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trans-Activation of PPARα and PPARγ by Structurally Diverse Environmental Chemicals[1]

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A large number of industrial chemicals and environmental pollutants, including trichloroethylene (TCE), di(2-ethylhexyl)-phthalate (DEHP), perfluorooctanoic acid (PFOA), and various phenoxyacetic acid herbicides, are nongenotoxic rodent hepatocarcinogens whose human health risk is uncertain. Rodent model studies have identified the receptor involved in the hepatotoxic and hepatocarcinogenic actions of these chemicals as peroxisome proliferator–activated receptor alpha (PPARα), a nuclear receptor that is highly expressed in liver. Humans exhibit a weak response to these peroxisome proliferator chemicals, which in part results from the relatively low level of PPARα expression in human liver. Cell transfection studies were carried out to investigate the interactions of peroxisome proliferator chemicals with PPARα, cloned from human and mouse, and with PPARγ, a PPAR isoform that is highly expressed in multiple human tissues and is an important regulator of physiological processes such as adipogenesis and hematopoiesis. With three environmental chemicals, TCE, perchloroethylene, and DEHP, PPARα was found to be activated by metabolites, but not by the parent chemical. A decreased sensitivity of human PPARα compared to mouse PPARα to *trans*-activation was observed with some (Wy-14,643, PFOA), but not other, peroxisome proliferators (TCE metabolites, trichloroacetate and dichloroacetate; and DEHP metabolites, mono[2-ethylhexyl]-phthalate and 2-ethylhexanoic acid). Investigation of human and mouse PPARγ revealed the transcriptional activity of this receptor to be stimulated by mono(2-ethylhexyl)phthalate, a DEHP metabolite that induces developmental and reproductive organ toxicities in rodents. This finding suggests that PPARγ, which is highly expressed in human adipose tissue, where many lipophilic foreign chemicals tend to accumulate, as well as in colon, heart, liver, testis, spleen, and hematopoietic cells, may be a heretofore unrecognized target in human cells for a subset of industrial and environmental chemicals of the peroxisome proliferator class.

Key Words: **PPAR; peroxisome proliferator chemical; trichloroethylene; DEHP.**

Peroxisome proliferator chemicals (PPCs)[3] comprise a broad class of environmental chemicals that stimulate liver hypertrophy and hyperplasia in rodents, leading to the formation of liver tumors (Rao and Reddy, 1987; Reddy *et al.*, 1980). PPCs include hypolipidemic fibrate drugs (e.g., clofibrate, nafenopin), chlorinated hydrocarbons such as TCE and PCE, industrial plasticizers (DEHP), perfluorinated fatty acids (PFOA) (Intrasuksri *et al.*, 1998; Kluwe, 1994; Rao and Reddy, 1987), and certain fatty acids, prostaglandins, and endogenous steroids (e.g., dehydroepiandrosterone-3β-sulfate) (Waxman, 1996). PPCs exert their effects on liver and certain other tissues by activation of the nuclear receptor protein PPARα (Issemann and Green, 1990), which stimulates the synthesis of peroxisomal enzymes and certain cytochrome P450 enzymes important in lipid metabolism, and increases the number and size of peroxisomes within liver and some other cell types (Schoonjans *et al.*, 1997; Waxman, 1999). In rodents and other sensitive species, this PPARα-dependent process (Lee *et al.*, 1995; Peters *et al.*, 1997) ultimately results in hepatocellular carcinoma (Holden and Tugwood, 1999; Masters and Crane, 1998). Mechanistically, PPC-activated PPARα induces the transcription of lipid-metabolizing enzymes, leading to increased production of DNA-damaging reduced-oxygen species (Fahl *et al.*, 1984; Kasai *et al.*, 1989; Reddy *et al.*, 1986). This process is associated with an alteration of the balance between hepatocyte proliferation, which is stimulated by PPCs, and hepatocyte apoptosis, which is suppressed following PPC exposure (Christensen *et al.*, 1998; Gill *et al.*, 1998; Roberts *et*

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[3] Abbreviations used: 2,4-D, 2,4-dichlorophenoxyacetic acid; CH, chloral hydrate; DCA, dichloroacetic acid; DEHP, di(2-ethylhexyl)phthalate; DMEM, Dulbecco's Minimal Essential Medium; DMSO, dimethyl sulfoxide; EHA, 2-ethylhexanoic acid; EOH, 2-ethylhexanol; FBS, fetal bovine serum; MCPA, 2-methyl-4-chlorophenoxyacetic acid; MEHP, mono-2-ethylhexylphthalate; PCE, tetrachloroethylene; PFOA, perfluorooctanoic acid; PPAR, peroxisome proliferator–activated receptor; PPC, peroxisome proliferator chemical; PPRE, PPC response element; TCA, trichloroacetic acid; TCE, trichloroethylene; TCE-OH, trichloroethanol; Wy-14,643, 4-chloro-6-(2,3-xylidino)-2-pyrimidinythiol acetic acid.

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al., 1998). Liver cell apoptosis is hypothesized to be a key mechanism whereby genetically damaged cells are eliminated prior to their clonal expansion, leading to fixation of PPC-induced mutations in initiated cells (Gonzalez *et al.*, 1998; James *et al.*, 1998).

Human cells are only weakly responsive to peroxisome proliferators (Cattley *et al.*, 1998; Holden and Tugwood, 1999), which may in part be due to their low level of PPARα (Palmer *et al.*, 1998; Tugwood *et al.*, 1997) and to species differences in PPARα responsiveness, as suggested by studies of the prototypic PPC Wy-14,643 (Mukherjee *et al.*, 1994). These species differences in receptor specificity, which are primarily mediated by the receptor's ligand-binding domain (Keller *et al.*, 1997), have thus far been described for only two PPCs, Wy-14,643 and the synthetic arachidonic acid analog 5,8,11,14-eicosatetraynoic acid. In contrast to PPARα, a second PPAR receptor, PPARγ, is highly expressed in a number of human tissues, including adipose tissue, colon, heart, liver, testis, spleen, and hematopoietic cells (Greene *et al.*, 1995; Vidal-Puig *et al.*, 1997). Although PPARγ is known to be activated by certain foreign chemicals, including troglitazone and other thiazolidinediones used as insulin sensitizers for treatment of type II diabetes (Lehmann *et al.*, 1995), the potential of PPARγ for interaction with PPCs or other environmental agents has not been examined. Studies on the responsiveness of PPARγ to environmental peroxisome proliferators may help establish the potential of these chemicals to perturb physiological processes dependent on PPARγ, such as adipogenesis and hematopoiesis (Brun *et al.*, 1997; Tontonoz *et al.*, 1998), and thereby help identify potential human health risks associated with exposure to these agents.

Di(2-ethylhexyl)phthalate (DEHP) is an industrial plasticizer and PPC that is commonly used to coat polyvinylchloride surfaces of plastics used in medical devices (intravenous drip bags, blood storage bags, medical tubing) and food packaging, to make their surfaces both tougher and more pliable (Blass, 1992). DEHP and related plasticizers readily leach from plastic surfaces and evaporate into the environment, and are major environmental contaminants in water, food, and soil. While the pathological consequences of moderate levels of DEHP exposure in human populations are uncertain, DEHP is an established reproductive toxicant (Melnick *et al.*, 1987; Tyl *et al.*, 1988) and hepatocarcinogen (Huber *et al.*, 1996) in rodents. In contrast to wild-type mice, PPARα-deficient mice fed DEHP do not develop liver tumors, indicating that PPARα is an essential mediator of this hepatocarcinogenic response (Ward *et al.*, 1998). By contrast, the testicular and renal toxicities associated with exposure to DEHP and its metabolites (Albro *et al.*, 1989; Richburg and Boekelheide, 1996; Rothenbacher *et al.*, 1998) are maintained in PPARα-deficient mice (Ward *et al.*, 1998). This indicates that PPARα is not required for DEHP toxicity in extrahepatic tissues, and suggests that a distinct receptor protein, such as PPARγ, may mediate the observed testicular and kidney toxicity.

PPARγ has recently received much attention due to its involvement in the regulation of adipocyte differentiation and its importance in the development of obesity linked to noninsulin-dependent diabetes mellitus, which can be treated with the synthetic PPARγ ligand troglitazone (Brun *et al.*, 1997). The possibility that mammalian PPARγ might be activated by foreign chemical PPCs is suggested by the finding that avian PPARγ_1 can mediate PPC-induced peroxisome proliferation in the uropygial gland of the duck, whereas PPARγ_2, a splice variant that is expressed in adipose tissue, is primarily responsible for the regulation of duck adipocyte differentiation (Ma *et al.*, 1998). The present study was undertaken to assess the ability of environmental chemicals of the PPC class to *trans*-activate PPARα and PPARγ cloned from both mouse and human tissues, and to compare receptor activation between species in order to determine the extent to which there are intrinsic species-dependent differences in receptor sensitivity. The PPCs presently examined for PPARα and PPARγ activation are chemicals of specific interest to Superfund clean-up efforts (Fay and Mumtaz, 1996; Johnson and DeRosa, 1997), and include TCE, PCE, and their oxidized metabolites; the plasticizer DEHP and its metabolites; the industrial chemical PFOA; and the phenoxyacetic acid herbicides 2,4-D and MCPA.

MATERIALS AND METHODS

Chemicals. TCE, PCE, TCA, DCA, TCE-OH, CH, EOH, MCPA, 2,4-D, and PFOA, were purchased from Aldrich Chemical Co. (Milwaukee, WI). TCA (Fisher Scientific, Pittsburgh, PA), troglitazone (Rezulin; Parke-Davis Pharmaceuticals, Ann Arbor, MI), and nafenopin (Ciba-Giegy, Basel, Switzerland) were obtained from the sources indicated. Wy-14,643, DEHP, EHA, and DMSO were purchased from Sigma Chemical Co. (St. Louis, MO). MEHP was purchased from TCI America (Portland, OR).

Plasmids. The Firefly luciferase reporter plasmid pHD(x3)-Luc, which contains three copies of nts -2956 to -2919 of the rat enoyl-CoA hydratase/3-hydroxyacyl CoA gene PPRE cloned into pCPS-Luc (Marcus *et al.*, 1993), was obtained from Dr. J. Capone (McMaster University, Ontario, Canada). The mouse PPARα expression plasmid pCMV-PPARα (CMV promoter) (Muerhoff *et al.*, 1992) was provided by Dr. E. Johnson (Scripps Research Institute, La Jolla, CA) and the mouse PPARγ expression plasmid pSV-Sport1-PPARγ_1 (SV40 promoter) (Zhu *et al.*, 1993) was provided by Dr. J. Reddy (Northwestern University Medical School, Chicago, IL). The human PPARα expression plasmid pSG5-PPARα (SV40 promoter) (Sher *et al.*, 1993) was obtained from Dr. F. Gonzalez (National Cancer Institute, Bethesda, MD) and a human PPARγ1 expression plasmid, pSG5-PPARγ_1 (SV40 promoter) (Kliewer *et al.*, 1997) was provided by Dr. S. Kliewer (Glaxo Wellcome Inc., Research Triangle Park, NC). The Renilla luciferase reporter plasmids pRL-CMV and pRL-TK were purchased from Promega (Madison WI).

Cell culture and transient transfections. COS-1 cells (American Type Culture Collection, Rockville, MD) were passaged in 100-mm tissue culture dishes (Greiner Labortechnik, Germany) in DMEM supplemented with 10% FBS (Gibco, Grand Island, NY) and 50 U/ml penicillin/streptomycin (Gibco). Cells were cultured overnight at 37°C and then reseeded at 2000 to 4000 cells/well of a 96-well tissue culture plate (Greiner Labortechnik) in DMEM containing 10% FBS. The cells were grown for 24 h and then were transfected as described below, using FuGENE 6 transfection reagent (Boehringer–Mannheim, Germany), which gave higher transfection efficiencies and more consistent results than calcium phosphate transfection methods.

A mixture of plasmid DNAs to be transfected was prepared, based on the following amounts of plasmid DNA per tissue culture well: 1–3 ng PPAR expression plasmid, 50 ng pHD(x3)-Luc reporter plasmid, and either 4 ng pRL-TK or 0.25 ng pRL-CMV, used to normalize transfection efficiences between samples. Salmon sperm DNA (Stratagene Inc., La Jolla, CA) was added as a carrier DNA to give 250 ng of total DNA per well. Plasmid DNAs purified on Qiagen columns were dissolved in 1× TE buffer (10 mM Tris–EDTA, pH 8). FuGENE 6 stock reagent (30 μl) was diluted into 1 ml of DMEM (without serum) to give sufficient reagent for 100 transfections (i.e., one 96-well tissue culture plate). The diluted FuGENE 6 reagent was vortexed gently, incubated 5 min at room temperature, then added dropwise to each plasmid DNA mixture at a ratio of 10 μl diluted FuGENE 6 per ~1 μl containing 250 ng DNA. FuGENE 6-DNA mixtures were incubated for 15 min at room temperature before addition to cells (see below). FuGENE 6 was maintained at a slight excess over DNA (ratio of ~1.2 μl FuGENE 6 per μg total DNA) in all experiments.

To initiate transfection, 10 μl of the final FuGENE 6-DNA mix was added directly to cells growing in 100 μl of DMEM, 10% FBS in each well of a 96-well tissue culture plate without changing the culture media. After 24 h, the media was replaced by DMEM without serum and containing the PPC chemicals to be evaluated for PPAR activation. PPCs were prepared fresh on the day of use. TCA, DCA, and EHA were directly dissolved in DMEM, while the other PPCs studied were diluted from a 1000-fold stock in DMSO, except for TCE and PCE, which were diluted from a 1000-fold stock prepared in acetone. Wy-14,643 (20 μM for mouse PPARα and 40 μM for human PPARα) or nafenopin (5 μM) were used as positive controls for activation of PPARα, and troglitazone (3 μM) was used as a positive control for PPARγ_1 activation. Basal PPAR activity associated with vehicle controls is presented for each experiment (graphed as the first set of bars in each figure) and reflects receptor activation by endogenous ligands (e.g., cellular fatty acids) present in the COS-1 cells. Control experiments were carried out with each of the PPC test chemicals using COS-1 cells transfected with the pHD(x3)-Luc reporter plasmid alone in the absence of PPARα or PPARγ expression plasmid. No *trans*-activation was observed, indicating the absence of significant endogenous PPARα or PPARγ in these cells (data not shown). Following PPC treatment, cells were washed once in cold phosphate-buffered saline (pH 7.4), and then lysed by incubation at 4°C in passive cell lysis buffer for 15–30 min (Promega). Firefly and Renilla luciferase activities were measured in the cell lysate using the Dual Luciferase Activity Kit (Promega).

Data analysis. Luciferase activity values were normalized for transfection efficiency using Renilla luciferase activity values obtained from the same cell extract ("relative luciferase activity"), except as noted. Firefly luciferase activities are reported as $\times 10^{-3}$ values. Data are presented as means ± SD luciferase activities for $n = 3$ separate determinations. Experiments were generally repeated at least three times with similar results. Statistical significance relative to vehicle controls, shown in each figure, was assessed by Student's *t* test, calculated using Excel 4.0 software.

RESULTS

Mouse and Human PPARα are trans-Activated by Wy-14,643 with Distinct Dose Dependencies

To examine the differences in the sensitivity of human and mouse PPARα to *trans*-activation by peroxisome proliferators, COS-1 cells transfected with human or mouse PPARα expression plasmids and a PPRE-luciferase reporter were stimulated for 24 h with the prototypical peroxisome proliferator Wy-14,643, at concentrations ranging from 4 nM to 20 μM. Wy-14,643 *trans*-activated PPARα from both species (Fig. 1). Wy-14,643 maximally stimulated human PPARα and mouse PPARα to similar extents (~ five- to sixfold); however, lower

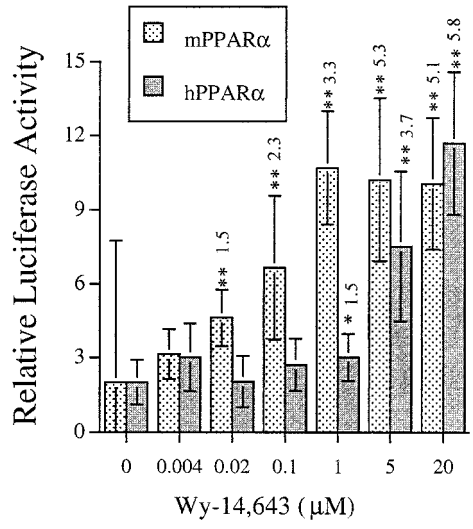

FIG. 1. Activation of mouse and human PPARα by Wy-14,643. COS-1 cells transfected with either mouse or human PPARα and the Firefly luciferase reporter plasmid pHD(x3)-Luc were treated for 24 h with increasing concentrations of Wy-14,643. Normalized luciferase reporter values shown are means ± SD, $n = 6$. Values of p compare vehicle-treated cells (DMSO vehicle alone) with cells treated with the PPAR activator Wy-14,643 (*$p < 0.05$, **$p < 0.005$). Fold-induction values are shown above the bar for each treatment that gave a significant increase in reporter activity.

concentrations of Wy-14,643 (1 μM) were required to saturate the response of mouse PPARα compared to human PPARα ($\geq$20 μM). Thus, there are intrinsic differences in the dose-responsiveness of human and mouse PPARα to Wy-14,643, a finding that is unlikely to be affected by potential differences in the absolute protein level of the two receptors achieved in the transfected COS-1 cells (see under Discussion). In control experiments performed in COS-1 cells transfected with pHD(x3)-Luc reporter in the absence of transfected PPAR receptor, no luciferase reporter activity was stimulated by Wy-14,643, or by any of the other PPARα or PPARγ_1 activators examined in this study (data not shown); thus, the responses to PPCs obtained in these studies are dependent on the transfected PPAR protein. An endogenous PPAR-like activity is present in COS-1 cells, and can be activated >20-fold by the PPAR activator 15-deoxy-δ-12,14-prostaglandin J2 (Zhou and Waxman, 1999). However, this endogenous PPAR-like activity is apparently unresponsive to Wy-14,643 or to the other PPCs investigated below.

Effect of TCE, PCE, and Their Metabolites on PPAR trans-Activation

Studies were undertaken to determine whether the peroxisome proliferative activity of TCE and PCE (Elcombe *et al.*, 1985; Goldsworthy and Popp, 1987) is mediated by the parent hydrocarbons or by one of their oxidative metabolites, and whether PPARα and PPARγ are differentially responsive to these PPCs. COS-1 cells were transiently transfected with

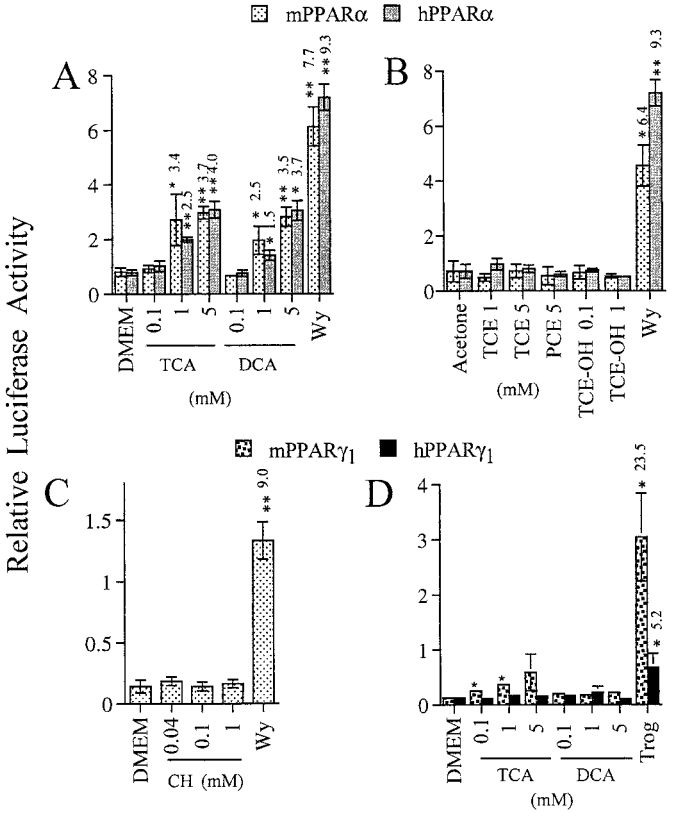

FIG. 2. Activation of PPARα and PPARγ$_1$ by TCE, PCE, and metabolites. COS-1 cells transfected as described in Fig. 1 with PPARα or PPARγ$_1$, mouse (m), or human (h), as indicated, were stimulated with the indicated concentrations (mM) of TCA or DCA (panels A, D); TCE, PCE, or TCE-OH (panel B); or CH (panel C), as described under Materials and Methods. Vehicle-treated cell values shown in the first pair of bars in each panel (DMEM, DMSO, or acetone) correspond to luciferase activities associated with endogenous COS-1 cell ligand(s). Normalized luciferase activities (relative activity values) are means ± SD values for $n = 3$ determinations. Wy: Wy-14,643, used as a positive control for PPARα activation (20 μM for mouse PPARα and 40 μM for human PPARα). Trog: troglitazone (3 μM), used as a positive control for PPARγ$_1$. Data presentation is as described in Fig. 1.

PPARα or PPARγ$_1$ expression plasmid together with PPRE reporter plasmid. Cells were treated for 24 h with TCE, PCE, or with the TCE and PCE metabolites (Davidson and Beliles, 1991; Miller and Guengerich, 1983) TCA, DCA, CH, or TCE-OH. Wy-14,643 was used as a positive control for PPARα activation and troglitazone was used as a positive control for PPARγ$_1$ activation in each experiment. Mouse and human PPARα were both activated by the acidic metabolites TCA and DCA (Fig. 2A), with no difference between species in terms of receptor sensitivity or maximal responsiveness (Table 1). The other metabolites tested, CH and TCE-OH, were inactive, as were the parent compounds TCE and PCE (Figs. 2B and 2C). In contrast to PPARα, PPARγ$_1$ displayed little or no responsiveness to TCA or DCA (Fig. 2D). TCA and DCA were toxic to the cells at ≥7 mM, precluding studies at higher concentrations.

Activation of PPARα and PPARγ by DEHP and Its Metabolites

We next examined whether PPARα or PPARγ$_1$ could be activated in the COS-1 cell transfection system by DEHP or its primary hydrolysis products (Albro and Lavenhar, 1989), MEHP and EOH. DEHP did not activate either PPARα or PPARγ$_1$ when tested at concentrations up to 2 mM (Figs. 3A and 3B). By contrast, MEHP activated both mouse and human PPARα in a process that was saturated at ~20 μM for receptor from both species (three- to fourfold activation; Fig. 3C). Interestingly, MEHP also activated PPARγ$_1$, both mouse and human, in a manner similar to PPARα (Fig. 3D). This responsiveness of PPARγ$_1$ to MEHP contrasts to this receptor's unresponsivness to the other PPCs examined earlier, Wy-14,643, TCA, and DCA. The *trans*-activation of PPARγ by MEHP was also seen in experiments using COS-1 cells transfected with an expression plasmid for hPPARγ$_2$ (data not shown), a splicing variant that differs from hPPARγ$_1$ by the presence of a 28–amino acid extension at its NH_2-terminus (Zhu *et al.*, 1995).

EHA is an acidic oxidation product of EOH, and thus is a secondary metabolite of DEHP (Albro and Lavenhar, 1989). EHA activated PPARα, but this required somewhat higher concentrations than MEHP for maximal receptor activation (Fig. 4A). By contrast, EHA did not activate either mouse or human PPARγ$_1$ (Fig. 4C). EOH did not activate either PPARα (Fig. 4B) or PPARγ1 (Fig. 4D).

Effect of PFOA on PPARα and PPARγ$_1$ Activity

The effects of the peroxisome proliferator and industrial chemical PFOA (Ikeda *et al.*, 1985; Sohlenius *et al.*, 1992) on PPARα and PPARγ$_1$ *trans*-activation were evaluated using the COS-1 cell transfection assay. PFOA maximally activated mouse PPARα at 5–10 μM, while human PPARα required somewhat higher PFOA concentrations for maximal activation (Fig. 5A). By contrast, mouse and human PPARγ$_1$ were unresponsive to PFOA when tested at a range of 0.5 to 40 μM (Fig. 5B). Toxicity of PFOA to the COS-1 cells precluded studies at higher concentrations.

Phenoxyacetic Acid Herbicides Do Not Directly Activate PPAR

To study the effects of the phenoxyacetic acid herbicides 2,4-D and MCPA on PPAR activation, COS-1 cells transfected with PPARα or PPARγ$_1$ plus the reporter plasmid pHD(x3)-Luc were treated with 2,4-D or MCPA. 2,4-D did not significantly *trans*-activate PPARα or PPARγ$_1$ from either species when tested at concentrations up to 800 μM (Figs. 6A and 6C). Similarly, MCPA stimulated little or no increase (≤ twofold) in PPARα or PPARγ$_1$ activity at concentrations up to 400 μM (Figs. 6B and 6D).

TABLE 1
Activation of Mouse and Human PPARα and PPARγ_1 by TCA and DCA

	mPPARα		hPPARα		mPPARγ_1	
Treatment[a]	Fold-activation	$p < 0.05$	Fold-activation	$p < 0.05$	Fold-activation	$p < 0.05$
Wy or Troglitazone	7.7	0.004	9.3	0.001	23.5	0.01
TCA						
0.1 mM	1.1	NS[b]	1.3	NS	2.0	0.04
1.0 mM	3.4	0.04	2.5	0.003	2.8	0.01
5.0 mM	3.7	0.006	3.7	0.001	4.5	NS
DCA						
0.1 mM	1	NS	1	NS	1.5	NS
1.0 mM	2.5	0.02	1.5	0.013	1.4	NS
5.0 mM	3.5	0.008	3.7	0.014	1.7	NS

[a] COS-1 cells transfected with the indicated PPARs were treated for 24 h with increasing concentrations of TCA and DCA. Fold-increases in reporter gene *trans*-activation activity compared with control cells (DMSO treatment only) are shown. Student's *t* tests were calculated to assess statistical significance ($p < 0.05$). Data are based on comparisons of mean ± SD values for $n = 3$ determinations. Wy-14,643 ('Wy') and troglitazone were used as positive controls for PPARα and PPARγ, respectively.

[b] NS = not significant.

DISCUSSION

A COS-1 cell-based transient *trans*-activation assay was used to characterize the activation of mouse and human PPARs by various PPCs, including several chlorinated hydrocarbons and other environmental chemicals of specific interest to Superfund clean-up efforts. COS-1 cells are suitable for these studies because they have little or no endogenous PPARα or PPARγ activity, although they exhibit a PPAR-like activity when stimulated with the prostaglandin metabolite 15-deoxy-δ-12,14-prostaglandin J2 (Zhou and Waxman, 1999). These studies were undertaken with the following three objectives: (1) to ascertain which PPCs directly activate PPARα vs which compounds may require further metabolism for their action; (2) to compare the responsiveness of PPARα cloned from human liver, which exhibits poor responses to PPCs, to that of the corresponding PPARα form cloned from mouse, a highly responsive species; and (3) to determine whether environmental PPCs can also activate PPARγ, which is expressed at high levels in a broad range of human tissues. Activation of these receptors was monitored using the luciferase reporter gene pHD(x3)-Luc, which incorporates three tandem copies of a strong PPAR response element derived from the 5′-flank of the rat hydratase/dehydrogenase promoter, and enabled us to detect reporter gene activity with high sensitivity using a luminescence assay.

Potential limitations that need to be considered when interpreting the findings presented in this study include the following: (1) Activation of the luciferase reporter gene involves a series of events subsequent to the initial PPAR ligand binding event. These include receptor activation and DNA binding, recruitment of coactivators, reporter gene transcription, and translation of luciferase mRNA. Accordingly, while a good correlation between receptor binding and luciferase activity measurements is generally observed in these types of studies, caution should be used in viewing the PPAR activation data presented in this report as comparative ligand binding data *per se*. (2) DNA sequences flanking a core PPAR response element can have a significant effect on the level and the PPAR form-specificity of the transcriptional response (Juge-Aubry *et al.*, 1997; Palmer *et al.*, 1995). Accordingly, it is possible that differing results would be obtained using PPREs derived from other PPAR-activated promoters. (3) Differences in PPAR expression plasmid and in the mRNA and protein stability of the fours PPARs examined in this study (PPARα and PPARγ_1 from both mouse and human) could result in different levels of each PPAR protein in the transfected COS-1 cells. Direct comparison of expression levels of the four PPARs was not possible, due to the lack of purified PPAR standards or Western blotting antibodies that are reactive with the PPARs in a species- and PPAR form-independent manner. This is not considered a significant limitation, however, because the data presented do not compare absolute receptor activities. Rather, the present studies compare the intrinsic dose-responsiveness to PPCs of each PPAR, a property that is expected to be insensitive to moderate changes in receptor protein levels.

Role of Metabolism in PPAR Activation by PPCs

In the case of three important environmental PPCs investigated in this study, TCE, PCE, and DEHP, PPARα was found to be activated by metabolites, but not by the parent foreign chemical. Two other environmental chemicals with peroxisome proliferative activity *in vivo*, the chlorinated phenoxyacetic acid herbicides 2,4-D and MCPA, were found to be inactive in the COS-1 cell-based PPAR activation assay, suggesting that metabolism may be required to activate these PPCs as well. Metabolites of 2,4-D include the glucuronide, glycine,

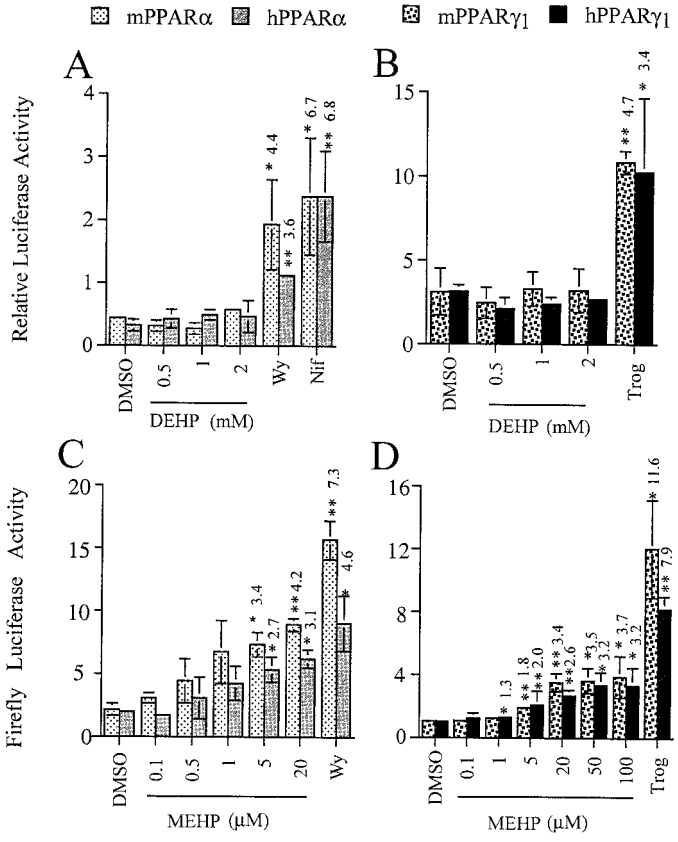

FIG. 3. Effect of DEHP and MEHP on PPARα and PPARγ_1 transcriptional activity. COS-1 cells transfected with the indicated PPARs and the reporter pHD(x3)-Luc were treated for 24 h with increasing concentrations of DEHP (mM) or MEHP (μM). Shown are normalized luciferase activites or Firefly luciferase activities, as indicated (means $\pm$ SD, $n = 3$). Nif: nafenopin (5 μM), used as a positive control for PPARα activation. Wy-14,643 and troglitazone concentrations were as specified in Fig. 2. Data presentation is as described in Figs. 1 and 2.

and taurine conjugates (Griffin *et al.*, 1997). Our finding that metabolism is required for activation of at least some PPCs highlights the potential significance of interindividual differences in PPC metabolic activity in the individual responses to PPCs. 2,4-D is a weak carcinogenic peroxisome proliferator in rodents, where it can promote renal cytotoxicity and neurosystemic defects (Mattsson *et al.*, 1997). MCPA is a rodent peroxisome proliferator that has been associated with increased risk to non-Hodgkin lymphoma and soft-tissue sarcomas in humans (Hardell and Eriksson, 1999; Lynge, 1998). Further investigation is required to delineate the role of PPAR in the toxicities associated with human exposure to these latter PPCs.

Species Differences in PPAR-Responsiveness for Some but Not All PPCs

Analysis of the PPC-responsiveness of human and mouse PPARα revealed that human PPARα is less sensitive than its mouse counterpart to some (Wy-14,643, PFOA) but not other foreign chemical PPCs (TCA, DCA, MEHP, EHA). In one earlier report, intrinsic differences between mouse and human PPARα receptor activation by Wy-14,643 were not seen (Kliewer *et al.*, 1994); however, in another study, rat PPARα was found to be more responsive to Wy-14,643 than human PPARα (Mukherjee *et al.*, 1994), in accord with our findings with mouse and human PPARα. This differential sensitivity of human PPARα to these PPCs cannot alone account for the greatly reduced peroxisome proliferative responses seen in human compared to rodent liver cells, and other factors, such as the much lower level of PPARα expression in human compared to rodent liver (Palmer *et al.*, 1998; Tugwood *et al.*, 1997), are also likely to be important.

The acidic TCE metabolites TCA and DCA both activated human and mouse PPARα, but not PPARγ_1, with similar dose dependencies seen for PPARα from each species. The relatively high (mM) concentrations required for PPARα activation by these compounds (cf., requirement for μM concentrations of MEHP and PFOA for receptor activation) is consistent

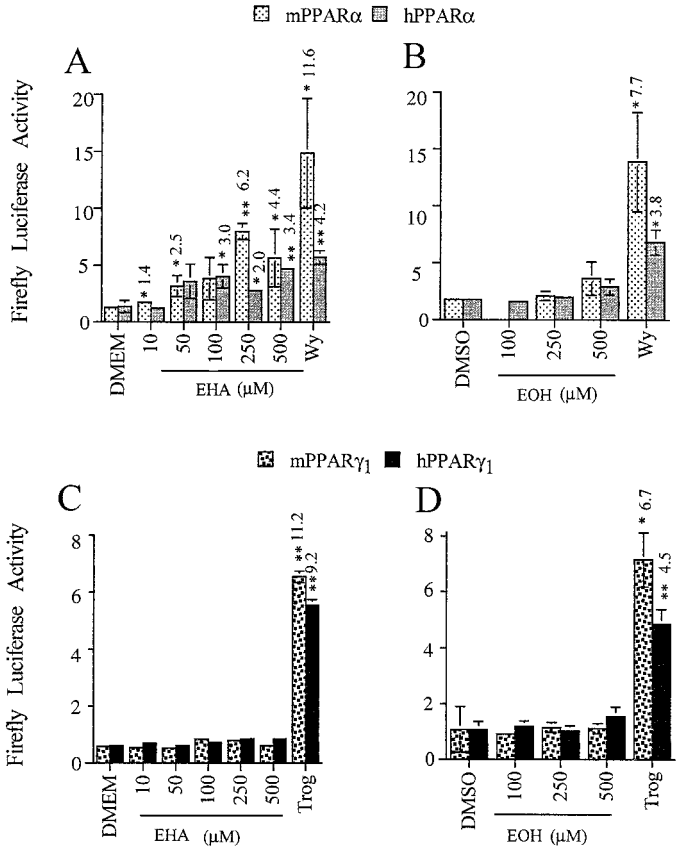

FIG. 4. Effect of DEHP metabolites, EHA and EOH, on PPARα and PPARγ_1 activity. Transfection of COS-1 cells; stimulation with the indicated μM concentrations of EHA, EOH, or vehicle control (DMEM or DMSO) for 24 h; reporter gene assays; and data presentation were as described in Figs. 1 and 2. Wy-14,643 was used as a positive control for PPARα activation (20 μM for mouse and 40 μM for human receptor) and troglitazone (3 μM) as a positive control for PPARγ.

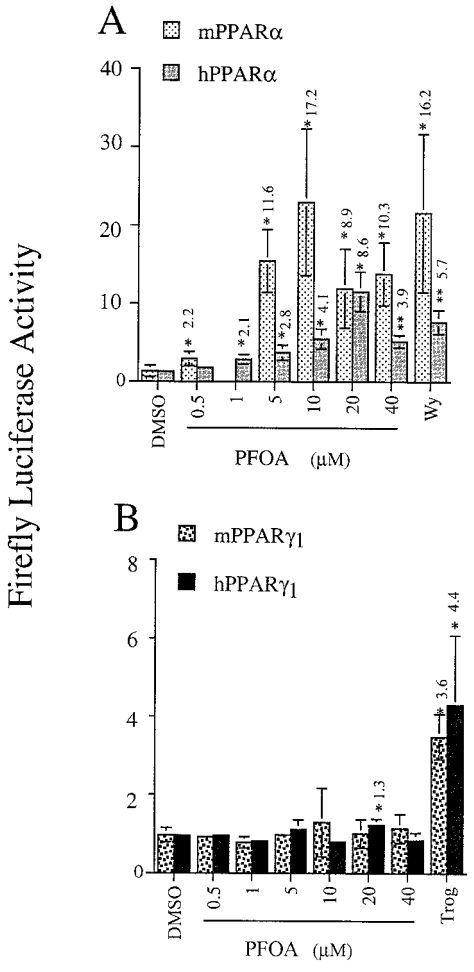

FIG. 5. Effect of PFOA on PPARα (panel A) and PPARγ_1 (panel B) activity. Transfection of COS-1 cells, stimulation with the indicated μM concentrations of PFOA for 24 h, reporter gene assays, and data presentation were as described in Figs. 1 and 2. Wy-14,643 and troglitazone positive controls were as specified in Fig. 4.

with the relatively weak peroxisome proliferative activity reported for TCE and PCE *in vivo* (Stott, 1988). The finding that human PPARα exhibits a similar sensitivity as mouse PPARα to activation by TCA and DCA contrasts with the decreased sensitivity of the human receptor to Wy-14,643, discussed earlier. Further studies are required to determine the structural basis for the species differences that characterize PPARα with respect to some, but not other, PPCs.

Activation of PPAR by DEHP and Its Metabolites

The plasticizer DEHP did not activate either PPARα or PPARγ_1 in our COS-1 cell transfection studies. This observation is consistent with earlier reports that metabolites of DEHP mediate the toxic actions of this plasticizer *in vivo* (Richburg and Boekelheide, 1996; Rothenbacher *et al.*, 1998; Sjoberg, 1986). DEHP is hydrolyzed in the liver by nonspecific esterases to yield phthalic acid, MEHP, and EOH; this latter metabolite is rapidly oxidized to EHA (Albro and Lavenhar, 1989). EOH is a mild dermal, respiratory, and gastrointestinal irritant in rats and rabbits, and may be involved in the formation of liver tumors in mice (Astill *et al.*, 1996). The secondary DEHP metabolite EHA is reported to be a more potent rodent peroxisome proliferator than EOH (Cornu *et al.*, 1992; Keith *et al.*, 1992), in agreement with our observation that EHA and MEHP, but not EOH, can activate PPARα. In the case of PPARγ_1, however, MEHP, but not EHA or EOH, stimulated receptor activity. Both human and mouse PPARγ_1 were activated by MEHP at concentrations as low as 5 μM. Our finding that MEHP and EHA can *trans*-activate PPARα is consistent with the finding that PPARα knock-out mice exposed to DEHP do not develop liver tumors (Ward *et al.*, 1998). However, those same mice were susceptible to DEHP-induced testicular and renal toxicities, which are thus independent of PPARα. In this regard, the *trans*-activation of PPARγ by MEHP characterized in the present study raises the possibility that PPARγ could be responsible for some of the testicular and renal toxicities associated with DEHP exposure. Further investiga-

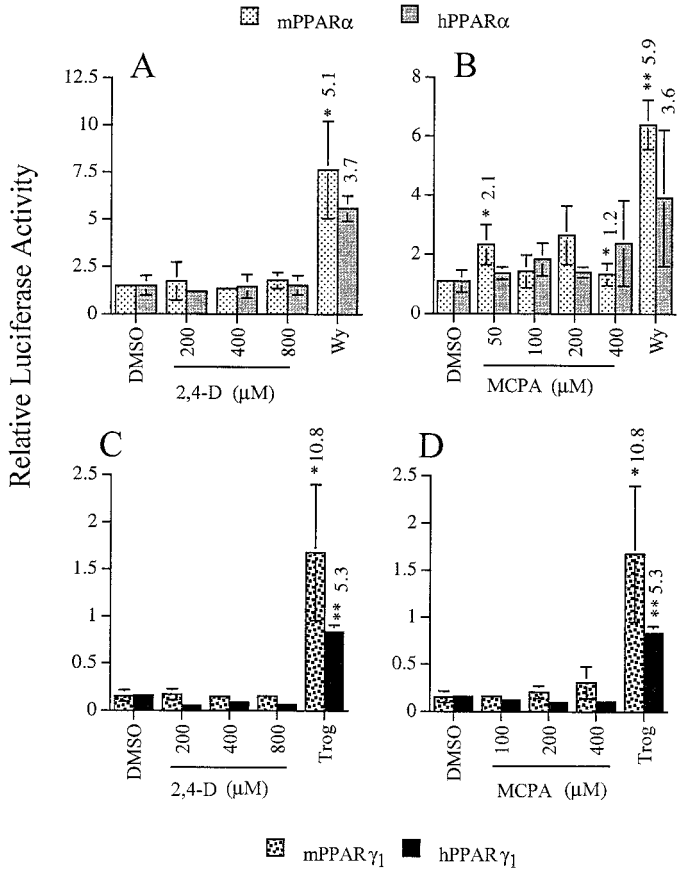

FIG. 6. Effect of the phenoxyacetic acid herbicides 2,4-D and MCPA on the activation of PPARα (panels A, B) and PPARγ_1 (panels C, D). COS-1 cell transfection, stimulation using the indicated μM concentrations of 2,4-D or MCPA, reporter gene assays, and data presentation were as described in Figs. 1 and 2. Wy-14,643 and troglitazone positive controls were as specified in Fig. 4.

tion, including evaluation of mice selectively deficient in PPARγ expression in these tissues, could help clarify this point.

In conclusion, we report that human PPARα is less responsive than mouse PPARα to some, but not all, PPCs. Thus, significant differences in responsiveness to peroxisome proliferators and industrial chemicals exist between species, as well as between the isoforms PPARα and PPARγ$_1$. TCE, PCE, and DEHP each require metabolism to exert their PPAR-dependent effects. A similar requirement for metabolism may characterize other PPCs, including the phenoxyacetic acid herbicides 2,4-D and MCPA, which did not *trans*-activate mouse or human PPARs, despite their established peroxisome proliferative activity *in vivo*. Finally, the phthalate MEHP was shown to activate PPARγ, a receptor form that is highly expressed in select extrahepatic human tissues, including adipose tissue, where lipophilic foreign chemicals tend to accumulate. This latter finding raises the distinct possibility that human PPARγ might be an important, heretofore unrecognized human target of foreign chemical PPCs. Further investigation, including animal studies that directly examine the susceptibility of PPARγ target genes in various tissues and species to MEHP and other PPCs, will be required to provide a full understanding of the role of this receptor in the pathophysiological and toxicological responses to PPCs.

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