

Differential Activation of Nuclear Receptors by Perfluorinated Fatty Acid Analogs and Natural Fatty Acids: A Comparison of Human, Mouse, and Rat Peroxisome Proliferator-Activated Receptor- α , - β , and - γ , Liver X Receptor- β , and Retinoid X Receptor- α

John P. Vanden Heuvel,^{*,1} Jerry T. Thompson,^{*} Steven R. Frame,[†] and Peter J. Gillies^{†,‡}

^{*}Department of Veterinary and Biomedical Science and The Center for Molecular Toxicology and Carcinogenesis, The Pennsylvania State University, University Park, Pennsylvania 16802; [†]DuPont Haskell Laboratory for Health and Environmental Sciences, Newark, Delaware 19714; and [‡]Department of Nutritional Sciences, The Pennsylvania State University, University Park, Pennsylvania 16802

Received March 24, 2006; accepted May 1, 2006

Administration of ammonium salts of perfluorooctanoate (PFOA) to rats results in peroxisome proliferation and benign liver tumors, events associated with activation of the nuclear receptor (NR) peroxisome proliferator-activated receptor- α (PPAR α). Due to its fatty acid structure, PFOA may activate other NRs, such as PPAR β , PPAR γ , liver X receptor (LXR), or retinoid X receptor (RXR). In this study, the activation of human, mouse, and rat PPAR α , PPAR β , PPAR γ , LXR β , and RXR α by PFOA (including its linear and branched isomers) and perfluorooctane sulfonate (PFOS) was investigated and compared to several structural classes of natural fatty acids and appropriate positive control ligands. An NR ligand-binding domain/Gal4 DNA-binding domain chimeric reporter system was used. Human, mouse, and rat PPAR α were activated by PFOA isomers and PFOS. PPAR β was less sensitive to the agents tested, with only PFOA affecting the mouse receptor. PFOA and PFOS also activated human, mouse, and rat PPAR γ , although the maximum induction of PPAR γ was much less than that seen with rosiglitazone, suggesting that PFOA and PFOS are partial agonists of this receptor. Neither LXR β nor the common heterodimerization partner RXR α was activated by PFOA in any species examined. Taken together, these data show that of the NRs studied, PPAR α is the most likely target of PFOA and PFOS, although PPAR γ is also activated to some extent. Compared to naturally occurring long-chain fatty acids, e.g. linoleic and α -linolenic acids, these perfluorinated fatty acid analogs were more selective and less potent in their activation of the NRs.

Key Words: nuclear receptors; transactivation; dietary fatty acids; fatty acid analogs.

Perfluorooctanoate (PFOA) is a member of a structurally diverse class of chemicals that induce hepatomegaly in rodents (Hess *et al.*, 1965; Ikeda *et al.*, 1985; Kennedy *et al.*, 2004; Moody and Reddy, 1978). This hepatomegaly is characterized by the subcellular proliferation of organelles such

as smooth endoplasmic reticulum, mitochondria, but most notably peroxisomes. The molecular biology underlying peroxisome proliferation has evolved considerably since the discovery of the first peroxisome proliferator-activated receptor (PPAR) (Issemann and Green, 1990). PPARs are now recognized as members of a superfamily of nuclear receptors (NRs) that form obligate heterodimers with the retinoid X receptor (RXR) (for review, see Francis *et al.*, 2003). PPARs exist in three isoforms, namely, PPAR α , PPAR β (also called PPAR δ), and PPAR γ . Upon ligand-mediated activation, the PPAR-RXR heterodimer undergoes conformational changes, which recruit various coactivators, e.g., Steroid receptor coactivator-1 and vitamin D(3) receptor-interacting protein, and interacts with peroxisome proliferation-response elements located in the promoter regions of target genes (Shearer and Hoekstra, 2003). In this regard, interaction of a ligand with the ligand-binding domain (LBD) of PPAR is just the first step in a complex series of events that underlie the combinatorial control of gene transcription. The phenomenon of ligand-specific conformational change leading to selective peroxisome proliferator-activated receptor modulation (SPPARM) has introduced yet another level of complexity to PPAR biology (Camp *et al.*, 2000; Duez *et al.*, 2005). Soon after the discovery of PPAR isoforms was the identification of other members of the NR superfamily, notably, liver X receptors (LXRs), farnesol X receptor, and pregnane X receptor (also referred to as the steroid and xenobiotic receptor). PPARs and LXR are particularly important as master regulators of lipid and lipoprotein metabolism (Li and Glass, 2004), an area of biology that PFOA has been reported to modify in some but not all rodent species (Haughom and Spydevold, 1992; Kudo *et al.*, 1999; Pastoor *et al.*, 1987; Xie *et al.*, 2003).

Although initially the purview of the toxicology community was based on the relationship between peroxisome proliferation and rodent tumors (for review, see Klaunig *et al.*, 2003), PPARs have now captured the attention of the medical

¹ To whom correspondence should be addressed. Fax: 814-863-1696. E-mail: jpv2@psu.edu.

community as therapeutic targets for the management of dyslipidemia, insulin resistance, and obesity (Duval *et al.*, 2002; Francis *et al.*, 2003; Fruchart and Duriez, 2002; Kersten *et al.*, 2000); more recently, the nutrition community has become more interested in PPARs with respect to the health benefits ascribed to polyunsaturated fatty acids (for review, see Kersten *et al.*, 2000). The current studies were conducted because of the recognition that fatty acid analogs have the potential to affect lipid metabolism via activation of these receptors (Intrasuksri *et al.*, 1998; Maloney and Waxman, 1999; Shipley *et al.*, 2004) and the need to translate this biology across species. The goal of these studies was to develop a cross-species (humans, mice, and rats) understanding of NR activation (PPAR α , PPAR β , PPAR γ , LXR β , and RXR α) in response to perfluorinated fatty acid analogs (PFOA and perfluorooctane sulfonate [PFOS]) and naturally occurring fatty acids (octanoate [Oct], oleate (OA), linoleate [LA], and α -linolenate [ALA]) on the basis of a common testing platform. In addition, since mixtures of linear and branched isomers of ammonium perfluorooctanoate (APFO) have been used in the production of commercial products (Kennedy *et al.*, 2004), various isomers were included in the study.

Numerous transactivation assays, reporter systems, and cell types have been used in the evaluation of NR ligands (Bocos *et al.*, 1995; Le Douarin *et al.*, 1996), each with positive and negative biological attributes and inherent sources of variability. Not surprisingly, there is considerable variability in the data reported in the literature even for identical and commonly tested ligands (Klaunig *et al.*, 2003). In order to facilitate the cross-species understanding and to limit the variability associated with complex signaling systems, a reductionism approach was employed to meet the goals of the study. To this end, a reporter assay system was used, whereby the LBDs of the human, mouse, and rat fatty acid-responsive NRs (PPAR α , PPAR β , PPAR γ , LXR β , and RXR α) were cloned in frame with the Gal4 DNA-binding domain. When a ligand binds to the LBD, a conformational change occurs allowing transcription coactivators to be recruited, inducing the expression of a Gal4 luciferase reporter gene. The data developed with this assay provide a molecular, although biologically constrained, basis for evaluating the effects of perfluorinated fatty acid analogs versus natural fatty acids on these fatty acid-responsive NRs.

MATERIALS AND METHODS

Positive controls for NR assays. Ciprofibrate (Cipro), purchased from Sigma Chemical Co. (St Louis, MO), was used as the positive control for PPAR α . Tetradecylthioacetic acid (TTA), purchased from Sigma Chemical Co., was used as the positive control for PPAR β . Rosiglitazone (Rosi), purchased from Cayman Chemicals (Ann Arbor, MI), was used as the positive control for PPAR γ . T0901317, purchased from Cayman Chemicals, was used as the positive control for LXR β . Methoprene acid, purchased from Cayman Chemicals, was used as the positive control for RXR α .

Natural fatty acids. Oct, OA, LA, and ALA were all purchased as the free acid from Sigma Chemical Co. Conjugated linoleic acid (CLA) was a kind gift from Pharnanutrients (Gurnee, IL).

Perfluorinated fatty acid analogs. The linear isomer of the ammonium salt of PFOA was provided by DuPont (Wilmington, DE); this form is designated throughout the text and in the figures as PFOA-linear. This molecule is a white to slightly opaque liquid and was provided as a 20% solution in water and stored at room temperature. A linear/branched form of the ammonium salt of PFOA was provided by the 3M Company, Specialty Materials Manufacturing Division (St Paul, MN); this form is designated throughout the text and in the figures as PFOA-linear/branched. This test material was 77.6% linear; the mole percentages of its branched contents were 12.6% internal monomethyl (non- α), 9% isopropyl, 0.2% *tert*-butyl, 0.1% gem-dimethyl, and 0.1% α monomethyl. The certificate of analysis indicated 2.01% lesser-homolog (C4–C7) impurities (Richard Payfer, 3M Company, 2 March 2000). Homolog distribution was as follows: C4, 0.01%; C5, 0.03%; C6, 0.43%; C7, 0.57%; C8, 97.99%; and C9, 0.16%. The sample also included 0.09% monohydro-APFO, 0.72% monounsaturated APFO, and 0.3% undefined material that was possibly substituted perfluorocyclo species (0.2% cyclopentyl and 0.1% cyclohexyl). This test material, a white solid, was 97.99% pure and was stored at room temperature. A multibranch form of APFO was provided by DuPont; this form is designated throughout the text and in the figures as PFOA-branched. Branched APFO, a white solid, was prepared by combining 58.3% ammonium 2,2,3,4,4,5,5,6,6,7,7,7-pentadecafluoro-3-(trifluoromethyl)-heptanoate (internal monomethyl non- α) with 41.7% ammonium 2,2,3,3,4,4,5,5,6,6,7,7,7-pentadecafluoro-6-(trifluoromethyl)heptanoate (isopropyl). The multibranch APFO was a solid and was stored at room temperature. Thermodynamically stable spatial models of the various natural and fatty acid analogs used in these studies are presented in Figure 1; these structures show a theoretical minimized energy configuration (minimum RMS gradient of 0.1; Chem3D, CambridgeSoft, Cambridge, MA). Given the increasing importance of SPPARM effects in understanding the pharmacology of PPARs, an appreciation of the spatial forms of these molecules is additive to understanding the binding/transactivation data generated in this study.

Plasmids. The LBD of human, mouse, or rat PPAR α , PPAR β , PPAR γ , LXR β , or RXR α was fused to the DNA-binding domain of the yeast transcription factor Gal4 under the control of the SV40 promoter. This plasmid was cotransfected with pFR, a plasmid which encoded the UAS-firefly luciferase reporter under the control of the Gal4 DNA response element. All plasmids were verified by sequencing and through examination of positive controls.

Cell culture and transactivation assays. Mouse 3T3-L1 fibroblasts (ATCC, Manassas, VA) were cultured in high-glucose Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Sigma Chemical Co.), 0.2 mg/ml streptomycin, and 200 U/ml penicillin (GIBCO, Grand Island, NY). Cells were transfected with plasmid DNA using Lipofectamine reagent (Invitrogen, Carlsbad, CA) and following the manufacturer's recommended procedures, using 3T3-L1 cells at approximately 80% confluence in 10-cm culture dishes. After 6 h, the DNA-Lipofectamine complex was removed, and the cells were maintained overnight in the culture medium. Following overnight culture, the transfected 3T3-L1 cells containing the chimeric receptor/reporter plasmids were split to multiwell cluster plates. The media was replaced 4 h after replating with DMEM (10% FBS) containing test compounds in dimethyl sulfoxide (DMSO, Sigma Chemical Co.) (0.1% final concentration). Concentrations of the chemicals are given in the figure captions. Twenty-four hours after treatment, the cells were lysed with passive lysis buffer (Promega, Madison, WI) for 30 min; luciferase activity was measured using the luciferase reporter assay kit (Promega) and a Turner TD-20/20 Luminometer (Turner BioSystems, Sunnyvale, CA) or a Tecan GeniosPro (Research Triangle Park, NC) according to the manufacturer's recommended procedures. The fold induction of normalized luciferase activity was calculated relative to the vehicle (DMSO)-treated cells and is the mean of three independent samples per treatment group.

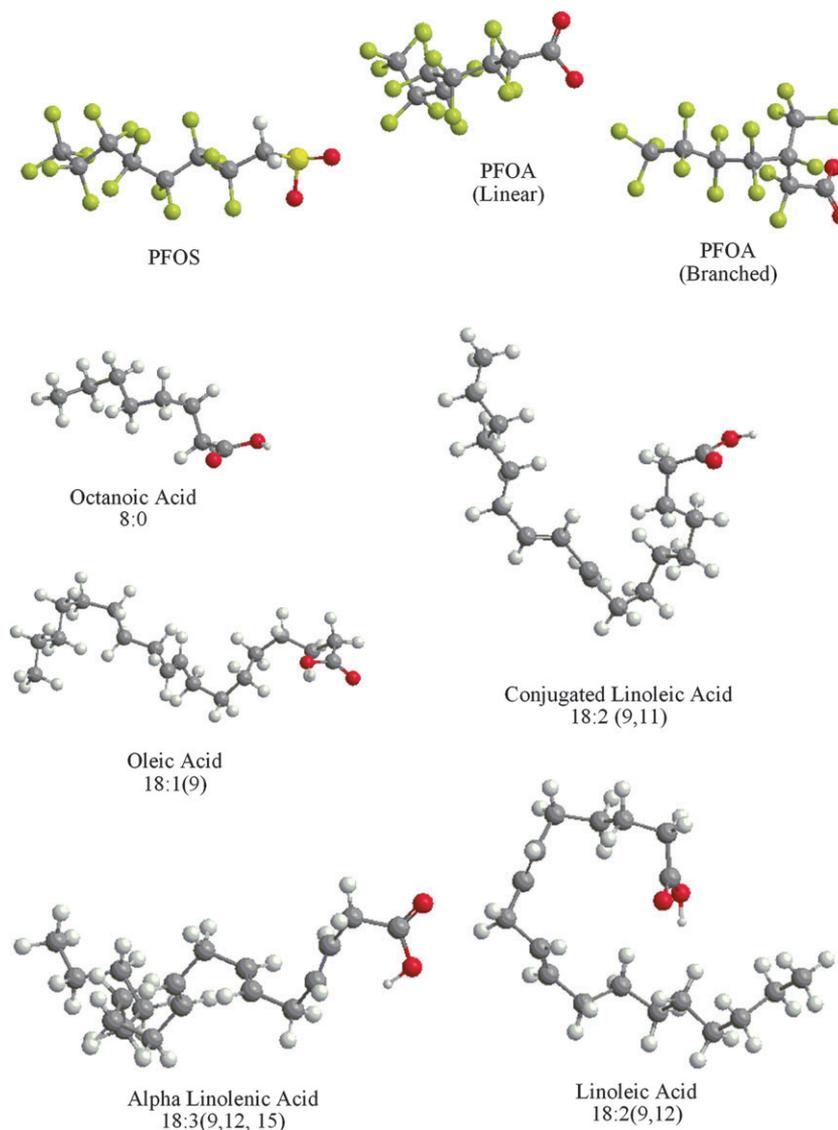


FIG. 1. Spatial models of natural fatty acids and fatty acid analogs. These thermodynamically stable spatial models show the theoretical minimized energy configuration (minimum RMS gradient of 0.1; Chem3D, CambridgeSoft) of the molecules tested.

Study design and statistical analysis. Each chemical was examined concurrently with a known positive control for each receptor. Due to the number of samples to be examined, the perfluorinated fatty acid analogs and the natural fatty acids were analyzed in separate experiments. It is important to note that the parameters measured, such as EC_{50} and peak values, are not constants and vary, often significantly, from experiment to experiment. Thus, only by comparing the activity of the perfluorinated acids relative to similar compounds assayed in the same experiment can meaningful judgments regarding potency and efficacy be made.

Differences between treatments were determined using ANOVA followed by Dunnett's *post hoc* test (Minitab, State College PA), and the lowest statistically significant dose (LSSD) was determined. The peak effect represents the highest average induction observed and is not based on modeling. Nonlinear regression and EC_{50} calculations were performed with Prism 4.0 (GraphPad Software, Inc., San Diego, CA). For each receptor, a dose-response was included, and EC_{50} and maximum induction were calculated by nonlinear regression (see Supplemental Data). Hierarchical clustering was performed

using GeneSpring (Agilent Technologies, Palo Alto, CA), with both chemical and receptor trees generated using standard correlation as a similarity measure.

RESULTS

The first set of experiments compared the relative ability of perfluorinated fatty acid analogs and natural fatty acids to bind and transactivate fatty acid-sensitive NRs from humans, mice, and rats. The results are presented in Figures 2–6, and the statistical analysis is shown in Table 1. Due to the day-to-day variability in both the efficacy (peak effect and maximum induction) and potency (LSSD and EC_{50}) observed in reporter assays, it is only appropriate to compare the fatty acids to positive controls run concurrently within that species

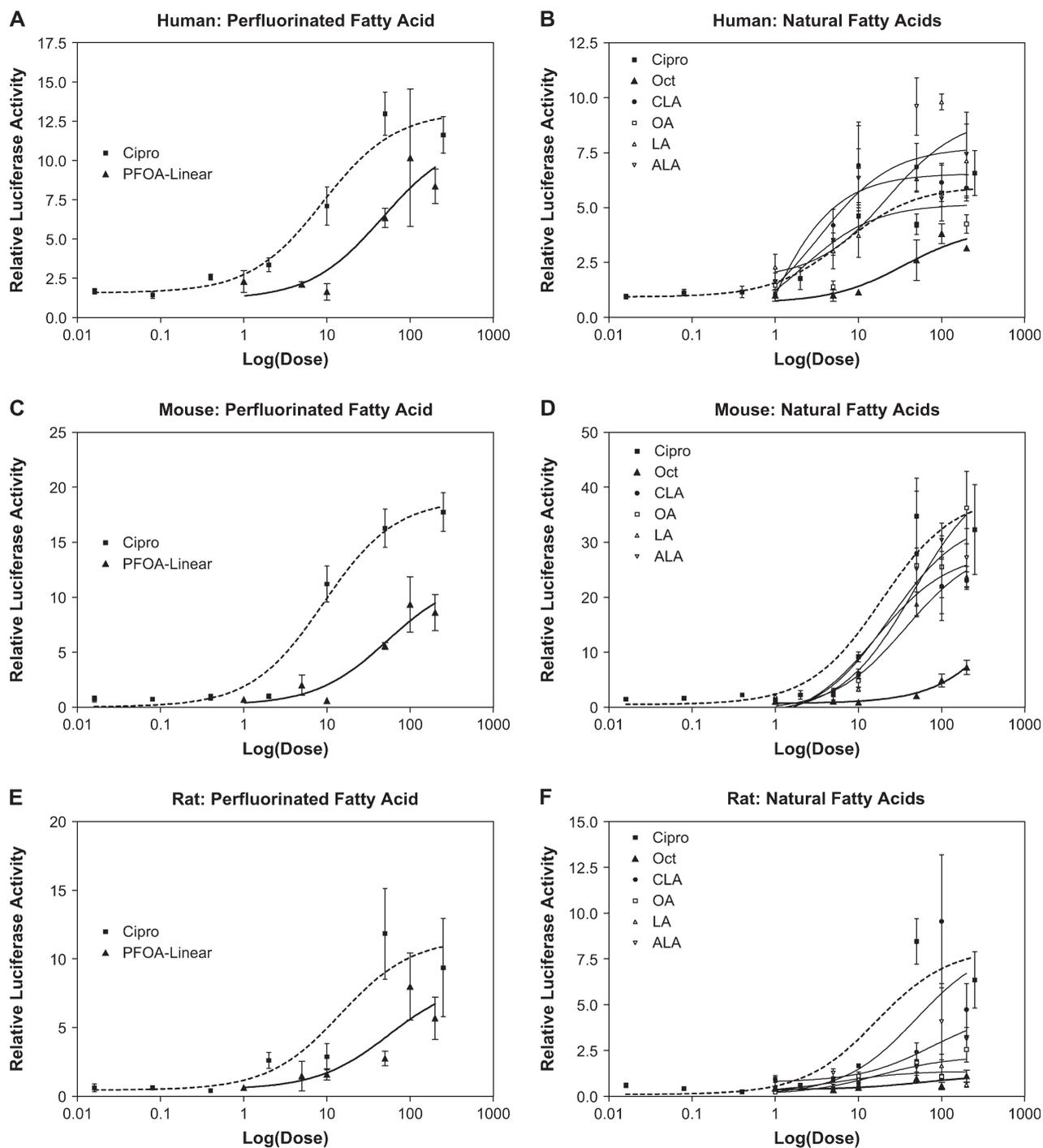


FIG. 2. Activation of PPAR α by fatty acids. The human, mouse, or rat Gal4-PPAR α expression plasmid was cotransfected with a luciferase reporter plasmid into 3T3-L1 cells. Transfected cells were treated in triplicate with increasing concentrations of the indicated fatty acids for 24 h. Cipro was used as a positive control for activation. Cell lysates were analyzed for luciferase activity. Fold induction of the normalized luciferase activity was calculated relative to DMSO (vehicle)-treated cells. Nonlinear regression was performed using GraphPad Prism 4.0 (GraphPad Software, Inc.). The best-fit nonlinear regression curve for the positive control is depicted with the dotted line. Concentrations examined are as follows: Cipro, 0.016, 0.08, 0.4, 2, 10, 50, 250 μ M; Fatty acids and PFOA-linear, 1, 5, 10, 50, 100, 200 μ M. DMSO, 0.1% vol/vol.

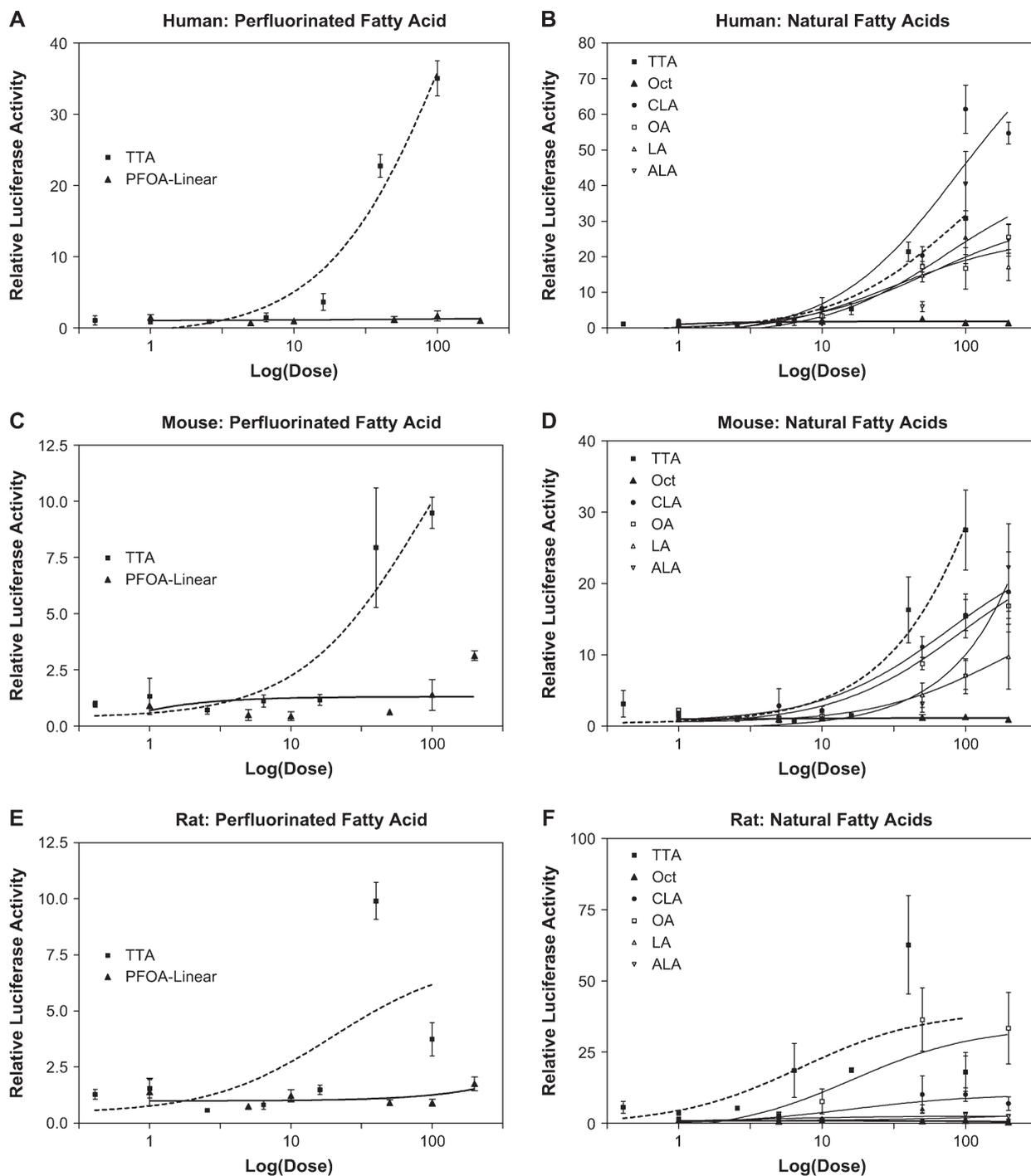


FIG. 3. Activation of PPAR β by fatty acids. The human, mouse, or rat Gal4-PPAR β expression plasmid was cotransfected with a luciferase reporter plasmid into 3T3-L1 cells. Concentrations examined are as follows: TTA, 0.4, 2.5, 6.4, 16, 40, 100 μ M. All other conditions are shown in the caption of Figure 2.

(the results of the nonlinear regression including EC₅₀ and peak effects are given in the Supplemental Data).

Peroxisome Proliferator-Activated Receptor- α

As shown in Figure 2A, PFOA-linear significantly activated human PPAR α albeit with lower potency and efficacy relative

to that of Cipro. Cipro had a peak effect of 13-fold with a significant increase seen at 0.4 μ M compared to 10-fold and 50 μ M for PFOA. The natural fatty acids (Fig. 2B) were similar or slightly more potent and efficacious than the positive control, with the exception of octanoic acid, which activated only slightly (threefold) and at higher concentrations (LSSD 50 μ M).

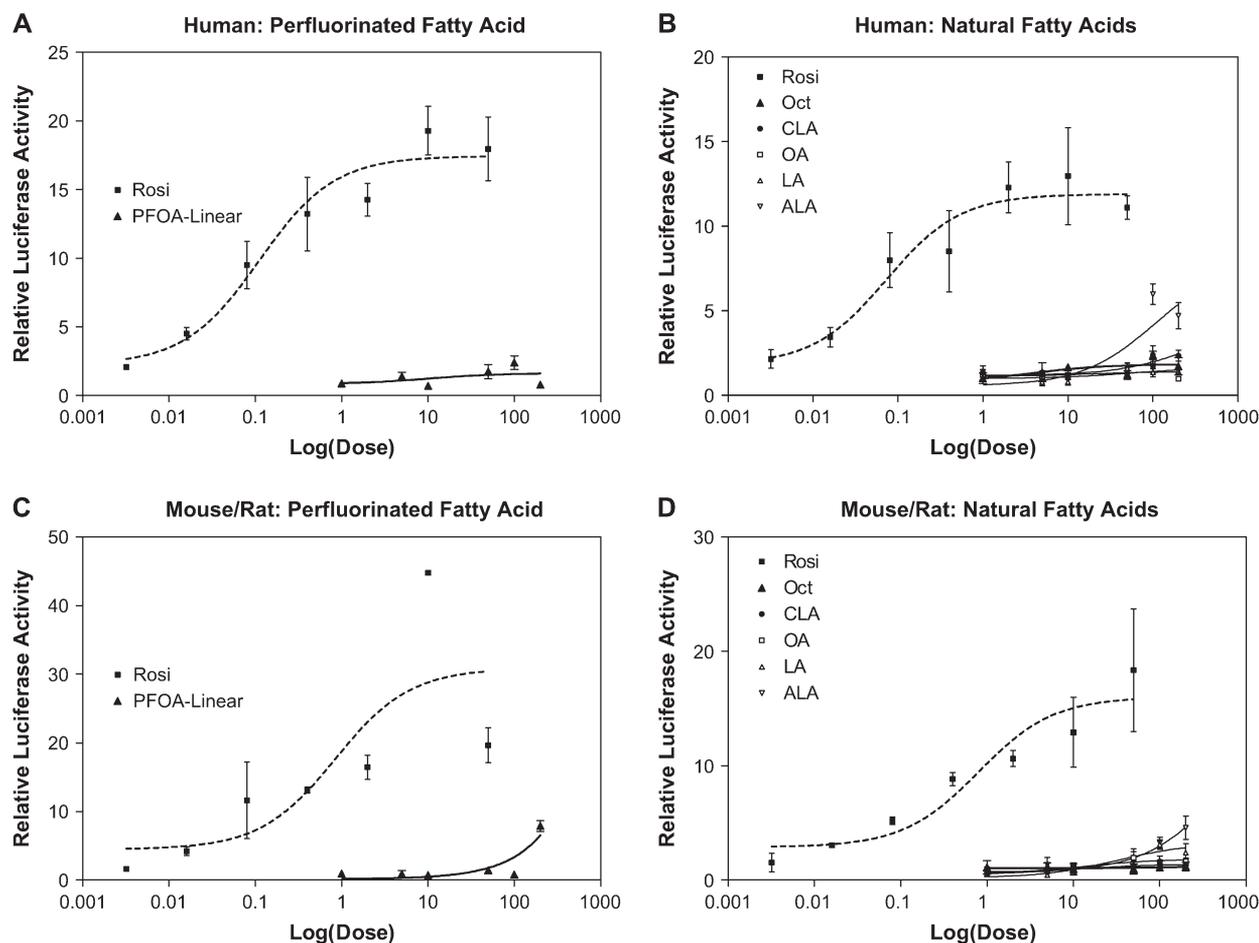


FIG. 4. Activation of PPAR γ by fatty acids. The human, mouse, or rat Gal4-PPAR γ expression plasmid was cotransfected with a luciferase reporter plasmid into 3T3-L1 cells. Concentrations examined are as follows: Rosi, 0.0032, 0.016, 0.08, 0.4, 2, 10, 50 μ M. All other conditions are shown in the caption of Figure 2.

All the longer chained fatty acids activated human PPAR α with the induction ranging from 6- to 10-fold, with significant activation observed at concentrations of 5–10 μ M. Similar trends for PFOA and natural fatty acids were also observed for the mouse (Figs. 2C and 2D), although relative to Cipro, the natural fatty acids were slightly less potent and efficacious. Rat PPAR α showed a similar ability to be activated by the positive control and by PFOA (Fig. 2E). However, in this instance, Oct, CLA, and LA did not activate rat PPAR α . To compare the human PPAR α agonist activity of the fatty acids and their analogs, the ratio of peak effect versus LSSD was used for ranking (Table 2), although similar trends were noted for the nonlinear regression values (Top/EC₅₀). The relative potency of human PPAR α agonism was LA, ALA, CLA > OA, Cipro > Oct, PFOA-linear.

Peroxisome Proliferator-Activated Receptor- β

As shown in Figure 3A, human PPAR β was not significantly affected by PFOA-linear, although this receptor was activated by TTA, CLA, OA, LA, and ALA with peak effects ranging

from 61-fold in the case of CLA to 25-fold for OA and LA (Fig. 3B). PFOA-linear significantly activated mouse PPAR β , although the level of activation was modest (two- to threefold, Fig. 3C) and only seen at the highest concentration. The mouse receptor was also activated by the long-chain normal fatty acids, as was the case for the human homolog. The rat receptor was not sensitive to PFOA-linear (Fig. 3E) and was less sensitive to the natural fatty acids (Fig. 3F) compared to that of mice and humans. The relative potency for human PPAR β agonism (Table 2) was CLA > TTA > ALA, LA, OA with PFOA being a nonagonist.

Peroxisome Proliferator-Activated Receptor- γ

Since the rat and mouse PPAR γ LBDs are identical, they are described as mouse/rat PPAR γ . PFOA-linear slightly activated human PPAR γ (Fig. 4A), and potency and efficacy were dramatically less than the antidiabetic drug Rosi (roughly 1/10th the efficacy and four orders of magnitude less potent). Human PPAR γ was also activated by ALA but not affected by the other natural fatty acids (Fig. 4B). Although we have

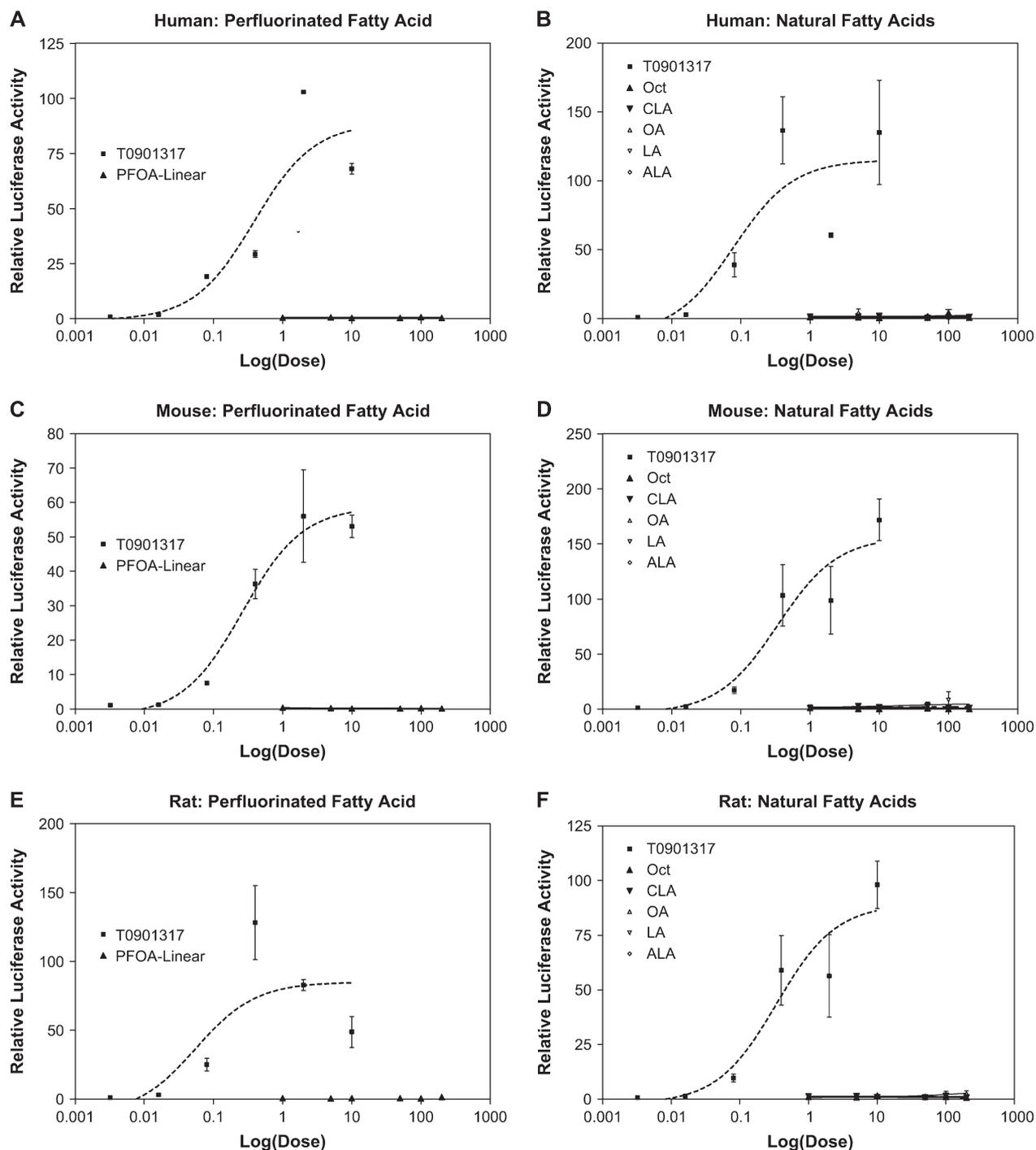


FIG. 5. Activation of LXR β by fatty acids. The human, mouse, or rat Gal4-LXR β expression plasmid was cotransfected with a luciferase reporter plasmid into 3T3-L1 cells. Concentrations examined are as follows: T0901317, 0.08, 0.4, 2, 10, 50 μ M. All other conditions are shown in the caption of Figure 2.

reported CLA to be a PPAR γ agonist (Belury and Vanden Heuvel, 1999; Houseknecht *et al.*, 1998), the present mixture contains a high percentage of 9Z11E-CLA, which has lower affinity for PPAR γ than either the 10E12Z- or 9Z11Z-CLA isomers (DeGrazia *et al.*, 2003). Similar trends were noted

for the rodent PPAR γ with PFOA-linear activating this receptor, although much less efficiently than Rosi (Fig. 4C). LA and ALA activated the mouse/rat PPAR γ (Fig. 4D). The rank-order agonism for human PPAR γ was Rosi \gg ALA > PFOA-linear.

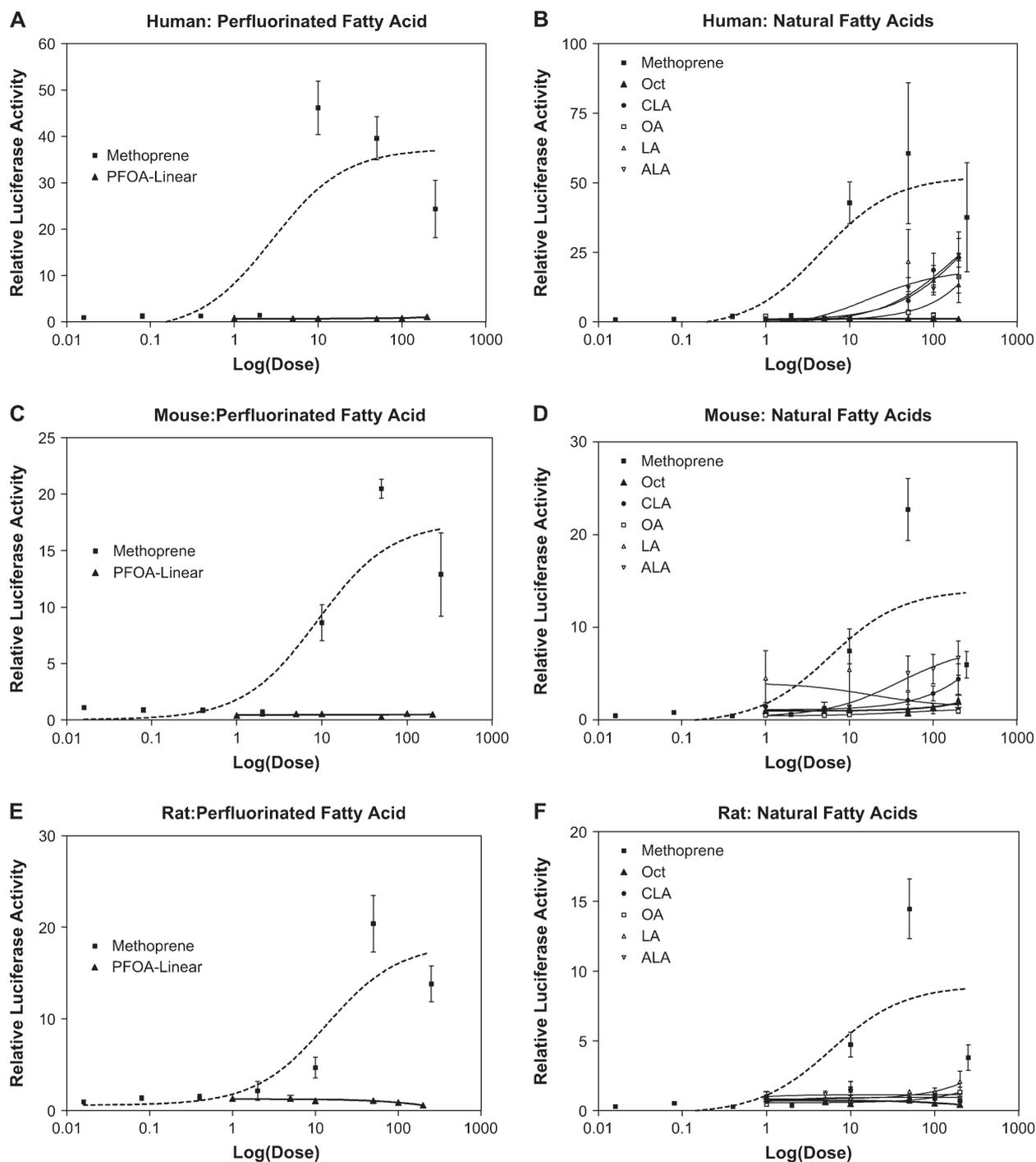


FIG. 6. Activation of RXR α by fatty acids. The human, mouse, or rat Gal4-RXR α expression plasmid was cotransfected with a luciferase reporter plasmid into 3T3-L1 cells. Concentrations examined are as follows: Methoprene, 0.016, 0.08, 0.4, 2, 10, 50, 250 μ M. All other conditions are shown in the caption of Figure 2.

Liver X Receptor- β

LXR β was not activated by any of the fatty acids or perfluorinated analogs (Fig. 5). PFOA-linear did not affect LXR β from humans, mice, or rats. In preliminary experiments, there was a suggestion of an inhibition of LXR activity by

the PFOA-linear, similar to that observed for Oct in the mouse (data not shown). To assess this possibility, these experiments were repeated in the presence or absence of an EC₅₀ of T0901317 or 3-hydroxycholesterol (data not shown). In these experiments, there was no significant effect of PFOA-linear

TABLE 1
Statistical Analysis of Dose-Response Curves

	Cipro (1)			PFOA-linear			Oct			CLA			OA			LA			ALA			Cipro (2)		
	Human	Mouse	Rat	Human	Mouse	Rat	Human	Mouse	Rat	Human	Mouse	Rat	Human	Mouse	Rat	Human	Mouse	Rat	Human	Mouse	Rat	Human	Mouse	Rat
ANOVA <i>p</i> value	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.061	<0.01	<0.01	0.341	<0.01	<0.01	<0.01	<0.01	<0.01	0.07	<0.01	<0.01	0.017	<0.01	<0.01	<0.01
LSSD	0.4	10	50	50	50	100	100	50		5	5		10	5	200	5	10		5	5	100	10	0.4	50
Peak effect	13	17.8	11.8	10.2	9.3	8	3.8	7.3		6.9	28		6.9	36.3	2.6	9.8	23.7		9.6	30.4	4.1	6.6	34.7	8.5
	TTA (1)			PFOA-linear			Oct			CLA			OA			LA			ALA			TTA (2)		
PPAR β	Human	Mouse	Rat	Human	Mouse	Rat	Human	Mouse	Rat	Human	Mouse	Rat	Human	Mouse	Rat	Human	Mouse	Rat	Human	Mouse	Rat	Human	Mouse	Rat
ANOVA <i>p</i> value	<0.01	<0.01	<0.01	0.962	<0.01	0.232	0.162	<0.01	0.143	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.015	<0.01	<0.01	0.023	<0.01	<0.01	<0.01
LSSD	40	40	40		N/A			0.4		10	50	50	50	50	10	50	50	50	50	100	N/A	10	50	0.016
Peak effect	35	9.5	9.9		2.8			34.7		61.5	18.8	10.1	25.6	16.9	36.4	25.5	9.7	5.1	40.3	22.2	2.9	30.8	27.5	62.6
	Rosi (1)			PFOA-linear			Oct			CLA			OA			LA			ALA			Rosi (2)		
PPAR γ	Human	Mouse	Rat	Human	Mouse/Rat		Human	Mouse/Rat		Human	Mouse/Rat		Human	Mouse/Rat		Human	Mouse/Rat		Human	Mouse/Rat		Human	Mouse/Rat	
ANOVA <i>p</i> value	<0.01	<0.01		<0.01	<0.01		0.06	0.959		0.107	0.098		0.35	0.104		0.29	<0.01		0.04	<0.01		<0.01	<0.01	
LSSD	0.003	0.016		100	200												50		100	100		0.08	0.08	
Peak effect	19.3	44.8		2.4	7.9												3.1		5.9	4.6		13	18.4	
	T0901317 (1)			PFOA-linear			Oct			CLA			OA			LA			ALA			T0901317 (2)		
LXR β	Human	Mouse	Rat	Human	Mouse	Rat	Human	Mouse	Rat	Human	Mouse	Rat	Human	Mouse	Rat	Human	Mouse	Rat	Human	Mouse	Rat	Human	Mouse	Rat
ANOVA <i>p</i> value	<0.01	<0.01	<0.01	0.195	0.069	0.927	0.084	<0.01	0.327	0.56	0.07	0.164	0.982	0.267	0.823	0.119	0.561	0.534	0.868	0.01	0.401	<0.01	<0.01	<0.01
LSSD	0.08	0.08	0.016					0.08											5			0.08	0.4	0.4
Peak effect	103	56	26.9					0.1											2.3			137	172	98
	Methoprene (1)			PFOA-linear			Oct			CLA			OA			LA			ALA			Methoprene (2)		
RXR α	Human	Mouse	Rat	Human	Mouse	Rat	Human	Mouse	Rat	Human	Mouse	Rat	Human	Mouse	Rat	Human	Mouse	Rat	Human	Mouse	Rat	Human	Mouse	Rat
ANOVA <i>p</i> value	<0.01	<0.01	<0.01	0.481	0.02	0.292	0.853	0.279	0.327	<0.01	<0.01	0.394	<0.01	0.086	0.823	<0.01	0.351	0.133	<0.01	<0.01	0.401	<0.01	<0.01	<0.01
LSSD	10	10	10		50					50	200		50			50			50	50		10	10	0.4
Peak effect	46.2	20.5	20.4		0.25					23.1	4.4		16.2			21.6			24	6.7		60.6	22.7	98

Note. The positive controls used for comparison with PFOA-linear are depicted with a (1), while the (2) signifies positive control for the regular fatty acids. Gray shading indicates that the *p* value was >0.05 and hence no LSSD nor peak effect is presented.

TABLE 2
Ranking of Agonist Activity for Human Receptors: Perfluorinated versus Natural Fatty Acids

Chemical	PPAR α		PPAR β		PPAR γ		LXR α		RXR β	
	Peak/LSSD ^a	Rank	Peak/LSSD	Rank	Peak/LSSD	Rank	Peak/LSSD	Rank	Peak/LSSD	Rank
Positive	1.00	5	1.00	2	1.00	1	1.00	1	1.00	1
PFOA-linear	0.01	7			0.000004	3				
Oct	0.06	6								
CLA	2.09	3	2.00	1					0.20	2
OA	1.05	4	0.17	4					0.05	5
LA	2.97	1	0.17	5					0.07	4
ALA	2.91	2	0.26	3	0.0004	2			0.08	3

^aPeak/LSSD for the positive control was set to 1; values for fatty acids and perfluorinated fatty acid analogs are relative to the positive control examined concurrently. If the compound did not significantly affect activity, it is denoted with gray shading and was not ranked.

under conditions designed to test for inhibition of LXR β activation.

Retinoid X Receptor- α

In addition to being the common heterodimerization partner for the other receptors in this study, RXR α is a fatty acid receptor in its own right. PFOA-linear did not activate RXR α , regardless of the species (Fig. 6). As was observed with LXR, initially the PFOA-linear showed some indication of being an inhibitor. However, PFOA-linear did not compete with 9-*cis*-retinoic acid or methoprene for activation of human, rat, or mouse RXR α (data not shown), thus allowing us to conclude that PFOA-linear was neither an agonist nor an antagonist of RXR α . The ω -3 fatty acids such as docosahexaenoic (DHA) and α -linolenic acids are known RXR ligands; herein, we confirmed that ALA is an RXR activator for both human and mouse receptors. In addition, CLA, LA, and OA were able to significantly activate the human subtype. The rank-order potency for agonism of human RXR α was methoprene > CLA > ALA, LA, OA.

Structure-Activity Relationship: Perfluorinated Fatty Acid Analogs

The data thus far indicated that PFOA-linear was an activator of both PPAR α and, to a lesser extent, PPAR γ . However, the level of activation and the potency for PPAR α , in particular compared to the pharmaceutical positive control, was much greater than that of PPAR γ . An additional dose-response study was performed to compare PPAR α activation for PFOA-linear to the major isomeric forms of PFOA (Fig. 7). The perfluorinated fatty acid analogs, with the exception of PFOA-linear/branched, significantly activated human PPAR α with an efficacy similar to Cipro (Fig. 7). The rank-order potency for human PPAR α was Cipro > PFOA-branched, PFOA-linear > PFOS (Table 3). The mouse PPAR α was significantly activated by all the fatty acid analogs and PFOS,

with the latter being the least efficacious. As was observed in Figure 2, the rat PPAR α was not as responsive as that of either mice or humans.

Similarly, the activation of PPAR γ by various perfluorinated acids was examined (Fig. 8). As was mentioned in Figure 4, the peak effect of PFOA-linear for human PPAR γ was modest compared to Rosi (2-fold vs. 20-fold); this was also true for PFOS (four- to fivefold), PFOA-branched (five- to sixfold), and PFOA-linear/branched (threefold). The mouse/rat PPAR γ was slightly more responsive to the perfluorinated acids, resulting in a peak effect of approximately eightfold with PFOA-linear. The rank-order agonism for human PPAR γ was Rosi \gg PFOS > PFOA-branched, -linear, -linear/branched.

Cluster Analysis of Receptor Activation Data

Based on the data presented in Figures 2–6, the perfluorinated fatty acid analogs are acting in a slightly more specific manner than regular fatty acids. For example, linoleic acid is capable of activating PPAR α , PPAR β , PPAR γ , and RXR α while PFOA-linear affects only PPAR α and PPAR γ . To formally assess this possibility, the activation of the human NRs by 100 μ M of each of the fatty acid analogs and normal fatty acids was compared by cluster analysis (Fig. 9). Interestingly, the profile of the NR activation was stratified by broad classification of fatty acid structures. The ω -3 polyunsaturated fatty acids (ALA and DHA, data not shown in previous figures) and the ω -6 polyunsaturated fatty acids (LA and CLA) clustered together and were closely related to the activation observed for the monounsaturated fatty acid, OA. The eight-carbon octanoate and the perfluorinated fatty acid analogs grouped together with PFOA-branched and -linear sharing the most in common, at least by this analysis. A similar pattern was observed when cluster analysis was performed at 50 or 200 μ M with the human receptors or 100 μ M with the mouse receptors (data not shown). Taken together, these data indicate that the perfluorinated acids are able to affect the activation of NRs, in

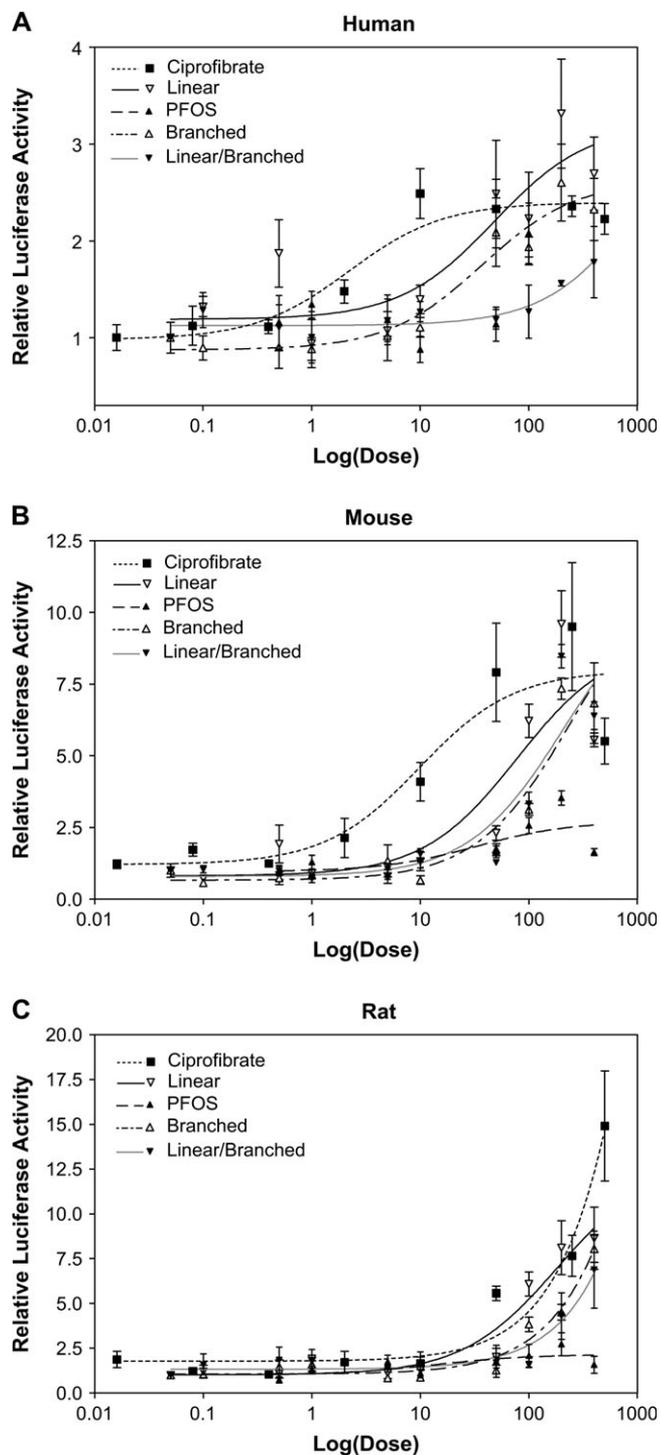


FIG. 7. Fatty acid analogs activate PPAR α . The human, mouse, or rat Gal4-PPAR α expression plasmid was cotransfected with a luciferase reporter plasmid into 3T3-L1 cells and was tested for activation by increasing the concentrations of the major isomeric forms of PFOA (linear, linear/branched, and branched) as well as PFOS. Cipro was used as a positive control. Concentrations examined are as follows: Cipro, 0.016, 0.08, 0.4, 2, 10, 50, 250, 500 μ M; Perfluorinated fatty acid analogs, 0.1, 0.5, 1, 5, 10, 50, 100, 200, 400 μ M. DMSO, 0.1% vol/vol.

TABLE 3
Ranking of Agonist Activity for Human Receptors:
Structure-Activity of Perfluorinated Fatty Acids

Chemical	PPAR α		PPAR γ	
	Peak/LSSD ^a	Rank	Peak/LSSD	Rank
Positive	1.00	1	1000.00	1
PFOS	0.08	4	0.15	2
PFOA-linear/branched			0.02	5
PFOA-branched	0.21	3	0.05	3
PFOA-linear	0.26	2	0.04	4

^aPeak/LSSD for the positive control was set to 1 for PPAR α and 1000 for PPAR γ ; values for fatty acids and perfluorinated fatty acid analogs are relative to the positive control examined concurrently. If the compound did not significantly affect activity, it is denoted with gray shading and was not ranked.

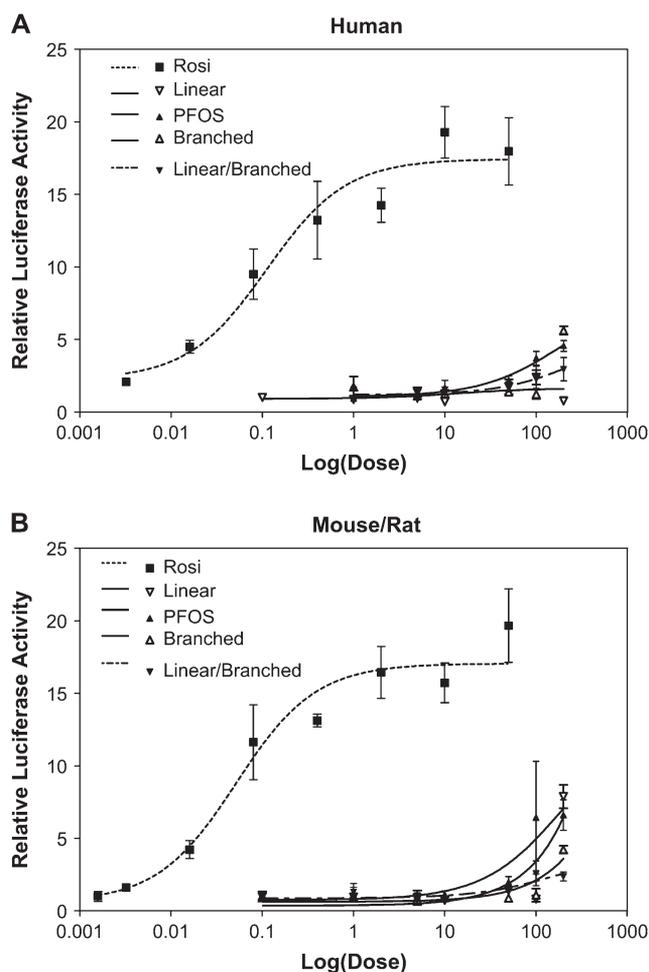


FIG. 8. Fatty acid analogs activate PPAR γ . The human, mouse, or rat Gal4-PPAR γ expression plasmid was cotransfected with a luciferase reporter plasmid into 3T3-L1 cells and was tested for activation by increasing the concentrations of the major isomeric forms of PFOA (linear, linear/branched, and branched) as well as PFOS. Rosi was used as a positive control. All other conditions are shown in the caption of Figure 2.

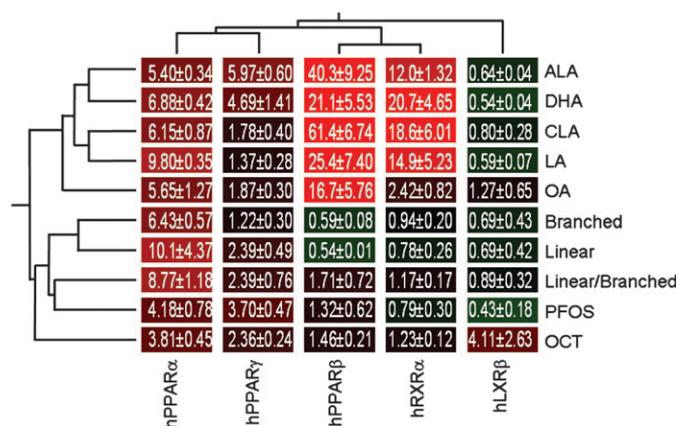


FIG. 9. Hierarchical clustering of NR activation by fatty acids. Data from relative activation of each receptor at 100 μ M were examined by K-means clustering using GeneSpring with standard correlation being the similarity measure. The blocks were shaded based on average expression with the mean and standard error shown within each block.

particular PPAR α , and that they have a unique activation profile when compared to normal fatty acids.

DISCUSSION

NRs constitute a superfamily of transcription factors that are regulated by a structurally diverse array of small lipophilic molecules ranging from xenobiotics to drugs to nutrients. With the identification of natural and synthetic ligands for these receptors, their once-orphan status has been replaced with a growing recognition of their roles as primary regulators of many aspects of lipid metabolism. Based on observations in the published literature that PFOA can affect fatty acid and cholesterol metabolism, the NRs selected for the studies reported herein centered on PPAR with its related α -, β -, γ -isoforms, LXR, and their obligate heterodimer partner, RXR. The β - and α -isoforms of LXR and RXR, respectively, were chosen for study on the basis of their ubiquitous tissue expression profiles.

The current studies were undertaken with three goals in mind. The first goal was to gain a broader understanding of the NRs activated by PFOA and to compare its activity with that of naturally occurring fatty acids. The second goal was to develop a cross-species understanding of the ability of PFOA to activate these receptors. Finally, the third goal was to evaluate the impact of isomeric forms of PFOA on receptor activation. An overarching strategy for achieving these goals was to undertake this work in a common testing platform that measured activation as the combination of the test ligand's ability to bind and transactivate chimeric receptors containing the LBDs of the NRs and species of interest. Within the limitations of the experimental design, the data show that PFOA exerts its primary effect on PPAR α , with a secondary

and lesser effect on PPAR γ . PFOA is a relatively weak ligand for PPAR α in comparison with naturally occurring fatty acids, and it is far less potent than Cipro, a representative member of the fibrates class of drugs. Finally, these observations generally hold true across several species including mice, rats, and humans.

The present study confirms and extends the work of others. As expected on the basis of ultrastructural and biochemical changes observed in rats, PFOA was found to be an activator of PPAR α . There are two reports in the literature describing the ability of PFOA to transactivate rat and human PPAR α expressed in CV-1 cells (Intrasuksri *et al.*, 1998) and mouse and rat PPAR α expressed in COS-1 cells (Maloney and Waxman, 1999). Neither study reported EC₅₀ values for the PFOA effect, and given the use of different cell types, incubation times, and positive controls, it is not easy to compare the data among the various studies. However, in all published reports, activation of PPAR α was observed in the 10–100 μ M range. Our studies provide new information in three areas. First, the major isomers of PFOA exhibited were all able to activate PPAR α to similar peak effect. Although the molecular pharmacology suggests that there may be some rank ordering to the PPAR α -mediated effect, this was not reflected in the biological end points such as peroxisomal β -oxidation and hepatomegaly where minimal differentiation of effect was observed among the various isomers in rats and mice (Loveless *et al.*, in press). It should be kept in mind, however, that direct comparisons between molecular and *in vivo* data are subject to multiple confounding variables such as PK/PD considerations and binding to competing proteins such as FABP (Luebker *et al.*, 2002). Second, a broader survey for effects on PPAR isoforms revealed that while the primary effect of PFOA was on PPAR α , there was some minor activity observed with respect to PPAR γ . Specifically, PFOA-linear was a very weak partial agonist of PPAR γ exhibiting EC₅₀ and maximal relative induction values that were 150- and 7-fold less, respectively, than that observed for Rosi. Similar activities were observed for the isomers of PFOA as well as PFOS. The effect of PFOA on PPAR γ is consistent with what has been reported in the patent literature (Elcombe and Wolf, 2002, 2004) but inconsistent with that reported in the scientific literature (Maloney and Waxman, 1999). Third, data are provided that LXR and RXR are unresponsive to PFOA.

Although it is tempting to draw analogies with perfluorinated and natural fatty acids, there are more differences than similarities between the two classes of fatty acids. First and foremost, the very low pK_a values of the perfluorinated fatty acid analogs prevent their conversion to acyl-CoA esters and thereby block their entry into lipid intermediary metabolism (Kuslikis *et al.*, 1992; Vanden Heuvel *et al.*, 1991a). From the perspective of NR pharmacology, the free forms of the polyunsaturated fatty acids are the proximate ligands of PPAR α ; however, natural fatty acids have an additional pharmacology related to their conversion to acyl-CoA derivatives with the

subsequent opportunity to interact with another NR, hepatic nuclear factor-4 α (HNF-4 α). Notably, while the acyl-CoA of a fatty acid may activate HNF-4 α , the same molecule may inhibit PPAR α . In this regard, the relative abundance of free fatty acids (natural and perfluorinated)/acyl-CoAs may be the biological determinant of the transcriptional activity of these fatty acid-sensitive NRs. Since HNF-4 α plays a major role in the regulation of lipid and lipoprotein metabolism (Hayhurst *et al.*, 2001) and in the recognition of the differential expression and responsiveness of these NRs in rodents versus man (Hertz *et al.*, 2003), studies of the effects of perfluorinated on HNF-4 α and the interplay between PPAR α and HNF-4 α may be worthy of pursuit. Interestingly, a recent study has shown that saturated fatty acids-CoA esters and CoA esters of fibrate drugs are high-affinity PPAR α ligands (Hostetler *et al.*, 2005). Once again, due to the molecular structure of the perfluorinated acids, this metabolism-dependent receptor interaction is not possible.

A comparison of the effects of PFOA with that of Oct offers some insights into the molecular differences between these fatty acids of equal chain length. As illustrated in Figure 1, these two fatty acids have different spatial size and orientations, due mainly to the electron-withdrawing ability and strength of the C-F bond. In the context of SPPARM, it is perhaps not surprising that the two fatty acids present with different activation profiles. While both fatty acids are PPAR α agonists, their profiles clearly differ across the species. For example, with respect to the human receptor, PFOA functions as a full agonist, whereas Oct functions as a partial agonist. When PFOA is compared to longer chain fatty acids with increasing numbers of double bonds, differences in spatial and molecular orientations between natural and this perfluorinated fatty acid are even more dramatic and have a significant impact on the molecular pharmacology of these fatty acids. While PFOA has a primary effect on PPAR α across species, it had little if any effect on the other NRs investigated in this study. In contrast, natural fatty acids, such as OA, LA, and ALA, readily transactivate several of the NRs in the RXR heterodimer family.

It should be kept in mind that the assay used in the present study measures the first of many steps in the complex regulation of gene transcription. There are several caveats related to the present data. First, comparing sensitivity across species based on reporter data may not be valid. For example, we have observed that human PPAR α is as sensitive to PFOA and fatty acid activations as the mouse receptor in terms of both potency and efficacy; however, this comparison is made between the two species under conditions wherein the receptors are equivalently expressed and in the same cellular milieu. Second, a chimeric receptor assay was employed where the ligand-binding activation domain is present but the kinase-regulated ligand-independent domain (a potential source of species differences) is removed. Thus, the chimeric report system is isolating the ligand-dependent activation and is

simplifying many of the dynamic factors that may contribute to species differences in toxicity observed *in vivo*. Third, the molecular techniques as used in the studies reported herein are designed to examine the potential for a particular pathway to be associated with a biological response but may not address the plausibility of that effect. For example, PFOA is found in highest concentrations in the liver and blood of rats (Vanden Heuvel *et al.*, 1991b). The fact that hepatic peroxisome proliferation is observed shows that PPAR α activation can be achieved *in vivo*. However, whether PFOA would achieve sufficient concentration to activate PPAR γ in the adipose tissue cannot be predicted. Finally, the simple chimeric system shows the ability to activate the Gal4-reporter construct but does not take into account the complexity of the ligand-dependent gene expression. *In lieu* of the complexity of SPPARM, gene expression profiling rather than the *in vitro* assessment of receptor biology parameters may be more informative in terms of explaining or predicting *in vivo* biological outcomes (Guruge *et al.*, 2006). Despite these limitations, the data presented herein offer new insights and contexts into the mechanism of action of PFOA and its various isomers.

SUPPLEMENTAL DATA

Supplementary data are available online at www.toxsci.oxfordjournals.org.

ACKNOWLEDGMENTS

This research was funded by DuPont Haskell Laboratory for Health and Environmental Sciences and the National Institutes of Health (DK49009 awarded to J.P.V.H.). Portions of this research were performed by Indigo Biosciences LLC (State College, PA). Preliminary results were presented at the Fluoros meeting held in Toronto, Canada, in July 2005, and all data have been shared with the EPA Scientific Advisory Board. The authors would like to thank Dr John Butenhoff (3M, Minneapolis, MN) and Drs Nancy Everds and Scott Lovelace (DuPont Haskell Laboratories) for their insightful comments during the preparation of the manuscript.

REFERENCES

- Belury, M. A., and Vanden Heuvel, J. P. (1999). Modulation of diabetes by conjugated linoleic acid. In *Advances in Conjugated Linoleic Acid Research* (M. P. Yurawecz, M. M. Mossoba, J. K. G. Kramer, M. W. Pariza, and G. J. Nelson, Eds.), Vol. 1, pp. 404–411. AOCS Press, Champaign, IL.
- Bocos, C., Gottlicher, M., Gearing, K., Banner, C., Enmark, E., Teboul, M., Crickmore, A., and Gustafsson, J. A. (1995). Fatty acid activation of peroxisome proliferator-activated receptor (PPAR). *J. Steroid Biochem. Mol. Biol.* **53**, 467–473.
- Camp, H. S., Li, O., Wise, S. C., Hong, Y. H., Frankowski, C. L., Shen, X., Vanbogelen, R., and Leff, T. (2000). Differential activation of peroxisome proliferator-activated receptor-gamma by troglitazone and rosiglitazone. *Diabetes* **49**, 539–547.

- DeGrazia, M. J., Thompson, J., Vanden Heuvel, J. P., and Peterson, B. R. (2003). Synthesis of a high-affinity fluorescent ligand PPAR γ ligand for high-throughput fluorescence polarization assays. *Bioorg. Med. Chem.* **11**, 4325–4332.
- Duez, H., Lefebvre, B., Poulain, P., Torra, I. P., Percevault, F., Luc, G., Peters, J. M., Gonzalez, F. J., Gineste, R., Helleboid, S., *et al.* (2005). Regulation of human apoA-I by gemfibrozil and fenofibrate through selective peroxisome proliferator-activated receptor alpha modulation. *Arterioscler. Thromb. Vasc. Biol.* **25**, 585–591.
- Duval, C., Chinetti, G., Trottein, F., Fruchart, J. C., and Staels, B. (2002). The role of PPARs in atherosclerosis. *Trends Mol. Med.* **8**, 422–430.
- Elcombe, C. R., and Wolf, C. R. (2002). Perfluorinated fatty acids for the treatment of diabetes, obesity, cardiovascular disease or as anti-tumour agents. UK Patent GB0220045.
- Elcombe, C. R., and Wolf, C. R. (2004). Perfluorinated fatty acids for the treatment of diabetes, obesity, cardiovascular disease or as anti-tumour agents. UK Patent GB 2392386.
- Francis, G. A., Fayard, E., Picard, F., and Auwerx, J. (2003). Nuclear receptors and the control of metabolism. *Annu. Rev. Physiol.* **65**, 261–311.
- Fruchart, J. C., and Duriez, P. (2002). HMG CoA reductase inhibitors and PPAR-alpha activators: Are their effects on high-density lipoprotein cholesterol and their pleiotropic effects clinically relevant in prevention trials? *Curr. Atheroscler. Rep.* **4**, 403–404.
- Guruge, K. S., Yeung, L. W. Y., Yamanaka, N., Miyazaki, S., Lam, P. K. S., Geisy, J. P., Jones, P. D., and Yamashita, N. (2006). Gene expression profiles in rat liver treated with perfluorooctanoic acid (PFOA). *Toxicol. Sci.* **89**, 93–107.
- Haughom, B., and Spydevold, O. (1992). The mechanism underlying the hypolipemic effect of perfluorooctanoic acid (PFOA), perfluorooctane sulphonic acid (PFOSA) and clofibrate acid. *Biochim. Biophys. Acta* **1128**, 65–72.
- Hayhurst, G. P., Lee, Y. H., Lambert, G., Ward, J. M., and Gonzalez, F. J. (2001). Hepatocyte nuclear factor 4alpha (nuclear receptor 2A1) is essential for maintenance of hepatic gene expression and lipid homeostasis. *Mol. Cell. Biol.* **21**, 1393–1403.
- Hertz, R., Ben-Haim, N., Petrescu, A. D., Kalderon, B., Berman, I., Eldad, N., Schroeder, F., and Bar-Tana, J. (2003). Rescue of MODY-1 by agonist ligands of hepatocyte nuclear factor-4alpha. *J. Biol. Chem.* **278**, 22578–22585.
- Hess, R., Staubli, W., and Riess, W. (1965). Nature of the hepatomegaly effect produced by ethyl-chlorophenoxy-isobutyrate in the rat. *Nature* **208**, 856–858.
- Hostetler, H. A., Petrescu, A. D., Kier, A. B., and Schroeder, F. (2005). Peroxisome proliferator-activated receptor alpha interacts with high affinity and is conformationally responsive to endogenous ligands. *J. Biol. Chem.* **280**, 18667–18682.
- Houseknecht, K. L., Vanden Heuvel, J. P., Moya-Camarena, S. Y., Portocarrero, C. P., Peck, L. W., Nickel, K. P., and Belury, M. A. (1998). Dietary conjugated linoleic acid normalizes impaired glucose tolerance in the Zucker diabetic fatty fa/fa rat. *Biochem. Biophys. Res. Commun.* **244**, 678–682.
- Ikeda, T., Aiba, K., Fukuda, K., and Tanaka, M. (1985). The induction of peroxisome proliferation in rat liver by perfluorinated fatty acids, metabolically inert derivatives of fatty acids. *J. Biochem. (Tokyo)* **98**, 475–482.
- Intrasuksri, U., Rangwala, S. M., O'Brien, M., Noonan, D. J., and Feller, D. R. (1998). Mechanisms of peroxisome proliferation by perfluorooctanoic acid and endogenous fatty acids. *Gen. Pharmacol.* **31**, 187–197.
- Issemann, I., and Green, S. (1990). Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. *Nature* **347**, 645–650.
- Kennedy, G. L., Jr, Butenhoff, J. L., Olsen, G. W., O'Connor, J. C., Seacat, A. M., Perkins, R. G., Biegel, L. B., Murphy, S. R., and Farrar, D. G. (2004). The toxicology of perfluorooctanoate. *Crit. Rev. Toxicol.* **34**, 351–384.
- Kersten, S., Desvergne, B., and Wahli, W. (2000). Roles of PPARs in health and disease. *Nature* **405**, 421–424.
- Klaunig, J. E., Babich, M. A., Baetcke, K. P., Cook, J. C., Corton, J. C., David, R. M., DeLuca, J. G., Lai, D. Y., McKee, R. H., Peters, *et al.* (2003). PPARalpha agonist-induced rodent tumors: Modes of action and human relevance. *Crit. Rev. Toxicol.* **33**, 655–780.
- Kudo, N., Mizuguchi, H., Yamamoto, A., and Kawashima, Y. (1999). Alterations by perfluorooctanoic acid of glycerolipid metabolism in rat liver. *Chem-Biol. Interact.* **118**, 69–83.
- Kuslikis, B. I., Vanden Heuvel, J. P., and Peterson, R. E. (1992). Lack of evidence for perfluorodecanoyl- or perfluorooctanoyl-coenzyme A formation in male and female rats. *J. Biochem. Toxicol.* **7**, 25–29.
- Le Douarin, B., vom Baur, E., Zechel, C., Heery, D., Heine, M., Vivat, V., Gronemeyer, H., Losson, R., and Chambon, P. (1996). Ligand-dependent interaction of nuclear receptors with potential transcriptional intermediary factors (mediators). *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **351**, 569–578.
- Li, A. C., and Glass, C. K. (2004). PPAR- and LXR-dependent pathways controlling lipid metabolism and the development of atherosclerosis. *J. Lipid Res.* **45**, 2161–2173.
- Loveless, S. E., Finlay, C., Everds, N. E., Frame, R. R., Gillies, P. J., O'Connor, J. C., and Kennedy, G. L. (2006). Comparative responses of rats and mice exposed to linear/branched, linear, and branched ammonium perfluorooctanoate (APFO). *Toxicology* **220**, 203–217.
- Luebker, D. J., Hanson, K. J., Bass, N. M., Butenhoff, J. L., and Seacat, A. M. (2002). Interactions of fluorochemicals with rat liver fatty acid binding protein. *Toxicology* **176**, 175–185.
- Maloney, E. K., and Waxman, D. J. (1999). Trans-activation of PPARalpha and PPARgamma by structurally diverse environmental chemicals. *Toxicol. Appl. Pharmacol.* **161**, 209–218.
- Moody, D. E., and Reddy, J. K. (1978). Hepatic peroxisome (microbody) proliferation in rats fed plasticizers and related compounds. *Toxicol. Appl. Pharmacol.* **45**, 497–504.
- Pastoor, T. P., Lee, K. P., Perri, M. A., and Gillies, P. J. (1987). Biochemical and morphological studies of ammonium perfluorooctanoate-induced hepatomegaly and peroxisome proliferation. *Exp. Mol. Pathol.* **47**, 98–109.
- Shearer, B. G., and Hoekstra, W. J. (2003). Recent advances in peroxisome proliferator-activated receptor science. *Curr. Med. Chem.* **10**, 267–280.
- Shiple, J. M., Hurst, C. H., Tanaka, S. S., DeRoos, F. L., Butenhoff, J. L., Seacat, A. M., and Waxman, D. J. (2004). Trans-activation of PPARalpha and induction of PPARalpha target genes by perfluorooctane-based chemicals. *Toxicol. Sci.* **80**, 151–160.
- Vanden Heuvel, J. P., Kuslikis, B. I., Shrago, E., and Peterson, R. E. (1991a). Inhibition of long-chain acyl-CoA synthetase by the peroxisome proliferator perfluorodecanoic acid in rat hepatocytes. *Biochem. Pharmacol.* **42**, 295–302.
- Vanden Heuvel, J. P., Kuslikis, B. I., Van Rafelghem, M. J., and Peterson, R. E. (1991b). Tissue distribution, metabolism, and elimination of perfluorooctanoic acid in male and female rats. *J. Biochem. Toxicol.* **6**, 83–92.
- Xie, Y., Yang, Q., Nelson, B. D., and DePierre, J. W. (2003). The relationship between liver peroxisome proliferation and adipose tissue atrophy induced by peroxisome proliferator exposure and withdrawal in mice. *Biochem. Pharmacol.* **66**, 749–756.