFOS required for PPARα activation is likely to be lower than indicated by the nominal EC_{50} values of 13–15 μM presented here, in view of the low aqueous solubility and availability of PFOS.

The activation of PPARα by PFOS is consistent with the hepatic peroxisome proliferative activity seen in a variety of studies where PFOS was administered to rodents over a relatively short time period. In one study, PFOS administered to male rats at 200 ppm (0.02% PFOS) in the diet for 2 weeks, resulting in a cumulative dose of ∼270 mg/kg, increased hepatic fatty ACOX activity ∼5-fold (Ikeda *et al.*, 1987). In another study, a 7-fold increase in fatty acid oxidation was observed in mouse liver after five days of dosing with PFOS (500 ppm in the diet, ∼125 mg/kg cumulative dose; Sohlenius *et al.*, 1992). In a third study, a ∼2-fold increase in lauroyl CoA oxidase activity was seen in male rats given a single ip injection of PFOS at 100 mg/kg, while hepatic peroxisome proliferative responses were weak or undetectable at comparable dosing levels of N-EtFOSE (Berthiaume and Wallace, 2002). In contrast to these effects of PFOS in rats, no peroxisome proliferation responses are seen in guinea pigs given cumulative doses of 160 mg/kg PFOS or 640 mg/kg N-EtFOSE over four days by oral gavage (Wallace *et al.*, 2001). These findings in short-term assays are consistent with the report that PFOS is almost as potent a rodent peroxisome proliferator as the established peroxisome proliferator perfluorooctanoic acid (Sohlenius *et al.*, 1992).

The induction of peroxisome proliferation by PFOS in rats appears to exhibit a threshold dose response in short-term assays that rapidly reach relatively high tissue concentrations of PFOS. A threshold dose-response would be consistent with the hypothesis that peroxisome proliferation is a receptor-dependent mechanism that exhibits nonlinearity and a threshold exposure requirement for gene induction responses (Lapinskas and Corton, 1997). In contrast, longer-term repeat-dose studies that achieve higher liver PFOS concentrations than necessary to induce peroxisome proliferation in a short-term assay show no significant increase in hepatic peroxisome proliferative response. For example, at four weeks into a chronic (two-year) PFOS bioassay, rats fed 20 ppm PFOS in the diet received cumulative doses of ∼52 and 50 mg PFOS/kg with corresponding serum PFOS concentrations of ∼ 42 ppm (∼84 μM) and ∼54 ppm (∼108 μM), in male and female rats, respectively (Seacat *et al.*, 2003). These serum concentrations were associated with ∼7-fold higher liver PFOS concentrations, 282 ppm and 373 ppm, respectively, indicating liver accumulation. However, only a moderate (∼2-fold) increase in hepatic palmitoyl CoA oxidase activity was observed in the male liver samples and no response was seen in female liver samples, suggesting a very weak peroxisome proliferative response. Indeed, after 14 weeks of dosing, PFOS concentrations were greater than 130 ppm (∼260 μM) in the serum and 450 ppm (∼900 μM) in the liver with no increase in palmitoyl CoA oxidase activity (Seacat *et al.*, 2003). The modest increase in palmitoyl CoA oxidase

activity seen in these studies suggests that the cumulative doses after 4 or 14 weeks of 20 ppm dietary PFOS may not have been achieved rapidly enough to produce the increases in palmitoyl CoA oxidase seen in the shorter-term exposure studies, despite similar cumulative doses. The presence of high tissue concentrations of PFOS without a sustained increase in palmitoyl CoA oxidase activity suggests that a mechanism may exist *in vivo* for an adaptive down-regulation of the hepatic peroxisome proliferation response to PFOS treatment, which could explain the low level of ACOX induction seen in the current *in vitro* study. Alternatively, the current ACOX results may not reflect what occurs *in vivo* and may occur by some other mechanism.

The present findings establish the responsiveness of mouse and human PPARα to perfluorooctane-based chemicals, and support other data indicating a role for PPARα as a mediator of some of the toxicities associated with PPARα activation, at least in rodent species. PFOS activation of PPARα was characterized by an EC_{50} of 13–15 μM in the luciferase reporter gene assay used in the present study. This concentration corresponds to a serum PFOS concentration of ∼ 5–8 ppm, or approximately 12–20% of the *in vivo* serum PFOS concentration that elicited only a weak peroxisome response in male rat liver after 4 weeks administration of 20 ppm dietary PFOS (Seacat *et al.*, 2003). Other mechanisms for PFOS-based toxicity are likely, given the other cellular responses activated by these compounds *in vitro*, such as the inhibition of mitochondrial bioenergetics (Starkov and Wallace, 2002) and effects on cell-cell communication via gap junctions, which occur in rat liver at PFOS concentrations ranging from 112 ppm to 810 ppm, without a concentration-dependent response in that range (Hu *et al.*, 2002). Presumably, these effects of PFOS involve mechanisms that are independent of PPARα.

Some inconsistencies and variations in the dose-response data for PPARα activation were encountered in the present study, in particular for FOSA. This variability made it difficult to establish precise dose-response curves for receptor activation by FOSA. Nevertheless, the present studies establish the capacity of this fluorochemical for PPARα activation, which is associated with hepatocarcinogenesis in rodents (Gonzalez *et al.*, 1998). Additional investigation established that the PPARα-activation capacity of FOSA, and PFOS, extends to the induction of endogenous PPARα target genes, as shown by the strong increase in expression of the peroxisomal enzymes PTL and PBE in FAO rat hepatoma cells that stably express PPARα. Interestingly, PFOS and FOSA stimulated only a low level increase in expression of ACOX expression in FAO-PPARα cells, even though this peroxisomal enzyme, a known PPARα target gene, was readily induced by treatment of the cells with the established PPARα ligand and *trans*-activator nafenopin. This indicates that ACOX activity, commonly used to monitor hepatic peroxisome proliferation, may be a relatively insensitive monitor of the effect of PFOS in rodent liver. Conceivably, PFOS and FOSA could bind to PPARα in a manner that leads to *trans*-activation of a subset of classic PPARα target genes, perhaps inducing a novel

metabolic profile in terms of hepatic peroxisomal enzyme expression. Additional studies, including more detailed enzymatic analysis and global mRNA expression profiling of livers obtained from PFOS-treated rats and mice, may help clarify this issue. Some inconsistencies in the concentration of PFOS and FOSA required to activate a PPARα luciferase reporter, induce mRNA and protein of PPARα-responsive genes were seen. These may be explained by interexperiment variability due to fluorochemical solubility issues, which were routinely encountered in this study. Although studies in human cell lines would further characterize the risks posed by exposure to fluorooctane-based chemicals, we expect that such experiments would yield a negative result, given extensive earlier studies demonstrating the unresponsiveness of human cells to PPARα activators (Cattley *et al.*, 1998; Lake, 1995; Roglans *et al.*, 2002).

Peroxisome proliferators and other chemicals may activate PPARs by two types of mechanisms: by binding directly to the receptor (i.e., as ligands), and by perturbing lipid metabolism and transport in a manner that stimulates the synthesis and/or release of endogenous PPAR ligands. We cannot distinguish between these two mechanisms in the case of the activation of PPARα by PFOS and FOSA. Previous investigations have shown, however, that PFOSAs can displace endogenous ligands from liver fatty acid binding protein (Luebker *et al.*, 2002), an intracellular lipid carrier that binds and transports fatty acids, acyl-CoA derivatives, and a variety of other hydrophobic molecules within hepatocytes. This fatty acid binding protein stimulates phospholipid synthesis and regulates lipid metabolism, and may protect the cell by maintaining intracellular free fatty acids at subtoxic concentrations. Consequently, the hypolipidemic and peroxisome proliferating effects of PFOSAs may, in part, be due to interference with the capacity of liver fatty acid binding protein to bind fatty acids, cholesterol, and other lipids. Displacement of fatty acids could, in turn, stimulate the activation of PPARα or PPARγ by these endogenous ligands. Further investigation, including a more direct examination of the ability of PFOSAs to bind directly PPARα should help resolve this issue.

Finally, extension of the present findings to include other PPAR forms would be of interest, in view of their importance in diverse physiological processes including adipogenesis (PPARγ; Schoonjans *et al.*, 1996), reverse cholesterol transport (Oliver *et al.*, 2001) and development (Peters *et al.*, 2000; PPARδ). In contrast to PPARα, which is expressed at comparatively low levels in human cells (Palmer *et al.*, 1998), PPARγ is expressed at high levels in a broad range of human tissues. These include adipose tissue, where many lipophilic foreign chemicals tend to accumulate, as well as colon, heart, liver, testis, spleen, and hematopoietic cells (Vidal-Puig *et al.*, 1997). Other biological responses linked to PPARγ ligands and activators, which may potentially be influenced by fluorochemicals that activate PPARγ, include colon tumorigenesis (Saez *et al.*, 1998) and the inhibition of human vascular endothelial cell differentiation and angiogenesis (Xin *et al.*, 1999). PPARγ

activation also leads to foam cell macrophage differentiation, although it is uncertain whether this latter PPARγ-dependent response is likely to contribute to generation of an atherosclerotic plaque (Rosen and Spiegelman, 2000). The extent to which PFOSAs alter these physiological and pathophysiological responses to endogenous or other foreign chemical PPARγ ligands is uncertain, and is an important area for further research.

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