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Activation of Mouse and Human Peroxisome Proliferator-Activated Receptors (PPARs) by Phthalate Monoesters

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Administration of phthalates is known to cause toxicity and liver cancer in rodents through the activation of peroxisome proliferator-activated receptors (PPARs), and the monoesters appear to be the active metabolites that function as ligands of PPARs. There is evidence that PPARs exhibit significant species differences in response to ligand activation. In this study, the activation of mouse and human PPARα, PPARβ, and PPARγ by a broad class of phthalate monoesters was investigated using a trans-activation assay, functional analysis of PPARα target gene expression, and a PPARγ-mediated differentiation assay. These studies demonstrated a range in the ability of various phthalate monoesters to activate PPARα, with the mouse PPARα generally being activated at lower concentrations and exhibiting a greater response than human PPARα. Similarly, a range in the trans-activation of mouse PPARβ by phthalate monoesters was also observed, but this effect was not found with human PPARβ. A number of phthalate monoesters activated both mouse and human PPARγ, with similar sensitivity being exhibited by both receptors. These studies show that the potency and efficacy of phthalate monoesters for the activation of PPARα and PPARγ increase with increasing side-chain length. These studies also show that mouse PPARα and PPARβ are generally activated at lower concentrations of phthalate monoesters than human PPARα and PPARβ, and that both mouse and human PPARγ exhibit similar sensitivity to phthalate monoesters. Lastly, there is a good relationship between the relative ability of phthalate monoesters to trans-activate PPARα and PPARγ, and the relative induction of PPARα target gene mRNA and PPARγ-mediated adipocyte differentiation, respectively.

Key Words: **peroxisome proliferator-activated receptors (PPARs); phthalate monoesters.**

Phthalate diesters are chemicals that are widely used, primarily as plasticizers in the manufacture of flexible vinyl products including medical devices as well as in some nonvinyl products including cosmetics (Blass, 1992). Phthalate diesters can leach from vinyl products and enter the surrounding environment. Recent examination of urinary levels of phthalate monoesters in a United States reference population indicated human exposure to several phthalate monoesters (Blount *et al*., 2000; Kato *et al*., 2004; Silva *et al*., 2004). The phthalate monoesters with the highest urinary levels were monoethyl phthalate (MEP; 9,463 ppb), monobenzl phthalate (MBenP; 464 ppb), and monobutyl phthalate (MButP; 306 ppb). Human exposure to phthalates is due primarily to ingestion of food contaminated from environmental sources (Kavlock *et al*., 2002a,b,c), although inhalation of airborne or dust-associated phthalates may also occur (Adibi *et al*., 2003). Dermal contact with products containing phthalates can also contribute to exposure, although high-molecular-weight phthalates are very poorly absorbed (Elsisi *et al*., 1989). Once ingested or otherwise absorbed, phthalate diesters are hydrolyzed to the corresponding monoesters by esterases in the liver and blood and by pancreatic lipases in the gastrointestinal tract (Huber *et al*., 1996).

Many of the adverse effects, including hepatocarcinogenesis, induced by monoesters of high-molecular-weight phthalates (e.g., $\geq$C6 side-chains) in rodents are thought to be mediated by peroxisome proliferator-activated receptors (PPARs) (Doull *et al*., 1999). PPARs are members of the nuclear receptor superfamily and consist of three isoforms, namely PPARα, PPARβ(δ), and PPARγ (Shearer and Hoekstra, 2003). In response to ligand activation, PPARs heterodimerize with retinoid-X-receptor-α (RXRα), interact with co-activators and peroxisome proliferator-response elements (PPREs) found in the promoter region of target genes, and modulate expression of target genes (Shearer and Hoekstra, 2003). Specific ligands have been identified for all three PPARs. For example, a broad class of chemicals collectively referred to as peroxisome proliferators (e.g., the fibrate class of hypolipidemic drugs, endogenous and dietary fatty acids, herbicides and phthalate monoesters), can all bind to, or specifically activate, PPARα (Berger and Moller, 2002). Each PPAR participates in regulating biological functions through modulation of specific target genes.

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PPARα regulates target genes that modulate fatty acid degradation, PPARγ regulates target genes that modulate glucose homeostasis, and PPARβ may regulate fatty acid metabolism in skeletal muscle (Berger and Moller, 2002; Fredenrich and Grimaldi, 2004; Wang *et al*., 2003). There is also evidence that all three PPARs modulate carcinogenesis. PPARα is required to mediate hepatocarcinogenesis induced by peroxisome proliferators in rodents, and activation of PPARγ has been shown to be anti-carcinogenic in a number of model systems, but the role of PPARβ in carcinogenesis is unclear (Gupta *et al*., 2004; Harman *et al*., 2004; Michalik *et al*., 2004; Stephen *et al*., 2004).

Previous work by others has shown that several phthalate monoesters (MEHP, MBenP, MButP) activate PPARα and PPARγ, and that lower concentrations are required for activation of mouse PPARα than human PPARα (Hurst and Waxman, 2003; Maloney and Waxman, 1999). The purpose of the present studies was three-fold: (1) to examine the ability of a broader class of phthalate monoesters and related substances of varying side-chain length and structures, to activate all three PPARs, (2) to determine if there is a species difference in receptor activation for this broader class of phthalate monoesters, and (3) to compare receptor activation observed in a trans-activation assay with PPAR-mediated biological changes, or PPAR-mediated alterations in target gene expression. It is important to point out that the broad class of monoesters examined for these studies represents the majority of commercially important phthalates.

MATERIALS AND METHODS

Chemicals. Mono-ethyl phthalate (MEP), mono-benzyl phthalate (MBenP), mono-isoheptyl phthalate (MIHP), mono-isononyl phthalate (MINP), mono-2-ethylhexyl adipate (MEHA), mono-isohexyl phthalate (MIHP2), mono-2-ethylhexyl phthalate (MEHP), mono-isodecyl phthalate (MIDP), and mono-n-octyl phthalate (MnOP) were synthesized by ChemSyn Laboratories (Lenexa, KS) and kindly provided by the Phthalate Esters Panel, American Chemistry Council. MButP was synthesized by Chem Service (West Chester, PA) and kindly provided by Dr. Kevin Gaido. Structures of the phthalate monoesters used for these studies are shown in Figure 1. Wy-14,643 (4-chloro-6-(2,3-xylidino)-2-pyrimidinythiol acetic acid) was purchased from ChemSyn Laboratories (Lenexa, KS). Troglitazone (Trog) was kindly provided by Dr. Takashi Yamoto (Sankyo, Shigouka, Japan). Dimethyl sulfoxide (DMSO) and tetradecylthioacetic acid (TTA) were purchased from Sigma Chemical Co. (St. Louis, MO).

Plasmids. The ligand-binding domain of mouse or human PPARα, PPARβ, or PPARγ was fused to the DNA-binding domain of the yeast transcription factor Gal4 under the control of the SV40 promoter. This plasmid also encoded the UAS-firefly luciferase reporter under the control of the Gal4 DNA response element.

Cell culture and trans-activation assay. 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, St Louis, MO), 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 culture medium. Following overnight culture, the transfected 3T3-L1 cells containing chimeric mouse or human PPARα-LBD/Gal4-DBD-(PPARα–ligand binding domain/Gal4–DNA binding domain), PPARβ-LBD/Gal4-DBD-, or PPARγ-LBD/Gal4-DBD-Gal4 luciferase reporter plasmids were split to multiwell cluster plates. The media was replaced, 4 h after replating, with serum-free DMEM containing phthalate monoesters at concentrations of 3, 10, 30, 100, or 200 μM to evaluate PPAR activation. Solutions of phthalate monoesters and positive controls were prepared fresh on the day the cells were treated. Wy-14,643 (25 μM) was used as a positive control for the activation of PPARα, tetradecylthioacetic

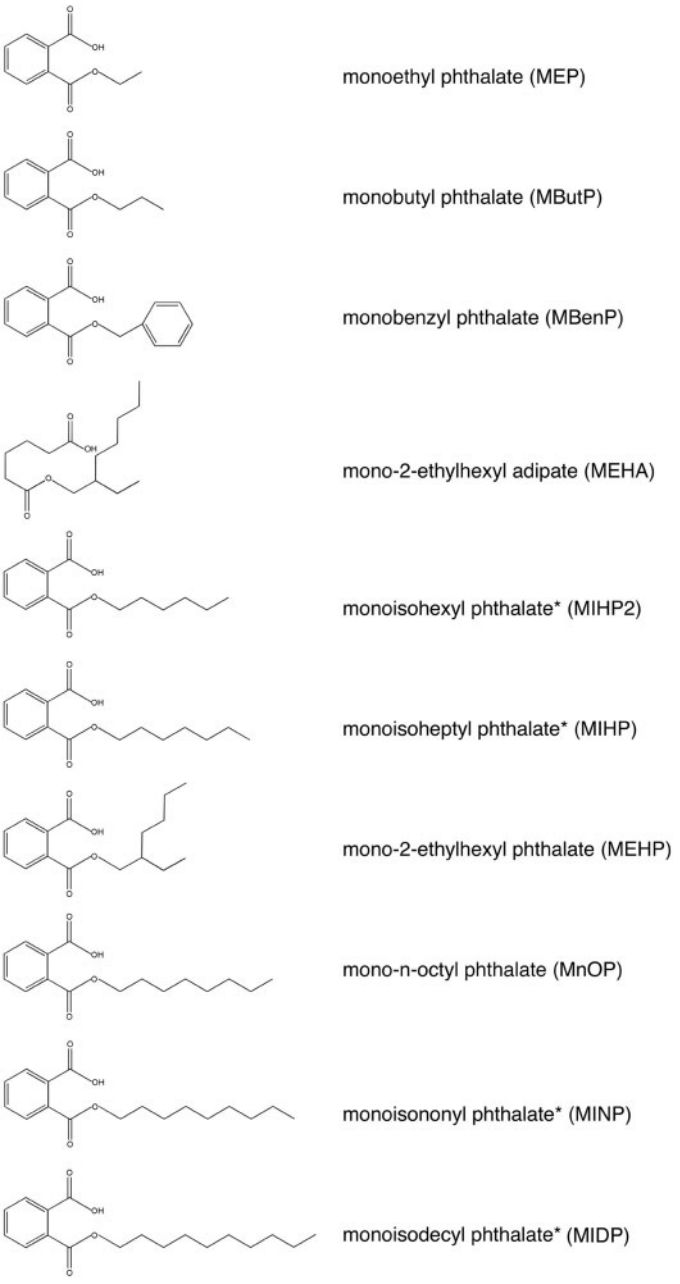

FIG. 1. Chemical structures of the phthalate monoesters tested for their ability to activate mouse and human PPARs. *The alkyl side-chains of these monoesters are isomeric with methyl branching.

acid (50 μM) was used as a positive control for PPARβ activation, and troglitazone (3 μM) was used as a positive control for PPARγ activation. Twenty-four h after the treatment of the transfected 3T3-L1 cells with phthalate monoesters, the cells were lysed at −20°C with passive lysis buffer (Promega, Madison, WI) for 30 min; luciferase activity was measured using the Luciferase reporter assay kit (Promega, Madison, WI) and a Turner TD-20/20 Luminometer (Turner BioSystems, Sunnyvale, CA) and manufacturer's recommended procedures. The protein concentration of the cell lysate was determined using the BCA protein assay kit (Pierce, Rockford, Illinois). Luciferase activity was normalized to the protein concentration of each sample. The fold induction of normalized luciferase activity was calculated relative to DMSO (vehicle)-treated cells, and represents the mean of three independent samples per treatment group.

Cell culture and Northern blot analysis of PPARα-dependent target mRNA induction in hepatoma cell lines. Rat hepatoma FaO cells were cultured in DMEM supplemented with 5% FBS, 100 units/ml of penicillin, and 100 μg/ml of streptomycin. Human hepatoma HepG2 cells were cultured in alpha Minimal Essential Medium (αMEM) supplemented with 5% FBS, 100 units/ml of penicillin, and 100 μg/ml of streptomycin. FaO and HepG2 cells were seeded in 6-well plates at 7×10^5 cells/well and treated for 48 h with either phthalate monoesters, DMSO (vehicle), or Wy-14,643 (100 μM). The test concentrations of phthalate monoesters were either 10 or 100 μM and corresponded to the lower or upper ends of the respective dose response curves obtained from the transactivation studies for those phthalate monoesters with significant PPARα activity. These concentrations were also used for phthalate monoesters that did not exhibit significant PPARα activity for comparative purposes. After treatment with the indicated phthalate monoester, total RNA was isolated using TRIZOL reagent (Invitrogen, Carlsbad, CA) and following the manufacturers recommended procedures. Five micrograms of total RNA were electrophoresed on 0.22 M formaldehyde denaturing agarose gel, transferred to a nylon membrane, and fixed using UV cross-linking. Membranes were hybridized in ULTRAhyb hybridization buffer (Ambion, Austin, TX) with random primed ^{32}P-labeled probes for known PPARα target genes including acyl-CoA oxidase (ACOX) and cytochrome P450 4A (CYP4A) or GAPDH (loading control) generated from the respective cDNA using Ready-To-Go DNA Labeling Beads (Amersham Biosciences, Piscataway, NJ) and following manufacturer's recommended procedures. Hybridization signals were obtained after scanning with a phosphorimager and were normalized relative to GAPDH. The fold induction of normalized ACOX and CYP4A mRNAs in both FaO and HepG2 cells were calculated relative to DMSO (vehicle)-treated cells and represent the mean of two independent samples per treatment group.

Cell culture and 3T3-L1 cell differentiation assay. Mouse 3T3-L1 fibroblasts were cultured in DMEM containing 10% FBS at 37°C/5% CO_2. The fibroblasts were later trypsinized and seeded at approximately 50% confluence in 12-well plates. Adipogenesis was induced 48 h postconfluence, using a standard differentiation assay (Green and Meuth, 1974). This consists of changing the culture medium to DMEM/4% FBS containing 1.0 μg/ml insulin, 1 μM dexamethasone, 100 μM isomethylbutylxanthine, and troglitazone (10 μM) or the indicated phthalate monoester and monitoring subsequent lipid accumulation. Four phthalate monoesters that had significant PPARγ activity based on the transactivation studies were used, and two phthalate monoesters that had no significant PPARγ activity were used for comparison. The media was replaced with DMEM/4% FBS containing 1.0 μg/ml insulin and troglitazone (10 μM), or the indicated phthalate monoester, every 48 h after the initiation of differentiation. The 3T3-L1 cells were fixed with formalin and stained with Oil Red O (Sigma Chemical Co, St. Louis, MO) 6 days after initiation of adipocyte differentiation. Briefly, cells were washed twice with phosphate buffered saline (PBS) and then fixed with formalin for 1 h at room temperature. The fixed cells were stained with Oil Red O (0.3 %) for 1 h. Cells were washed three times with water, visualized with a Nikon Eclipse microscope, and photographed.

Statistical analysis. Differences between treatments were determined using ANOVA followed by Dunnett's post hoc test (Prism 4.0, GraphPad Software, Inc., San Diego, CA). Significant differences were determined when $p \leq 0.05$.

RESULTS

Activation of Mouse and Human PPARα, PPARβ, and PPARγ by Phthalate Monoesters

Phthalate monoesters of varying side-chain length and different structures were investigated for their ability to activate mouse and human PPARs. MEP, a C2 alkyl side-chain phthalate monoester (Fig. 1), did not consistently activate either mouse or human PPARα (Fig. 2). MButP, a C3 alkyl side-chain phthalate monoester (Fig. 1), activated mouse PPARα at a concentration ≥100 μM, and human PPARα at a concentration of 200 μM (Fig. 2). MBenP, which has a benzyl side-chain (Fig. 1), caused increased luciferase activity with concentrations ≥100 μM for mouse PPARα and with a concentration of 200 μM for human PPARα (Fig. 2). MEHA, an adipic acid monoester of di-2-ethylhexyl adipate (DEHA; Fig. 1), caused increased luciferase activity with concentrations ≥100 μM for mouse PPARα (Fig. 2). However, while a significant increase in luciferase activity was observed with a concentration of 100 μM MEHA with human PPARα, no difference in activity was observed with 200 μM MEHA for human PPARα (Fig. 2). MIHP2, a branched C6 (isohexyl) alcohol side-chain phthalate monoester (Fig. 1), caused a dose-dependent increase in luciferase activity with concentrations of ≥30 μM for mouse PPARα, in contrast to human PPARα, where an increase in luciferase activity was only observed with a concentration of 200 μM (Fig. 2). MEHP, the active metabolite of the plasticizer di-2-ethylhexyl phthalate (DEHP), and MIHP, a branched C7 (isoheptyl) alcohol side-chain phthalate monoester (Fig. 1), caused a dose-dependent increase in luciferase activity with concentrations of ≥10 μM for mouse PPARα, and ≥30 μM for human PPARα (Fig. 2). A dose-dependent increase in luciferase activity was observed for MnOP, a linear C8 alkyl side-chain phthalate monoester (Fig. 1), with concentrations of ≥10 μM for both mouse and human PPARα (Fig. 1). MINP, a branched C9 (isononyl) alcohol side-chain phthalate monoester (Fig. 1), caused a dose-dependent increase in luciferase activity with concentrations of ≥3 μM for mouse PPARα, while a dose-dependent increase in luciferase activity was only observed at concentrations ≥10 μM for human PPARα (Fig. 2). MIDP, a branched C10 (isodecyl) alcohol side-chain phthalate monoester (Fig. 1), caused a dose-dependent increase in luciferase activity with concentrations of ≥3 μM for mouse PPARα (Fig. 1), while a concentration of ≥30 μM was required to elicit a dose response for human PPARα (Fig. 2). In general, lower concentrations of phthalate monoesters were required to activate mouse PPARα as compared to human PPARα, and the magnitude of the response was significantly greater for mouse PPARα than for human PPARα (Table 1).

Phthalate monoesters did not activate human PPARβ, although mouse PPARβ was responsive to some of these chemicals. Similar to results observed for PPARα, MEP was ineffective at activating mouse PPARβ-dependent luciferase

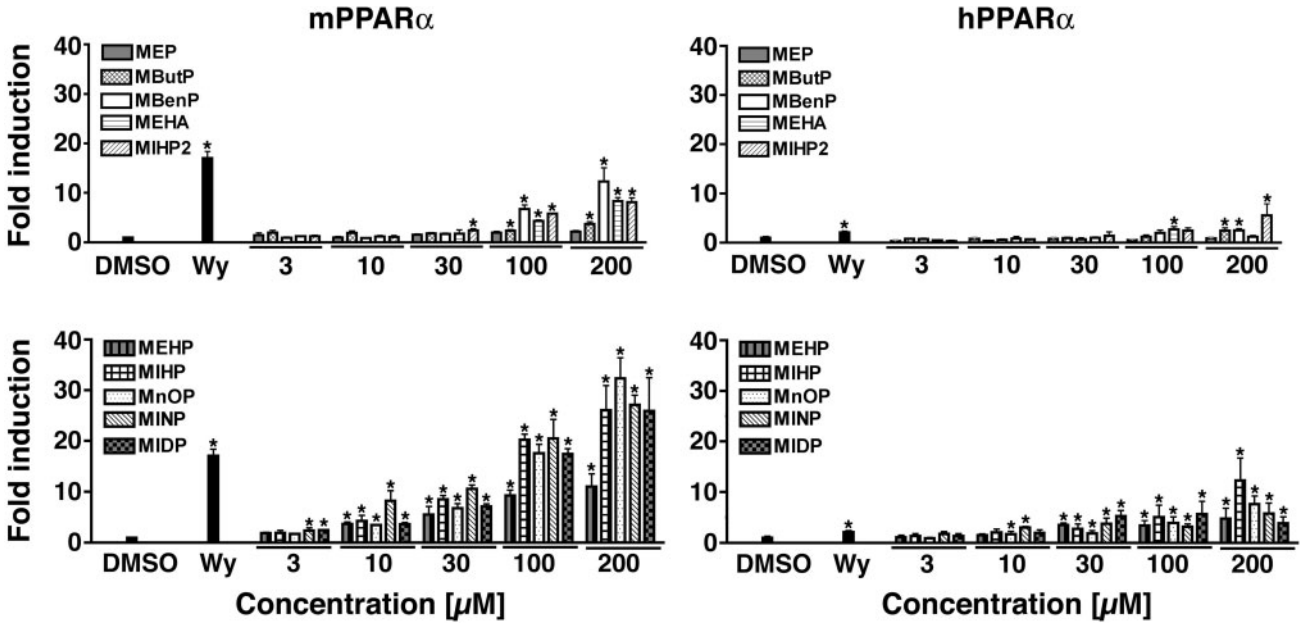

FIG. 2. Activation of mouse PPARα (mPPARα), and human PPARα (hPPARα) by phthalate monoesters. Transfected 3T3-L1 cells containing either the mouse or human PPARα-LBD/Gal4-DBD-Gal4-luciferase reporter plasmid were treated with the indicated phthalate monoester at a concentration of either 3, 10, 30, 100, or 200 μM for 24 h. Wy-14,643 (Wy) at a concentration of 25 μM was used as a positive control. All luciferase activity values were normalized to the amount of protein in each cell lysate. The fold induction of normalized luciferase activity was calculated relative to DMSO (vehicle)-treated cells and represents the mean of three independent samples per treatment group; *p*-values were calculated using ANOVA followed by Dunnett's post hoc. Significant differences determined when $p \leq 0.05$.

TABLE 1
Comparative Summary of Phthalate Monoester Activation of mouse and Human PPARα

	Mouse PPARα		Human PPARα	
Monoester	Lowest activation concentration[a]	Maximal fold-induction[b]	Lowest activation concentration	Maximal fold-induction
MEP	—	—	—	—
MButP	100 μM	3.7 ± 0.5	200 μM	2.4 ± 0.6
MBenP	100 μM	12.3 ± 2.8	200 μM	2.5 ± 0.4
MEHA	100 μM	8.3 ± 0.7	100 μM	2.7 ± 0.6
MIHP2	30 μM	8.2 ± 0.8	200 μM	5.6 ± 2.3
MEHP	10 μM	11.1 ± 2.4	30 μM	4.8 ± 2.1
MIHP	10 μM	26.1 ± 4.8	30 μM	12.3 ± 4.4
MnOP	10 μM	32.4 ± 4.0	10 μM	7.6 ± 1.6
MINP	3 μM	27.1 ± 1.9	10 μM	5.8 ± 2.1
MIDP	3 μM	26.9 ± 6.6	30 μM	3.9 ± 1.2

[a]The lowest phthalate monoester concentration where a statistically significant increase in reporter activity was observed.

[b]The maximal fold-induction observed with this phthalate monoester.

activity (Fig. 3). While a concentration of 3 μM MButP caused an increase in mouse PPARβ-dependent luciferase activity, a dose-dependent increase in activity was only observed between 100 and 200 μM (Fig. 3). Concentrations ≥100 μM of MBenP and MEHA were required to cause a dose-dependent increase in luciferase activity with the mouse PPARβ construct, and concentrations ≥30 μM MIHP2 caused a similar dose-dependent increase in activity (Fig. 3). MEHP and MIHP caused increases in luciferase activity with mouse PPARβ, but only at a concentration of 200 μM (Fig. 3). MnOP and MIDP caused dose-dependent increases in mouse PPARβ-dependent luciferase activity at a concentration ≥100 μM, while MINP was did not activate this PPAR isoform (Fig. 3). A comparative summary of these results is presented in Table 2.

No change in mouse PPARγ-dependent luciferase activity was observed for MEP, and although increased luciferase activity was observed using 10 μM MEP for human PPARγ-transfected cells, no dose response was found (Fig. 4). MButP did not cause any changes in luciferase activity using either mouse or human PPARγ (Fig. 4). MBenP caused a dose-dependent increase in luciferase activity at a concentration ≥100 μM for mouse PPARγ, while increased activity was only observed at a concentration of 200 μM for the human PPARγ (Fig. 4). Whereas concentrations ≥30 μM MEHA effectively increase luciferase activity using the mouse PPARγ construct, no significant increases in activity were observed with MEHA using the human PPARγ construct (Fig. 4). Increased luciferase activity caused by MIHP2 was only observed at a concentration of 200 μM with mouse PPARγ, and no change in activity was found when human PPARγ was used (Fig. 4). Increased luciferase activity was found using concentrations of MEHP

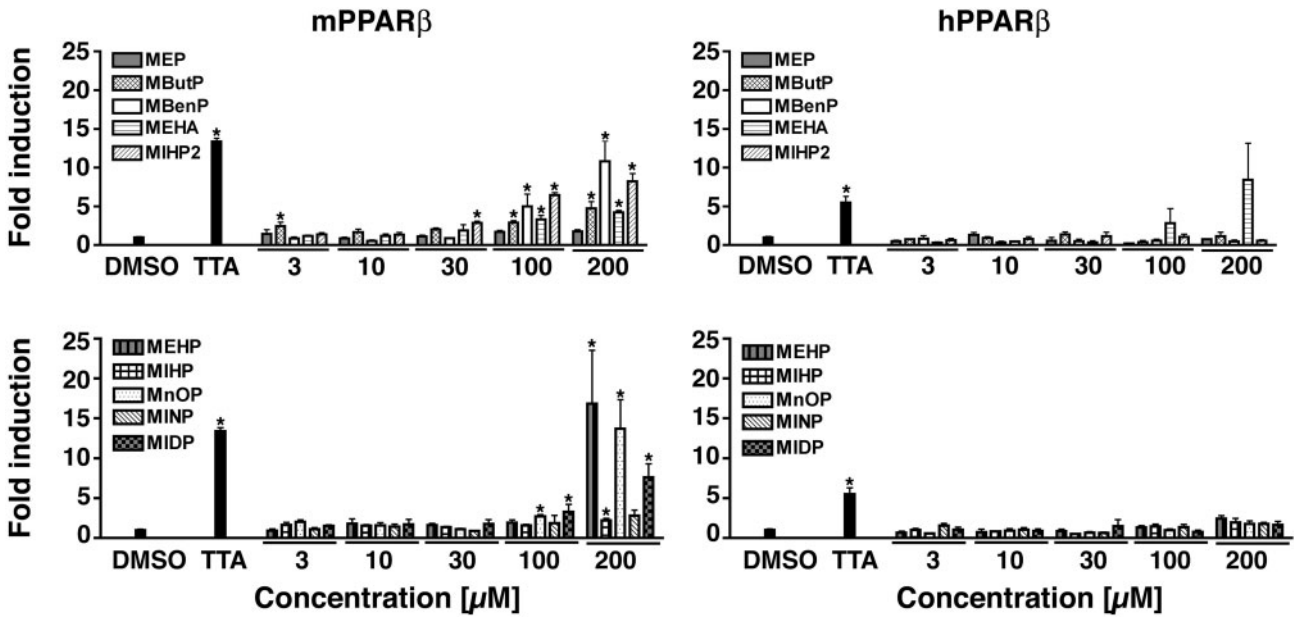

FIG. 3. Activation of mouse PPARβ (mPPARβ), and human PPARβ (hPPARβ) by phthalate monoesters. Transfected 3T3-L1 cells containing either the mouse or human PPARβ-LBD/Gal4-DBD-Gal4-luciferase reporter plasmid were treated with the indicated phthalate monoester at a concentration of either 3, 10, 30, 100, or 200 μM for 24 h. Tetradecylthioacetic acid (TTA) at a concentration of 50 μM was used as a positive control. All luciferase activity values were normalized to the amount of protein in each cell lysate. The fold induction of normalized luciferase activity was calculated relative to DMSO (vehicle)-treated cells, and represents the mean of three independent samples per treatment group; *p*-values were calculated using ANOVA followed by Dunnett's post hoc. Significant differences determined when $p \leq 0.05$.

TABLE 2
Comparative Summary of Phthalate Monoester Activation of Mouse and Human PPARβ

	Mouse PPARβ		Human PPARβ	
Monoester	Lowest activation concentration[a]	Maximal fold-induction[b]	Lowest activation concentration	Maximal fold-induction
MEP	—	—	—	—
MButP	3 μM	4.7 ± 0.9	—	—
MBenP	100 μm	10.8 ± 2.7	—	—
MEHA	100 μM	4.2 ± 0.3	—	—
MIHP2	30 μM	8.3 ± 1.0	—	—
MEHP	200 μM	16.8 ± 6.7	—	—
MIHP	200 μM	2.2 ± 0.3	—	—
MnOP	100 μM	13.7 ± 3.3	—	—
MINP	—	2.8 ± 0.7	—	—
MIDP	100 μM	7.6 ± 0.7	—	—

[a]The lowest phthalate monoester concentration where a statistically significant increase in reporter activity was observed.

[b]The maximal fold-induction observed with this phthalate monoester.

≥30 μM with mouse PPARγ, while concentrations ≥10 μM resulted in a dose-dependent increase in luciferase activity with human PPARγ (Fig. 4). Concentrations ≥100 μM MIHP were required to cause increased luciferase activity for both the mouse and human PPARγ (Fig. 4). MnOP caused a dose-dependent increase in luciferase activity at concentrations ≥10 μM and 100 μM for mouse and human PPARγ, respectively (Fig. 4). MINP and MIDP both caused a dose-dependent increase in mouse and human PPARγ-dependent luciferase activity at concentrations of ≥3 and 30 μM, respectively (Fig. 4). In general, comparable (yet moderately variable) concentrations of phthalate monoesters were required to effectively activate both mouse and human PPARγ, and the magnitude of the response was essentially similar between both the mouse and human PPARγ (Table 3).

Induction of PPARα-Dependent Target Gene mRNA in Rat Hepatoma FaO Cells and Human Hepatoma HepG2 Cells

The induction of mRNA encoding two PPARα target genes, acyl CoA oxidase (ACOX) and cytochrome P450 4A (CYP4A) was measured by Northern blot analysis, using RNA from rodent liver FaO cells and human liver HepG2 cells following treatment with phthalate monoesters, at concentrations ranging from the low and high end of the dose-response curves generated from trans-activation studies. Consistent with the results of the trans-activation studies, longer side-chain phthalate monoesters were more potent in inducing ACOX and CYP4A mRNAs than shorter side-chain phthalate monoesters in the rodent liver FaO cells (Fig. 5A). Human liver HepG2 cells were nonresponsive to

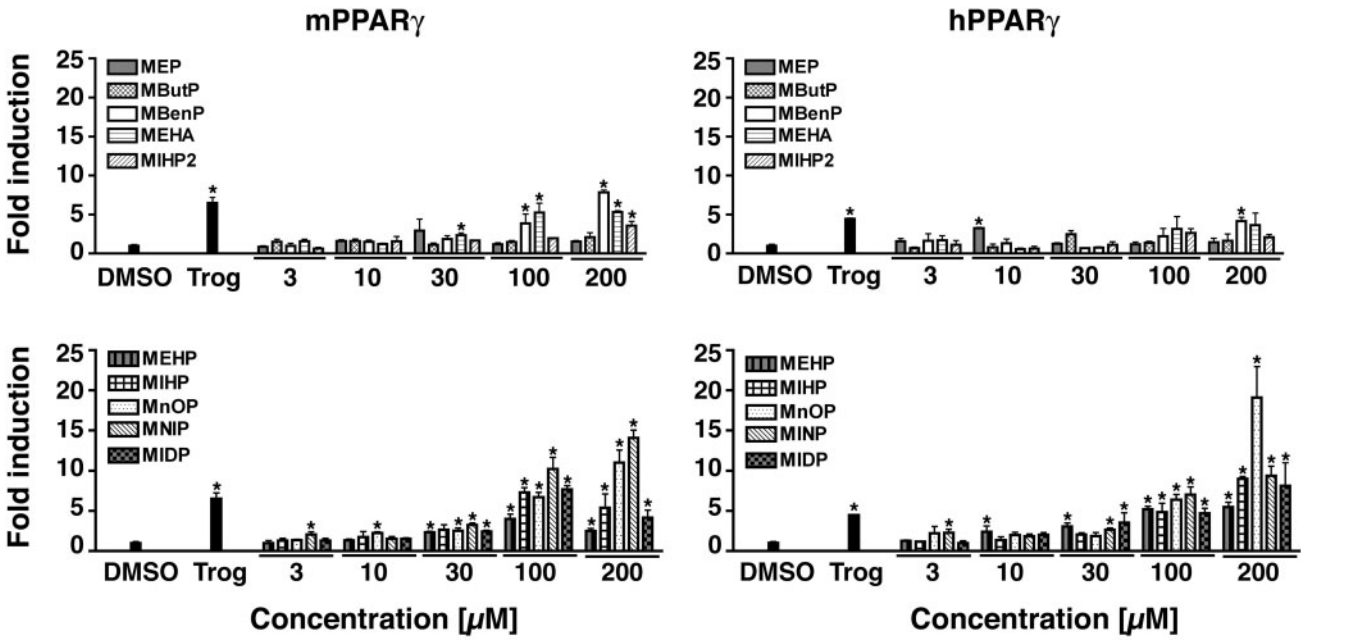

FIG. 4. Activation of mouse PPARγ (mPPARγ) and human PPARγ (hPPARγ) by phthalate monoesters. Transfected 3T3-L1 cells containing either the mouse or human PPARγ-LBD/Gal4-DBD-Gal4-luciferase reporter plasmid were treated with the indicated phthalate monoester at a concentration of either 3, 10, 30, 100, or 200 μM for 24 h. Troglitazone (Trog) at a concentration of 3 μM was used as a positive control. All luciferase activity values were normalized to the amount of protein in each cell lysate. The fold induction of normalized luciferase activity was calculated relative to DMSO (vehicle)-treated cells, and represents the mean of three independent samples per treatment group; *p*-values were calculated using ANOVA followed by Dunnett's post hoc. Significant differences determined when $p \leq 0.05$.

TABLE 3
Comparative Summary of Phthalate Monoester Activation of Mouse and Human PPARγ

	Mouse PPARγ		Human PPARγ	
Monoester	Lowest activation concentration[a]	Maximal fold-induction[b]	Lowest activation concentration	Maximal fold-induction
MEP	—	—	10 μM*	3.3 ± 0.1
MButP	—	—	—	—
MBenP	100 μM	7.8 ± 0.4	200 μM	4.2 ± 0.5
MEHA	30 μM	5.3 ± 0.2	—	—
MIHP2	200 μM	3.6 ± 0.5	—	—
MEHP	30 μM	2.5 ± 0.3	10 μM	5.5 ± 0.6
MIHP	100 μM	5.4 ± 1.7	100 μM	6.7 ± 1.0
MnOP	10 μM	11.0 ± 1.6	100 μM	19.1 ± 3.9
MINP	3 μM	14.1 ± 1.0	30 μM	9.3 ± 1.2
MIDP	30 μM	4.2 ± 0.9	3 μM	8.1 ± 2.8

[a]The lowest phthalate monoester concentration where a statistically significant increase in reporter activity was observed.

[b]The maximal fold-induction observed with this phthalate monoester.

*No further increase in reporter activity was observed at higher concentrations.

activation by phthalate monoesters for the induction of ACOX and CYP4A mRNA (Fig. 5B). These results are consistent with the species difference exhibited by mouse and human PPARα in the trans-activation studies, where the magnitude of the response to phthalate monoesters was greater for mouse PPARα than human PPARα.

Induction of PPARγ-Dependent Adipogenesis by Phthalate Monoesters

3T3-L1 fibroblasts terminally differentiate into adipocytes in a PPARγ-dependent manner when treated with PPARγ agonists in the presence of a differentiation cocktail consisting of dexamethasone, isobutylmethylxanthine, and insulin (Brun *et al.*, 1996). Lipid accumulation, indicative of PPARγ-dependent adipogenesis, was measured using Oil Red O staining. Consistent with the results of the trans-activation studies for the activation of PPARγ, the longer side-chain phthalate monoesters were more potent in inducing adipogenesis than the shorter side-chain phthalate monoesters (Fig. 6). Strong induction of adipogenesis was seen with MEHP, MINP, MIDP, and MnOP at 50 μM, while no induction of adipogenesis was seen with MButP and MBenP at 50 μM (Fig. 6).

DISCUSSION

This study was undertaken to characterize the hierarchy of potency for the activation of PPARs by a broad class of phthalate monoesters and the species differences between mouse and human PPARs for activation by this broad class of phthalate

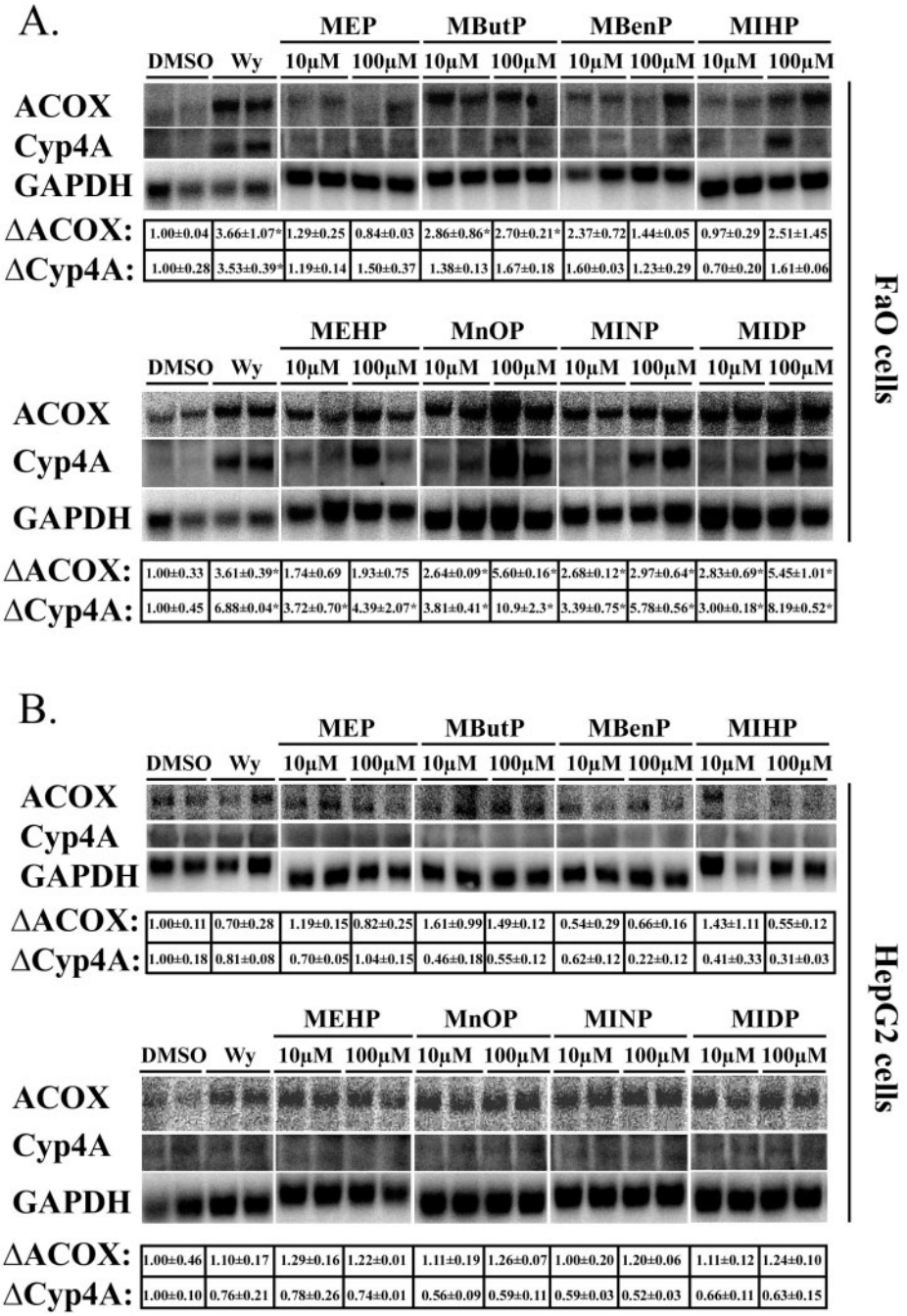

FIG. 5. Induction of PPARα target gene mRNAs acyl CoA oxidase (ACOX) and cytochrome P450 4A (CYP4A) in rodent FaO and human HepG2 liver cell lines. (A) Induction of PPARα target genes in rodent FaO cells treated with short and long side-chain phthalate monoesters. Note significant induction by longer side-chain phthalate monoesters in rodent FaO cells. (B) Induction of PPARα target genes in human HepG2 cells treated with short and long side-chain phthalate monoesters. Note the lack of induction by short and long side-chain phthalate monoesters in human HepG2 cells. The fold induction of normalized mRNA expression was calculated relative to DMSO (vehicle)-treated cells, and represents the mean of two independent samples per treatment group; *p*-values were calculated using ANOVA followed by Dunnett's post hoc. Significant differences determined when $p \leq 0.05$.

monoesters. These studies utilized expression vectors containing both the PPAR-LBD/Gal4-DBD coding sequence, and the Gal4-luciferase reporter in the same plasmid. This one-hybrid model is a sensitive method to detect PPAR activation, because the chimeric receptor does not require heterodimerization with RXRα and is independent of other variables that could influence reporter activity. In contrast, transfections that use multiple expression vectors (e.g., PPARs, PPRE-reporter, etc) introduce

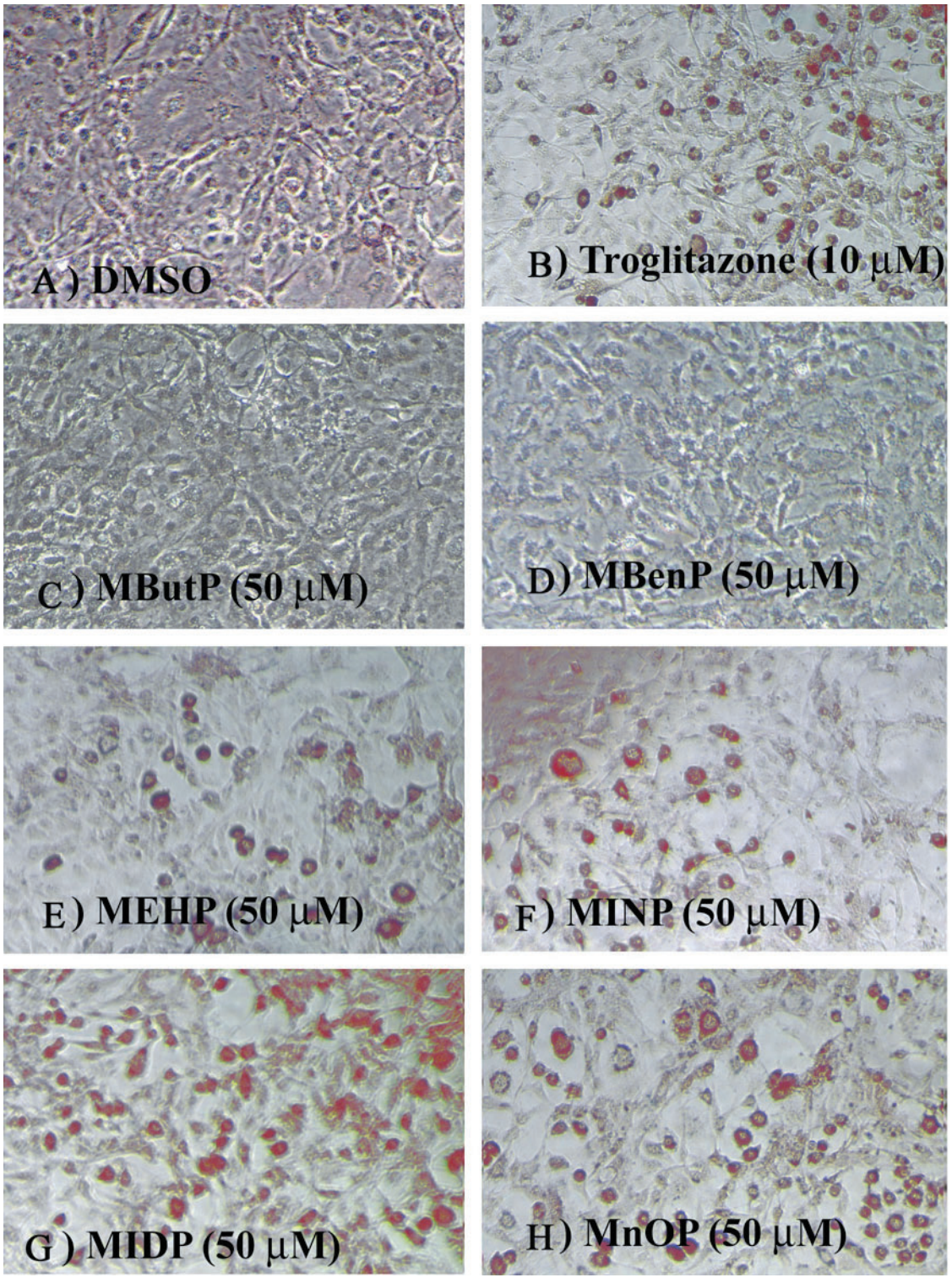

FIG. 6. Induction of PPARγ-dependent adipogenesis by phthalate monoesters in 3T3-L1 fibroblasts. 3T3-L1 fibroblasts were cultured with DMEM/4% FBS containing 1.0 μg/ml insulin, 1 μM dexamethasone and 100 μM isomethylbutylxanthine under the following treatment conditions (A) DMSO (vehicle); (B) Troglitazone (10 μM); (C) MButP (50 μM); (E) MBenP (50 μM); (F) MEHP (50 μM); (G) MINP (50 μM); (H) MIDP (50 μM); and (I) MnOP (50 μM). The media was replaced with DMEM/4% FBS containing 1.0 μg/ml insulin and troglitazone (10 μM), or the indicated phthalate monoester every 48 h after initiation of differentiation. The cells were fixed 6 days after initiation of adipogenesis and stained with Oil Red O. Note, lipid accumulation, indicative of adipogenesis in cells treated with phthalate monoesters that had significant PPARγ activity in the trans-activation studies, and the lack of lipid accumulation in samples treated with phthalate monoesters lacking significant PPARγ activity in the trans-activation studies.

more variability, due to differences in transfection efficiency and the requirement for other co-factors that could influence reporter activity. Based on results obtained with this model system, the hierarchy of potency for the activation of PPARα among the different phthalate monoesters was determined to be MnOP > (MIDP ≈ MINP ≈ MIHP) > (MEHP ≈ MIHP2) > MBenP > MEHA > (MButP ≈ MEP). These findings are relatively consistent with data from others examining the ability of MEHP, MBenP, MButP monomethyl, and mono-n-butyl to trans-activate mouse PPARα (Hurst and Waxman, 2003). The activation potency for PPARα increased with increasing side-chain length of the phthalate monoesters, which is consistent with analysis for mouse PPARα by others (Lampen *et al*., 2003). A significant species difference between receptor activation was observed, with mouse PPARα being activated by lower concentrations of phthalate monoesters and exhibiting greater luciferase activity as compared to human PPARα, which is also consistent with previous reports (Hurst and Waxman, 2003; Maloney and Waxman, 1999).

There was a reasonably good association between the hierarchy of potency and species differences in PPARα activity and the induction of ACOX and CYP4A mRNAs in the hepatoma cell lines. For example, MEP and MButP did not effectively trans-activate mouse PPARα, and no changes in ACOX or CYP4A mRNA were detected in rat hepatoma FaO cells treated with these phthalate monoesters. In contrast, relatively good dose responses in mouse PPARα trans-activation were found for MIHP, MEHP, MnOP, MINP, and MIDP between 10 and 100 μM, and treatment of rat hepatoma FaO cells with these chemicals at these concentrations caused a dose-dependent induction of ACOX and CYP4A mRNAs. Others have shown that treating stably transfected rat hepatoma FaO cells expressing higher than endogenous levels of mouse PPARα with 10–300 μM MButP or MBenP causes dose-dependent increased expression of ACOX protein, but no significant differences in mRNA encoding ACOX (Hurst and Waxman, 2003). Therefore, the results from the present studies are similar to previous reports, as no differences in ACOX mRNA levels were detected in response to either MButP or MBenP in rat hepatoma FaO cells. The reason why Hurst and Waxman (2003) detected higher levels of ACOX protein is uncertain, but could be due to higher than endogenous levels of mouse PPARα present in the cell line used for these studies. While marginally increased trans-activation of human PPARα was observed at concentrations typically greater than 30 μM for some of the phthalate monoesters including MIHP, MEHP, MnOP, MINP, and MIDP, no changes in the level of mRNA encoding ACOX or CYP4A were found in HepG2 cells cultured in the presence of these chemicals at 10 or 100 μM. However, it is important to point out that, while statistically significant increases in reporter activity were observed for MIHP, MEHP, MnOP, MINP, and MIDP between 10 and 100 μM using the human PPARα construct, the fold increase was significantly smaller as compared to that observed using the mouse PPARα construct. Therefore, the lack of increases in PPARα target gene mRNA in human HepG2 cells as compared to rodent FaO cells is still relatively consistent with the species differences observed between the human and mouse trans-activation assay. This is also consistent with other reports demonstrating the lack of induction of PPARα target genes in HepG2 cells in response to peroxisome proliferators (Cornu-Chagnon *et al*., 1995; Duclos *et al*., 1997; Hsu *et al*., 2001; Lawrence *et al*., 2001; Savas *et al*., 2003).

Previous studies have demonstrated significant differences in the response of humans and rodents to phthalates; rodents are generally sensitive, while humans and other primates are more refractory to the PPARα-mediated pathological effects of phthalates (Doull *et al*., 1999; Hall *et al*., 1999; Kamendulis *et al*., 2002; Klaunig *et al*., 2003; Kurata *et al*., 1998; Pugh *et al*., 2000). Several investigators (Barber *et al*., 1987; Smith *et al*., 2000) have assessed the effects of a range of phthalates on rodent liver. The *in vivo* data are reasonably consistent with the *in vitro* PPARα trans-activation data in at least a qualitative sense; low-molecular-weight phthalates (e.g., <C6 side-chains) have little effect on either liver weight or ACOX induction, whereas these effects are seen with the higher molecular weight species. Under *in vivo* conditions, the most active phthalates are DEHP, DINP, and DIDP. Interestingly, MnOP, which was the most active PPARα agonist with the *in vitro* trans-activation assay of the present study, does not appear to influence PPARα-dependent processes under *in vivo* conditions (Lake *et al*., 1984; Mann *et al*., 1985). This is possibly due to rapid conversion of DnOP under *in vivo* conditions to lower-molecular-weight metabolites (Albro and Moore, 1974). Results from the transactivation studies show that the mouse PPARα ligand-binding domain is generally more sensitive than its human counterpart to phthalate monoesters. Examination of PPARα target gene expression in a rodent and human liver cell line provides further evidence that rodents are more sensitive than humans to phthalate monoesters. The reason for this apparent species difference is uncertain, but there is evidence that differences in expression levels of liver PPARα, mutations, or polymorphisms in target gene response elements, or mutant PPARα isoforms may contribute to this effect (Klaunig *et al*., 2003). However, mice that express human PPARα in liver at similar levels to those reported in humans (in the absence of mouse PPARα expression) do not exhibit increased hepatocellular proliferation in response to a potent PPARα agonist (Cheung *et al*., 2004). This suggests that there are likely fundamental structural differences in the PPARα (e.g., ability to recruit co-activators) that mechanistically explain the species differences observed after exposure to PPARα agonists such as phthalate monoesters.

Results from these studies have some relevance for human risk assessment, since humans are routinely exposed to phthalate monoesters, as shown by the presence of these compounds in human urine and serum (Barr *et al*., 2003; Blount *et al*., 2000; Kato *et al*., 2004; Koch *et al*., 2003; Silva *et al*., 2004; Takatori *et al*., 2004). Based on these studies, it is known that the urinary levels of the shorter side-chain phthalate monoesters are found at

the higher levels (e.g., as high as ~3800 ppb for MEP; 95th percentile) as compared to the longer side-chain phthalate monoesters (e.g., ~500 ppb for MEHP; 95th percentile). Additionally, the concentration of total MEHP detected in urine and serum may be relatively comparable, although serum levels may actually be lower, since the current technology for measuring serum phthalate monoesters is hampered by the presence of these compounds in many of the reagents used for this analysis in addition to the presence of esterases in serum (Takatori *et al*., 2004). In a pharmacokinetic study in rats, oral doses of 30, 500, and 1000 mg DEHP/kg were associated with peak blood concentrations of MEHP of 10, 210, and 390 μM, respectively (Kessler *et al*., 2004), a concentration range similar to that used in the present studies. Rats administered DEHP orally in repeated administration studies exhibit significantly elevated liver weight and induction of ACOX at doses ranging from approximately 100 to 2000 mg/kg/day (Barber *et al*., 1987). These observations, in addition to results obtained from the present studies, indicate that in rodents there is a qualitative relationship between effective concentrations under *in vitro* and *in vivo* conditions. Assuming the same relationship pertains to humans, blood concentrations similar to or higher than those that are effective in rodents, would be required to produce effective interactions with PPARα. However, data from the human population at large indicate that, at least under ambient conditions, blood concentrations are well below this range. Serum concentrations of MEHP in the U.S. population range from 2.8 to 15.2 ng/ml with a geometric mean of 3.9 ng/ml (~14 nM) (Kato *et al*., 2004). Thus, at least for DEHP, the average concentration in humans is approximately three orders of magnitude below minimally effective *in vitro* concentrations capable of activating PPARα. Maximum serum levels of phthalate monoesters in humans within the general population are about two orders of magnitude below the effective concentrations required to activate PPARα. For example, the maximum serum concentrations of MEP, MBP, and MEHP in a reference U.S. human population are 73.3 (0.4 μM), 139.0 (0.6 μM) and 34.8 ng/ml (0.1 μM), respectively (Silva *et al*., 2003). Further, internal exposure to DEHP/MEHP can be much higher in patients undergoing some specific procedures, and in some cases the blood concentrations could reach ~100 μM. Combined, these observations suggest that activation of PPARα is not likely to be a significant effect in response to phthalate monoester exposure in the general population, but is theoretically possible under certain conditions.

Results from the present studies also demonstrate that some phthalate monoesters can activate mouse PPARβ, and that human PPARβ appears to be less responsive to this effect. However, not all phthalate monoesters consistently activated PPARβ. For example, no increase in PPARβ-dependent reporter activity was detected in response to MEP, and the lack of a consistent dose-response curve for MIHP, MEHP, and MINP, within a known concentration range where solubility is not a confounding variable, suggests that these chemicals do not activate this PPAR isoform. In contrast, relatively good dose-response curves for mouse PPARβ activation were found for MButP, MBenP, MIHP2, MIDP, and MnOP, and this effect was essentially lacking when the human PPARβ isoform was used for transactivation. Additionally, the efficacy of activation was greater for MIHP2, MIDP, and MnOP as compared to the MButP and MBenP, suggesting that the phthalate monoesters with longer side-chains function better as PPARβ ligands, similar to that observed for PPARα activation. Since isoC6, isoC10, and normal C8 side-chain monoesters effectively activated mouse PPARβ, whereas isoC7, isoC8, and isoC9 side-chain monoesters were inactive, these data suggest that the structure-activity relationships are more subtle as compared to those observed for activation of PPARα. While others have shown that human PPARβ can be activated by MEHP using a co-transfected reporter and PPAR constructs, a direct comparison with a mouse PPARβ construct under these conditions was not provided (Lampen *et al*., 2003). The reason for the difference between these results and the present studies cannot be determined from this work. The relevance of phthalate monoester exposure and PPARβ activation is unclear. While results from the present trans-activation studies suggest that human PPARβ would not likely be activated, data from another group suggests that human PPARβ can be activated by MEHP (Lampen *et al*., 2003). Interestingly, activation of PPARβ is associated with both positive and negative biological effects. For example, there are recent reports that intestinal cancer and breast and prostate cancer cell line growth can be enhanced by treating with a PPARβ ligand (Gupta *et al*., 2004; Stephen *et al*., 2004); however the specificity of these effects has not been examined in a null mouse model. In contrast, recent reports have also shown that PPARβ ligands can enhance serum levels of HDL cholesterol (Oliver *et al*., 2001), promote epithelial cell differentiation (Schmuth *et al*., 2004; Westergaard *et al*., 2001), and in the absence of PPARβ expression, skin and colon carcinogenesis is exacerbated (Harman *et al*., 2004; Kim *et al*., 2004). These results suggest that ligand activation of PPARβ could function to prevent atherosclerosis and epithelial cancers. Therefore, until the specific biological function of PPARβ is determined, the relevance of the present results is uncertain. However, based on observations made in the average human population described above, it is unlikely that human exposure would result in tissue concentrations capable of activating PPARβ.

The hierarchy of potency observed in the trans-activation studies for the activation of PPARγ by the phthalate monoesters was determined to be MnOP > MINP > (MIDP ≈ MIHP) > (MEHP ≈ MBenP ≈ MEHA) > (MIHP2) > (MButP ≈ MEP), with both species exhibiting similar responsiveness; this hierarchy also correlated well with the ability of the various phthalate monoesters to induce PPARγ-dependent adipogenesis in 3T3-L1 fibroblast cell line. The hierarchy of potency and the similarity in species responsiveness for the activation of PPARγ observed in this study is consistent with previous reports (Hurst and Waxman, 2003; Maloney and Waxman, 1999). Similar to

the biological role of PPARβ, the function of PPARγ is unclear, as there are conflicting reports in the literature. PPARγ agonists have been used for years as drugs to reduce blood glucose in type II diabetics, although the use of some agonists was associated with human mortality (Isley, 2003). Additionally, several studies have suggested that activation of PPARγ potentiates carcinogenic effects, as ligand treatment resulted in exacerbated carcinogenesis in APC^{min} mice (Lefebvre *et al*., 1998; Saez *et al*., 1998), and overexpression of an oncogene and a ligand-independent form of PPARγ leads to exacerbated mammary tumorigenesis in bi-transgenic mice (Saez *et al*., 2004). In contrast, other reports have shown that PPARγ ligands can significantly reduce the number of aberrant crypt foci and colon polyps induced by azoxymethane in rats, inhibit growth of transplanted tumors in nude mice, and inhibit growth of colon tumor cell lines (reviewed in Michalik *et al*., 2004). Additionally, tumor multiplicity is significantly greater in azoxymethane-treated heterozygous PPARγ-null mice, and loss of function mutations are reported in human cases of colorectal cancer (reviewed in (Michalik *et al*., 2004)). Therefore, while the present studies suggest that phthalate monoesters can activate both mouse and human PPARγ, further research is needed to determine the biological relevance of PPARγ activation that might occur in response to exposure to phthalate monoesters.

Lastly, while many ligands for PPARs (such as phthalate monoesters) function by activating specific PPARs, it is becoming increasingly clear that many ligands for nuclear receptors can be promiscuous and activate other nuclear receptors. Thus, while results from these studies demonstrate that phthalate monoesters can activate PPARs and that species differences exist in the ability to activate specific PPAR, it is still possible that these chemicals lead to events that are mediated by other nuclear receptors, and/or through events that are not dependent on receptor activation, per se, and this remains to be examined.

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