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Activation of PPARα and PPARγ by Environmental Phthalate Monoesters

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Phthalate esters are widely used as plasticizers in the manufacture of products made of polyvinyl chloride. Mono-(2-ethylhexyl)-phthalate (MEHP) induces rodent hepatocarcinogenesis by a mechanism that involves activation of the nuclear transcription factor peroxisome proliferator-activated receptor-alpha (PPARα). MEHP also activates PPAR-gamma (PPARγ), which contributes to adipocyte differentiation and insulin sensitization. Human exposure to other phthalate monoesters, including metabolites of di-*n*-butyl phthalate and butyl benzyl phthalate, is substantially higher than that of MEHP, prompting this investigation of their potential for PPAR activation, assayed in COS cells and in PPAR-responsive liver (PPARα) and adipocyte (PPARγ) cell lines. Monobenzyl phthalate (MBzP) and mono-*sec*-butyl phthalate (MBuP) both increased the COS cell transcriptional activity of mouse PPARα, with effective concentration for half-maximal response (EC_{50}) values of 21 and 63 μM, respectively. MBzP also activated human PPARα (EC_{50} = 30 μM) and mouse and human PPARγ (EC_{50} = 75–100 μM). MEHP was a more potent PPAR activator than MBzP or MBuP, with mouse PPARα more sensitive to MEHP (EC_{50} = 0.6 μM) than human PPARα (EC_{50} = 3.2 μM). MEHP activation of PPARγ required somewhat higher concentrations, EC_{50} = 10.1 μM (mouse PPARγ) and 6.2 μM (human PPARγ). No significant PPAR activation was observed with the monomethyl, mono-*n*-butyl, dimethyl, or diethyl esters of phthalic acid. PPARα activation was verified in FAO rat liver cells stably transfected with PPARα, where expression of several endogenous PPARα target genes was induced by MBzP, MBuP, and MEHP. Similarly, activation of endogenous PPARγ target genes was evidenced for all three phthalates by the stimulation of PPARγ-dependent adipogenesis in the 3T3-L1 cell differentiation model. These findings demonstrate the potential of environmental phthalate monoesters for activation of rodent and human PPARs and may help to elucidate the molecular basis for the adverse health effects proposed to be associated with human phthalate exposure.

Key Words: **PPAR; MEHP; phthalate monoesters.**

Peroxisome proliferator chemicals (PPCs) include hypolipidemic drugs, phthalates, endogenous steroids, herbicides, and solvents. These structurally diverse chemicals induce a pleiotropic set of responses in rat and mouse liver, including hepatomegaly, induction of enzymes involved in fatty acid β-oxidation, and an increase in the size and number of peroxisomes (Reddy *et al.*, 1980). Long-term exposure of rodents to PPCs is associated with increased risk of developing hepatocellular carcinoma (Reddy *et al.*, 1980). These effects of PPCs are mediated by peroxisome proliferator-activated receptor-alpha (PPARα), a ligand-activated transcription factor that belongs to the nuclear receptor superfamily. Heterodimers between PPARα and retinoid X receptor bind to and *trans*-activate peroxisome proliferator response elements (PPREs) found in the 5′-regulatory region of PPC-activated genes, such as acyl-CoA oxidase (ACOX), peroxisomal bifunctional enzyme, and cytochrome P450 4A, a fatty-acid ω-hydroxylase (Reddy and Hashimoto, 2001). The role of PPARα in PPC-induced hepatic proliferative responses is evident from studies of PPARα null mice, which do not exhibit the characteristic hepatomegaly, liver peroxisome proliferation, and target gene activation seen in PPC-treated wild-type (wt) mice (Lee *et al.*, 1995). Lipid homeostasis and fatty acid metabolism are altered in these mice (Aoyama *et al.*, 1998), and the hepatocarcinogenic effects of 4-chloro-6-(2,3-xylidino)-2-pyrimidinythiol acetic acid (Wy-14,643), a potent peroxisome proliferator, are abolished (Peters *et al.*, 1997). Thus, the major hepatic effects of PPCs, including their hepatocarcinogenic effects, are mediated by PPARα-dependent gene transcription and signaling events. By contrast, two other PPAR forms help regulate diverse physiological processes in several other tissues, with PPARγ essential for adipogenesis and PPARδ playing a role in development (Berger and Moller, 2002).

Strong species differences in the response to PPCs have been observed, with rats and mice being quite sensitive to PPCs and humans, guinea pigs, and other species being refractory (Gonzalez *et al.*, 1998). The relative insensitivity of human liver cells to PPCs reflects several factors, including the lower levels of PPARα in human liver, as compared with rodents (Palmer *et al.*, 1998), and species differences in amino acid sequence within the ligand binding domain (Keller *et al.*, 1997), which may contribute to the decreased intrinsic sensitivity of human PPARα, compared with its rodent counterparts

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seen with some but not all PPCs (Maloney and Waxman, 1999).

PPARγ is predominantly expressed in adipose tissue and at lower levels in skeletal muscle, liver, and heart (Kliewer *et al.*, 2001). PPARγ plays a key role in adipocyte differentiation and is the primary molecular target of a novel class of thiazolidinedione drugs used to treat non-insulin–dependent diabetes mellitus (Lehmann *et al.*, 1995). In addition to PPARγ, factors involved in the transcriptional control of adipogenesis include CCAAT/Enhancer-binding proteins (C/EBPs) and signal transducer and activators of transcription (STATs) (Morrison and Farmer, 1999; Nanbu-Wakao *et al.*, 2002). PPARγ has also been implicated in promoting macrophage differentiation and the formation of atherosclerotic lesions in humans (Berger and Moller, 2002). Given the extensive cross-talk between PPAR and other transcription factors and signaling pathways controlling adipogenesis and other physiological processes (Shipley and Waxman, in press; Zhou and Waxman, 1999; Zhou *et al.*, 2002), perturbation of these highly regulated processes by environmental chemicals that interact with PPARγ may potentially have significant pathophysiological consequences.

Phthalate esters are widely used as plasticizers in the manufacture of products made of flexible polyvinyl chloride products, including medical bags and food packaging, and can also be found in a variety of industrial fixatives, detergents, cosmetics, and solvents (Blass, 1992). Phthalates are ubiquitous environmental contaminants, and the potential for human exposure by oral, dermal, inhalation, and intravenous means is high (Huber *et al.*, 1996). Di-(2-ethylhexyl)-phthalate (DEHP), the most important phthalate ester in commercial use, is a rodent reproductive toxicant, a teratogen, and a liver carcinogen (Doull *et al.*, 1999). The hepatotoxicological effects of DEHP are hypothesized to involve peroxisome proliferation (Lake *et al.*, 1975) induced by DEHP's monoester hydrolysis product, mono-(2-ethylhexyl)-phthalate (MEHP; Lhuguenot *et al.*, 1988; Maloney and Waxman, 1999). The testicular toxicity of DEHP is independent of PPARα (Ward *et al.*, 1998) but may conceivably be mediated by another PPAR form (PPARγ or PPARδ). Recently, urinary phthalate monoester concentrations were found to be exceptionally high in a human reference population (Blount *et al.*, 2000). Particularly high levels were reported for monoethyl phthalate (6790 μg/g urinary creatinine), mono-*n*-butyl phthalate (M(n)BuP; 2760 μg/g), and monobenzyl phthalate (MBzP; 544 μg/g), whereas urinary levels of MEHP were much lower, at 192 μg/g (Blount *et al.*, 2000). M(n)BuP and MBzP are hydrolytic metabolites of the environmental phthalate diesters dibutyl phthalate and butyl benzyl phthalate, which are potential reproductive and developmental toxicants (Kavlock *et al.*, 2002a,b). Human exposure to phthalate monoesters is, thus, substantially higher and more prevalent than previously suspected. Presently, DEHP receives the most attention concerning health risks associated with phthalate exposure. However, the results of Blount *et al.* (2000) indicate that other phthalate monoesters need to be considered when carrying out human health risk-assessment analyses for this class of compounds. As a first step toward this goal, this study set out to determine whether these environmentally relevant phthalate monoesters can activate mouse or human PPARα and PPARγ when assayed in transfection studies and in intact cellular systems with endogenous receptors and target genes. Our findings extend previous studies on the effects of MEHP (Lovekamp-Swan *et al.*, 2003; Maloney and Waxman, 1999) and demonstrate significant activation of both PPAR forms by phthalate monoesters, most notably MBzP and mono-*sec*-butyl phthalate (MBuP), in addition to MEHP. The potential toxicological implications of these findings are discussed in the context of the roles played by PPARα and PPARγ in lipid homeostasis and the regulation of energy metabolism.

MATERIALS AND METHODS

Chemicals. MBzP, MBuP, diethyl phthalate, and monomethyl phthalate were purchased from Aldrich Chemical Co. (Milwaukee, WI). MEHP (TCI America, Portland, OR), M(n)BuP (Chem Service, West Chester, PA), phthalic acid (Sigma Chemical Co., St Louis, MO), and troglitazone (Sankyo Co., Japan) were obtained from the sources indicated.

Plasmids. The mouse PPARα expression plasmid pCMV-PPARα was provided by Dr. E. Johnson (Scripps Research Institute, La Jolla, CA). The mouse PPARγ expression plasmid pSV-Sport1-PPARγ_1 was provided by Dr. J. K. Reddy (Northwestern University Medical School, Chicago, IL). The human PPARα expression plasmid pSG5-PPARα was obtained from Dr. F. Gonzalez (National Cancer Institute, Bethesda, MD). The human PPARγ_1 expression plasmid pSG5-PPARγ_1 was obtained from Dr. S. Kliewer (GlaxoSmithKline, Research Triangle Park, NC). The firefly luciferase reporter plasmid pHDx3-luc contains three copies of a PPRE derived from the rat enoyl CoA hydratase/3-hydroxyacyl CoA promoter (nts –2956 to –2919) cloned into pCPS-Luc and was obtained from Dr. J. Capone (McMaster University, Ontario, Canada). This reporter plasmid is based on a known PPARα target gene but also responds to PPARγ. The renilla luciferase reporter plasmid pRL-CMV was purchased from Promega (Madison, WI). The Moloney murine leukemia virus-derived expression vector pBabe-Puro containing a full-length cDNA insert encoding mouse PPARα was obtained from Dr. B. M. Spiegelman (Dana-Farber Cancer Institute, Boston, MA).

Cell culture and transient transfections. COS-1 cells (ATCC, Manassas, VA) were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine system (FBS; Sigma, St. Louis, MO) and 50 U/ml penicillin/streptomycin (Gibco) in 75 cm^2 tissue culture flasks. Cells were cultured overnight at 37°C, then trypsinized and reseeded at 30,000 cells/well in a 48-well plate (Corning Inc., Corning, NY) in DMEM containing 10% FBS. The cells were transfected 24 h later, using FuGENE 6 transfection reagent (Boehringer-Mannheim, Germany), as previously described (Maloney and Waxman, 1999). The transfection mixture contained 90 ng pHD(x3)-luc, 5 ng PPAR expression plasmid, and 1 ng pRL-CMV per well in a volume of 15 μl of DMEM containing 0.3 μl of FuGENE 6. Salmon sperm DNA (Stratagene Inc., La Jolla, CA) was added as carrier DNA to give 250 ng total DNA per well. The media was replaced 16–18 h later with serum-free DMEM containing the PPCs or phthalates to be tested for PPAR activation. Stock solutions of PPCs and phthalates dissolved in dimethyl sulfoxide (DMSO) were prepared fresh on the day of cell treatment. MEHP (20 μM) or Wy-14,643 (5 μM) was used as a positive control for phthalate activation of mouse and human PPARα (Maloney and Waxman, 1999). Troglitazone (3 μM) was used as a positive control for the activation of PPARγ. Following PPC or phthalate treatment for 24 h, cells were lysed by incubation at 4°C in 200 μl

passive lysis buffer (Promega) for 20 min. Firefly and renilla luciferase activities were measured in the cell lysate, using a dual reporter assay system (Promega) and a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA). Luciferase activity values were normalized for transfection efficiency, using Renilla luciferase activity assayed in the same cell lysates (firefly luciferase/renilla luciferase). Firefly/renilla ratios calculated for PPC- or phthalate-treated cells were then expressed relative to untreated DMSO controls. The range of renilla luciferase activity for 5 μl of cell lysate was typically 150,000–400,000 light units. Data shown in each figure are presented as mean ± SED ($n = 3$ replicates). Each figure is representative of two to three independent replicate experiments. Effective concentration for half-maximal response (EC_{50}) values were calculated using GraphPad Prism software, version 3.0 (GraphPad, San Diego, CA).

Construction of FAO cells expressing PPARα by retroviral infection (FAO-PPARα cells). Rat hepatoma FAO cells obtained from Dr. J. Vanden Heuvel (Pennsylvania State University, University Park, PA) were grown in DMEM containing 5% FBS. Transfection of the packaging cell line Bosc 23 with mouse PPARα-encoding pBabe-Puro retroviral plasmid DNA, harvesting of the retroviral supernatant, and infection of the rat FAO hepatoma cells were carried out using methods described previously (Jounaidi *et al.*, 1998). Pools of puromycin-resistant FAO cells were selected using 2 μg/ml puromycin for 2 weeks. Drug-resistant clones (FAO-PPARα cells) were grown and analyzed for nafenopin responsiveness by Western blot analysis of the PPARα target gene peroxisomal 3-ketoacetyl-CoA thiolase (PTL).

Western blotting. Whole-cell extracts were prepared from FAO or FAO-PPARα cells dissolved in 1× passive lysis buffer (Promega) containing Complete (Roche Diagnostics, Mannheim, Germany) cocktail of protease inhibitors. Cells were lysed on ice for 30 min, and insoluble materials were pelleted by centrifugation (30 min at 15,000 x g). Protein concentrations were determined using a commercially available protein assay kit (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard. Protein was electrophoresed on 10% Laemmli SDS gels (40 μg protein/lane), electrotransferred onto nitrocellulose membranes, then probed with rabbit polyclonal anti-ACOX or anti-PTL antibody (1:10,000 dilution), generously provided by Drs. T. Hashimoto and J. K. Reddy (Northwestern University, Chicago, IL), as described earlier (Zhou *et al.*, 2002). Antibody binding was visualized on X-ray film by enhanced chemiluminescence using the ECL kit from Amersham Pharmacia Biotech (Piscataway, NJ). Scans of Western blots were obtained using a Microtek ScanMaker (Carson, CA) V6USL scanner and Ofoto software (Emeryville, CA). Protein band intensities were quantitated using ImageQuant, v1.2 software (Molecular Dynamics, Piscataway, NJ).

Quantitation of mRNA levels by real-time PCR. Relative cellular levels of rat 18S rRNA and PTL, ACOX, peroxisomal bifunctional enzyme (PBE), and urate oxidase mRNAs were quantified by real-time PCR analysis using the ABI 7900 Prism Sequence Detection System (Applied Biosystems, Foster City, CA). Total RNA was extracted using TRIZOL reagent (Gibco BRL, Carlsbad, CA) from FAO-PPARα cells that were seeded in 6-well plates at 7×10^5 cells/well and treated for 48 h with nafenopin (100 μM) or the indicated phthalate monoesters beginning 4 h after cell plating. The RNA obtained was treated with DNase I (1 U/ml) for 1 h to remove contaminating DNA. SYBR Green real-time PCR assays were used to quantify the following rat mRNAs: PTL (forward primer 5′-GGC-ACA-AGG-GCA-TCC-AAT-C-3′, reverse primer 5′-GTG-CGC-TGT-CTT-TGG-TTC-AA-3′); ACOX (forward primer 5′-CCT-CTG-TCG-ACC-TTG-TTC-GG-3′, reverse primer 5′-ACG-ACC-ACG-TAG-TGC-CAA-TG-3′); PBE (forward primer 5′-GCC-TTG-GGC-TGT-CAC-TAT-CG-3′, reverse primer 5′-CAA-GCC-GAC-ACG-AGC-CTT-T-3′); urate oxidase (forward primer 5′-ACT-GCA-AGT-GGC-GCT-ACC-A-3′, reverse primer 5′-CCC-AGG-TAG-CCT-CGA-AAT-CC-3′); and 18S rRNA (forward primer 5′-CGC-CGC-TAG-AGG-TGA-AAT-TC-3′, reverse primer 5′-CCA-GTC-GGC-ATC-GTT-TAT-GG-3′). For reverse transcription reactions, 0.4 μg RNA was transcribed into cDNA, using random hexamer primers and MuLV reverse transcriptase (Applied Biosystems). Each real-time PCR reaction contained SYBR Green PCR Master Mix and 300 nM of each primer in a volume of 4 μl and was carried out in triplicate. The PCR program was 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s. No PCR amplification was observed in control reactions that omitted reverse transcriptase or the cDNA template. Relative levels of PTL, ACOX, PBE, and urate oxidase mRNA were calculated for each cDNA sample after subtracting the threshold cycle (C_T) for 18S RNA (determined in triplicate for each cDNA) from the C_T values (determined in triplicate) for PTL, ACOX, PBE, and urate oxidase to adjust for small differences in the amount of cDNA template present in each sample (ΔC_T). The average ΔC_T for untreated FAO-PPARα cells was then subtracted from the corresponding ΔC_T for phthalate-treated cells ($\Delta\Delta C_T$), and the values were back-transformed ($2^{-\Delta\Delta CT}$) to calculate the amounts of each RNA in the treated cells, relative to untreated controls.

3T3-L1 cell differentiation assay. Mouse 3T3-L1 fibroblasts (ATCC, Manassas, VA) were grown in DMEM containing 10% FBS. Cells were seeded in 12-well plates at ~60% confluence. Two days postconfluence, adipogenesis was induced by changing the media to DMEM/10% FBS containing 1.67 μM insulin, 1 μM dexamethasone, 0.5 mM isomethylbutylxanthine, and a PPARγ activator. Troglitazone (10 μM) was used a positive control for PPARγ-dependent adipocyte differentiation. Phthalate monoester or troglitazone was added to the culture medium at the time of initiation of differentiation (2 days postconfluence) and with each subsequent medium change (every 48 h). Six days after initiation of adipocyte differentiation, the 3T3-L1 cells were fixed with formalin and stained with Oil Red O (Green and Kehinde, 1974). Briefly, cells were washed twice with PBS, then fixed with 10% formalin in phosphate buffer for 1 h at room temperature. The fixed cells were stained with Oil Red O (3 mg/ml) for 15 min. Cells were washed three times with water, visualized with a Nikon TMS-F light microscope, and photographed.

Color photomicrographs of 3T3-L1 adipocyte differentiation were converted to grayscale images using the "Color Range" tool in Adobe Photshop 6.0. This tool was used to select red color corresponding to a RGB value of 154, 0, 0. The range of reds selected was expanded using the "Fuzziness" option, which was set to the maximum value of 200. The selected color range was copied and pasted in a new Photoshop document and converted to grayscale. Dark stained lipid droplets shown in the final image indicate an increase in adipocyte differentiation.

Statistical analysis. GraphPad Prism v3.0 was used to perform all statistical analyses. All data were log-transformed, and a one-way ANOVA followed by Dunnett's post hoc test was used to determine whether differences between phthalate treatments were significantly different from the control values, with $p < 0.05$ as the limit of significance.

RESULTS

trans-*Activation PPARα and PPARγ by MEHP*

The *trans*-activation of PPARα by MEHP, the monoester hydrolysis product of DEHP, was investigated in COS-1 cells transfected with mouse or human PPARα expression plasmid and a PPRE-luciferase reporter. Cells were then treated for 24 h with MEHP (0.03–60 μM), which was previously shown to activate PPARα (Maloney and Waxman, 1999). MEHP activated PPARα-dependent reporter activity ~2- to 2.5-fold, relative to DMSO (control)-treated cultures with both the mouse and human receptor (Figs. 1A and 1B). Dose-response studies showed mouse PPARα to be ~5-fold more sensitive to MEHP ($EC_{50} = 0.6$ μM) than human PPARα ($EC_{50} = 3.2$ μM) (Fig. 2). Further investigation demonstrated that MEHP activates mouse PPARγ and human PPARγ transcriptional activity 3- to 4-fold (Figs. 1C and 1D), with EC_{50} values of 10.1 μM and 6.2 μM, respectively (Fig. 2).

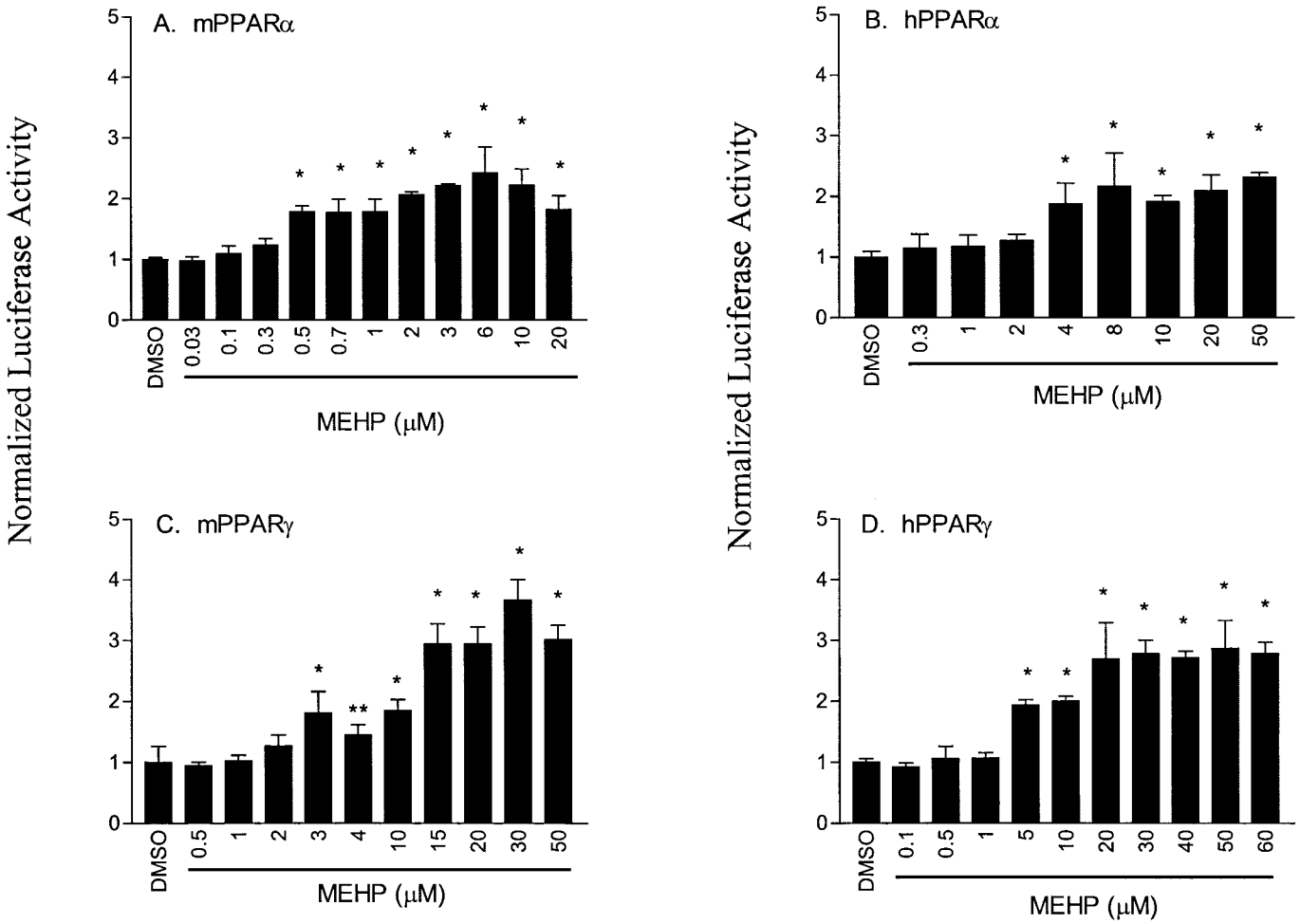

FIG. 1. Activation of mouse and human PPARα and PPARγ by MEHP. COS-1 cells were transfected with expression plasmid encoding the indicated PPAR isoform, a PPRE-Luc reporter plasmid, and a renilla luciferase internal control plasmid. Treatment of cells with the indicated concentrations of MEHP for 24 h and determination of firefly luciferase activity normalized to the renilla luciferase internal control were carried out as described in Materials and Methods. Data shown are luciferase reporter values normalized to untreated DMSO controls, mean ± SD, $n = 3$ based on a single representative experiment. *$p < 0.01$; **$p < 0.05$ from DMSO-control values by ANOVA.

Effect of Phthalate Monoesters on PPARα Activity

We next examined several other phthalate monoesters for their ability to activate mouse and human PPARα. MBzP (Fig. 3A) and MBuP (Fig. 4A) both increased the transcriptional activity of mouse PPARα up to ~3- to 3.5-fold, with EC_{50} values of 21 μM (MBzP) and 63 μM (MBuP; data not shown). MBzP also activated human PPARα (Fig. 3B), but the activation was less robust than that of mouse PPARα, suggesting a reduced responsiveness of the human receptor. Treatment of the cells with Wy-14,643, an established PPARα ligand and potent PPARα activator, resulted in 5- to 8-fold induction of mouse PPARα activity. Monomethyl phthalate activated mouse PPARα by 2-fold; however, no *trans*-activation of human PPARα was detected (data not shown). In contrast to the activation seen with the *sec*-butyl ester MBuP, mouse and human PPARα were unresponsive to M(n)BuP at concentrations up to 300 μM. The dimethyl and diethyl esters of phthalic acid were inactive when assayed for mouse and human PPARα *trans*-activation (data not shown).

FAO-PPARα Cells Are Responsive to Phthalate Monoesters

Initial experiments with rat liver FAO cells demonstrated that these cells were weakly responsive to PPCs, as revealed by Western blot analysis to detect induction of the PPARα target genes and peroxisomal enzymes ACOX and PTL. To increase the sensitivity of this liver cell line to PPCs, FAO cells expressing 4-fold higher levels of PPARα mRNA (FAO-PPARα cells) were generated by retroviral transduction (see Materials and Methods). The resultant stable cell line FAO-PPARα was then compared with wt FAO cells with respect to responsiveness to the PPCs Wy-14,643, nafenopin, and MEHP (48-h treatment). Cell extracts were analyzed on Western blots

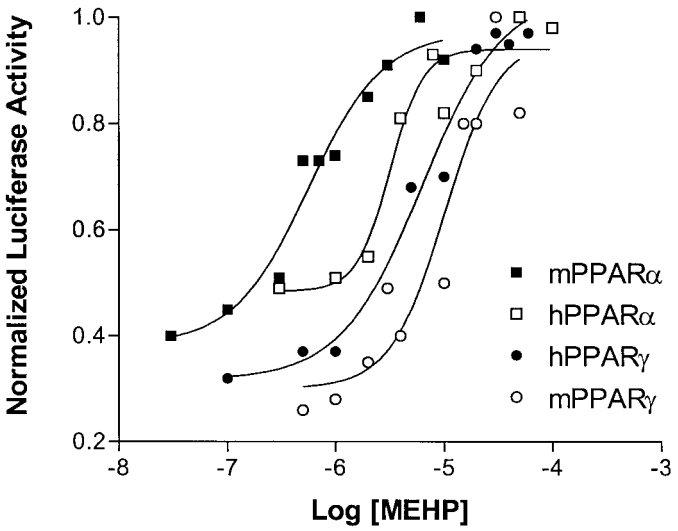

FIG. 2. Dose-response for activation of PPARα and PPARγ by MEHP. COS-1 cell transfection, treatment with MEHP, and determination of relative luciferase values were carried out as described in Materials and Methods. Data shown are based on normalized luciferase reporter values, such as those shown in Figure 1. Maximal activation for each receptor was arbitrarily set as 1. EC_{50} values were calculated using nonlinear regression analysis (GraphPad Prism v3.0).

probed for ACOX and PTL. Wt FAO cells responded to MEHP and nafenopin by induction of the 52-kDa form of ACOX (Fig. 5). Little or no induction of PTL was detected, despite the strong induction of this PPARα target gene seen in rat liver after treatment with the PPC ciprofibrate (lane 2 vs. lane 1). In contrast, ACOX and PTL were both strongly increased in FAO-PPARα cells treated with Wy-14,643, MEHP, or nafenopin (Fig. 5, lanes 6, 8, 10 vs. untreated control in lane 4). Therefore, we used FAO-PPARα cells as a model to study the activation of endogenous PPARα target genes by the *trans*-activating phthalate monoesters identified in Figures 3 and 4. Treatment of FAO-PPARα cells with MBzP for 48 h resulted in dose-dependent increases in ACOX protein (4-fold) and PTL protein (6-fold; Fig. 6). Both protein products of the ACOX gene were induced. However, induction of ACOX was less robust with MBuP (2- to 3-fold), as compared with MBzP (4-fold). These findings demonstrate that both phthalate monoesters are able to activate PPARα and stimulate expression of endogenous PPARα target genes in a liver cell model.

Induction of PPARα-Responsive Genes in FAO-mPPARα Cells

The induction of fatty-acid β-oxidation genes in FAO-mPPARα cells following MBzP or MBuP was confirmed by real-time PCR analysis. PTL mRNA was induced ~7-fold at 300 μM MBzP (Fig. 7). However, no induction of PTL mRNA was observed with MBuP, in agreement with the protein data shown in Figure 6. Induction of PTL mRNA in response to MBzP treatment was substantially lower than that achieved in cells treated with Wy-14,643 or nafenopin. PBE mRNA was induced up to ~7 to 10-fold in FAO-mPPARα cells treated with MBzP or MBuP. Although ACOX mRNA was induced with Wy-14,643 or nafenopin (4- to 7-fold increase), no significant increase was seen with MBzP or MBuP. By contrast, the protein data shown in Figure 6 indicate induction of the 52- and 72-kDa protein bands was observed with both MBzP and MBuP. Finally, urate oxidase mRNA, which encodes a peroxisomal enzyme that is not responsive to PPCs, was not induced by Wy-14,643, nafenopin, MBzP, or MBuP (Fig. 7).

trans-*Activation of Mouse and Human PPARγ by Phthalate Monoesters*

Transient transfections were carried out in COS-1 cells to assay the responsiveness of mouse and human PPARγ to phthalate monoesters. MEHP activated both mouse and human PPARγ (Figs. 1C and 1D), with EC_{50} values of 10.1 μM and 6.2 μM, respectively (Fig. 2). MBzP also stimulated a 3-fold increase in mouse and human PPARγ activity, as compared with a 10- to 15-fold activation by the potent PPARγ ligand troglitazone (Figs. 3C and 3D), with EC_{50} values of 75 and 100 μM, respectively (data not shown). MBuP activated mouse PPARγ ~2- to 3-fold at 300 μM (Fig. 4B) but induced little or no increase (< 2-fold) in human PPARγ activity (data not shown). Mouse and human PPARγ were also unresponsive to M(n)BuP, monomethyl phthalate, and diethyl phthalate when tested at concentrations up to 300 μM (data not shown).

Effect of MBzP and MBuP on Adipocyte Differentiation

We next investigated the effect of MBzP and MBuP on endogenous PPARγ function. We selected the 3T3-L1 preadipocyte differentiation model to characterize the ability of phthalate monoesters to activate endogenous PPARγ in an intact cell system. 3T3-L1 preadipocytes differentiate into mature fat cells in a PPARγ-dependent manner when treated with PPARγ activators in the presence of a cocktail of hormonal inducers (dexamethasone, isobutylmethylxanthine, and insulin; Brun *et al.*, 1996) and can be used to assay for PPARγ ligands/activators. PPARγ-dependent adipogenesis can be visualized by staining of accumulated fat droplets with Oil Red O. To test the effects of environmental phthalate monoesters in this model, 3T3-L1 cells were treated with differentiation cocktail for 6 days in the presence of increasing concentrations of MEHP, MBzP, or MBuP. The established PPARγ activator troglitazone served as positive controls for PPARγ-dependent differentiation (Fig. 8, panel C vs. panel B). Strong induction of differentiation was seen with MEHP (50 μM; panel D). Moreover, a dose-dependent increase in adipocyte differentiation was observed with both MBzP (Fig. 8, panel E and panel F vs. panel B) and MBuP (Fig. 8, panel G and panel H vs. panel B). At the highest concentration of phthalate tested, the extent of differentiation induced by MBuP was less than that of MBzP

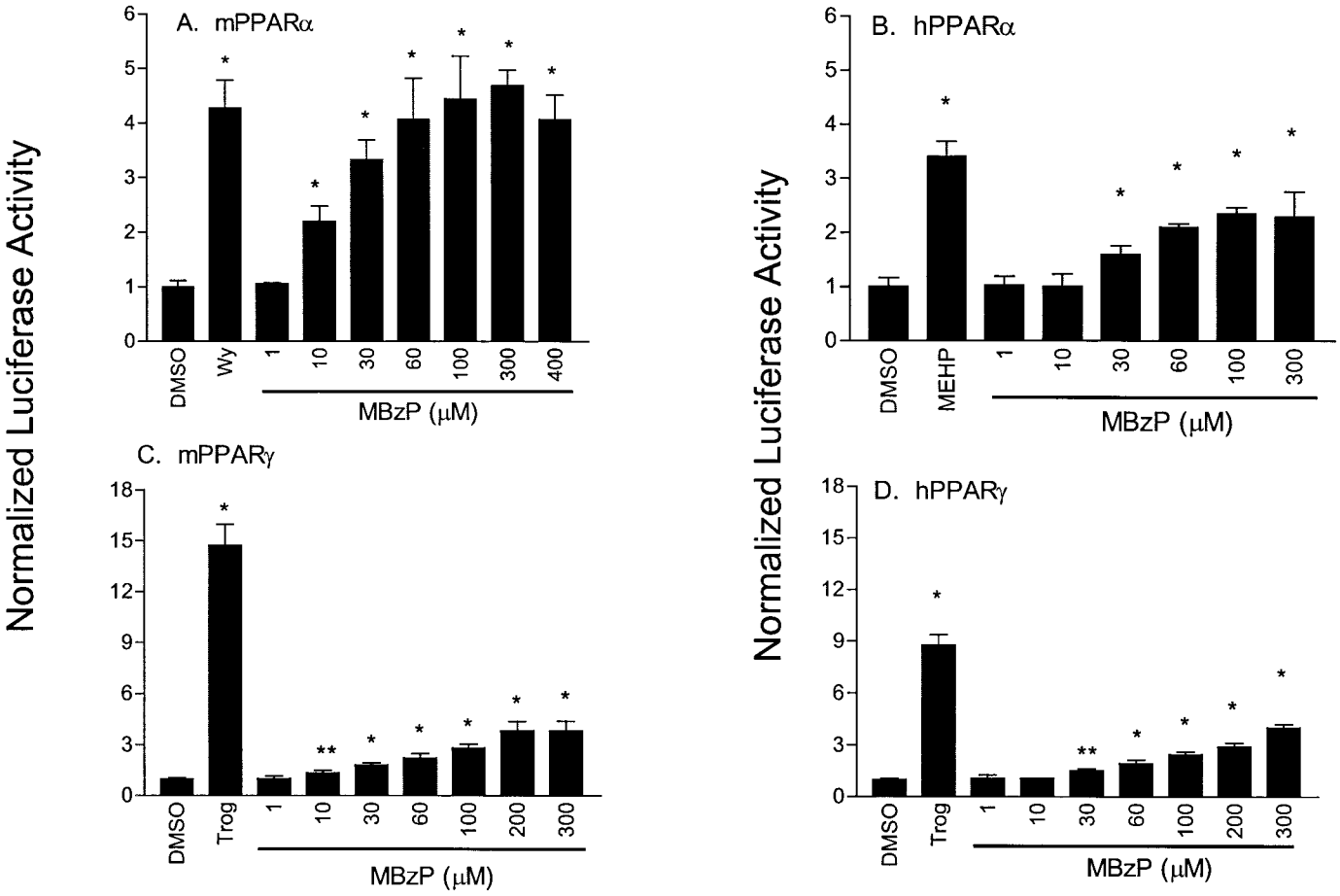

FIG. 3. Activation of mouse and human PPARα and PPARγ by MBzP. COS-1 cell transfection, treatment with MBzP at the indicated concentrations, and determination of relative luciferase activities were carried out as described in Materials and Methods. Data shown are normalized luciferase reporter values normalized to untreated DMSO controls, mean ± SD, $n = 3$. Wy: Wy-14,643 (5 μM) and MEHP (20 μM) were used as positive controls for mouse PPARα activation, as shown. Troglitazone (Trog; 3 μM) was used as a positive control for PPARγ activation. $*p < 0.01$; $**p < 0.05$ from DMSO-control values by ANOVA.

(Fig. 8). MBzP and MBuP are, thus, both able to activate PPARγ in an intact cell system.

DISCUSSION

Environmental exposure to DEHP, its active monoester hydrolysis product MEHP, and other phthalate esters occurs when these compounds leach from plastic, leading to the contamination of food, water, and soil (Albro and Lavenhar, 1989). Although DEHP is produced in the largest quantity, human exposure to phthalate monoesters derived from other phthalates is apparently much greater than that of DEHP and MEHP (Blount *et al.*, 2000). Rodent model studies demonstrate a causal link between exposure to phthalates and toxicity to liver, kidney, and testis, in addition to reproductive toxicity and teratogenicity, in some cases mediated by the nuclear receptor PPARα. The objective of this study was to determine the potential of environmental phthalate monoesters for activation of PPARα and PPARγ, using cell-based *trans*-activation assays and by monitoring PPAR target gene expression (PPARα) or PPAR-dependent adipocyte differentiation (PPARγ).

Long-term administration of phthalates leads to rodent hepatocarcinogenesis (Reddy *et al.*, 1980) by a mechanism that is dependent on PPARα (Peters *et al.*, 1997). Hepatic peroxisome proliferation and the associated hepatocarcinogenic response are not caused by DEHP itself but by its bioactive metabolite, MEHP (Albro *et al.*, 1989). In this study, MEHP activated mouse and human PPARα at low micromolar levels, with the mouse receptor ~5-fold more sensitive to MEHP (EC_{50} = 0.6 μM) than human PPARα (EC_{50} = 3.2 μM). Mouse and human PPARγ were also activated by MEHP in the micromolar range (EC_{50} = 6–10 μM), as determined in cell-based transient *trans*-activation assays. These assays enabled us to compare the phthalate monoester responsiveness of PPAR from a species that is highly responsive to classic peroxisome proliferator

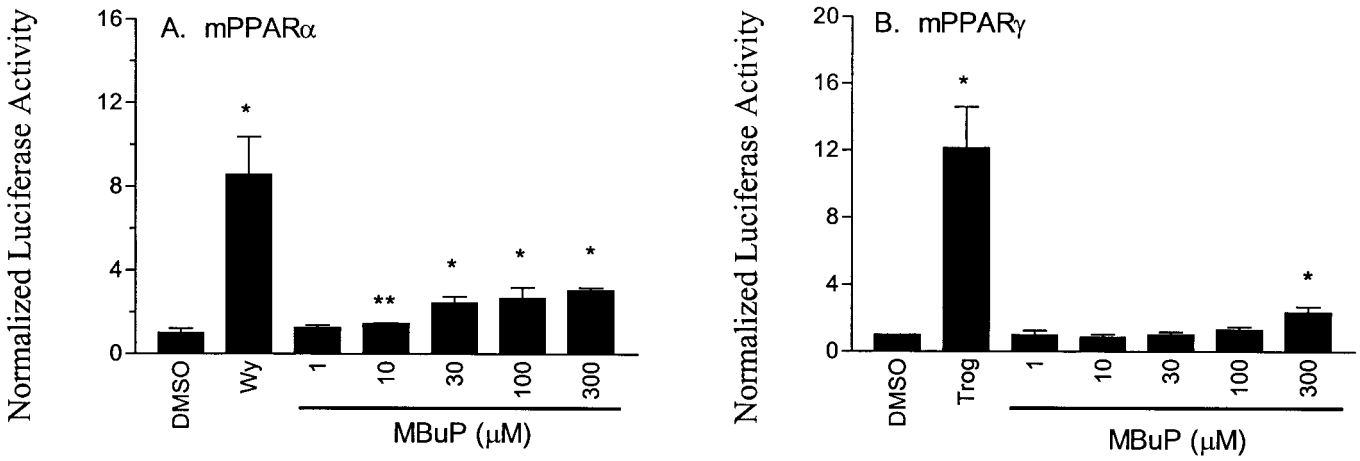

FIG. 4. Activation of mouse PPARα and PPARγ by MBuP. COS-1 cell transfection, treatment with MBuP at the indicated concentrations, and determination of relative luciferase activities were carried out as described in Materials and Methods. Data shown are normalized luciferase reporter values normalized to untreated DMSO controls, mean ± SD, $n = 3$. Wy: Wy-14,643 (5 μM was used as a positive control for mouse PPARα activation. Troglitazone (Trog; 3 μM) was used as a positive control for mouse PPARγ activation. $*p < 0.01$; $**p < 0.05$ from DMSO-control values by ANOVA.

chemicals (mouse) with that of a species that is poorly responsive (humans). Potential limitations of this assay system include the possibility that differences in PPAR plasmid expression, relative mRNA levels, and/or stability of mouse and human PPARα and PPARγ proteins may result in differences in the absolute levels of each PPAR protein in the transfected cells. Because PPAR isoform and species-specific antibodies were not available, it was not possible to determine the precise expression levels of the four PPAR proteins (mouse and human PPARα and PPARγ) included in this study. However, such differences would not alter the intrinsic ability of the phthalate esters to activate each PPAR, as discussed elsewhere (Maloney and Waxman, 1999).

Although the observed peroxisome proliferation and other hepatic toxicities of PPCs such as DEHP and MEHP are dependent on PPARα (Lee *et al.*, 1995), the testicular, renal, and developmental toxicities exhibited by DEHP are independent of PPARα (Ward *et al.*, 1998) and may conceivably be mediated by other PPAR forms, such as PPARγ. Decreased testosterone production is observed in mice fed diets containing DEHP or MEHP (Oishi and Hiraga, 1980a; Oishi and Hiraga, 1980b), and testosterone secretion by Leydig cells in the testis is inhibited following phthalate monoester treatment (Jones *et al.*, 1993), suggesting that Leydig cells, which express PPARα and PPARδ (Braissant *et al.*, 1996) but not PPARγ (Gazouli *et al.*, 2002), may be the target for MEHP's testicular toxicity. However, the inhibitory effects of MEHP on testosterone production are dependent on PPARα (Gazouli *et al.*, 2002) and are, thus, distinct from the PPARα-independent testicular toxicity of MEHP. Rather, the testicular toxicity of MEHP seems more likely to be associated with the Sertoli cell toxicant effect of these phthalates, which leads to a disruption of germ cell apoptosis (Richburg and Boekelheide, 1996).

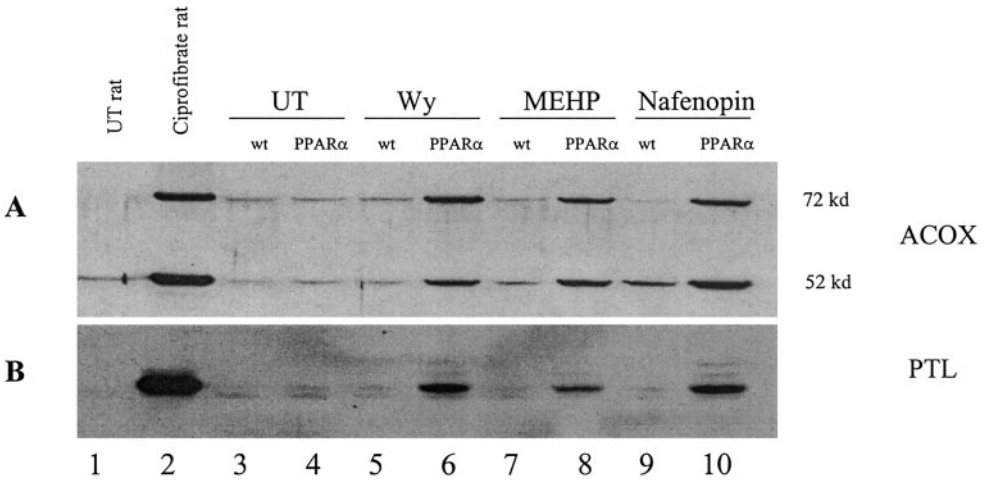

FIG. 5. Peroxisomal enzyme induction in FAO and FAO-PPARα cells treated with peroxisome proliferators. Shown is a Western blot of rat liver microsomes (lane 1, UT [untreated]; lane 2, ciprofibrate-induced) or FAO cell extracts probed with antibody to ACOX or PTL, as described under Materials and Methods. Data shown are for FAO cells (lanes 3, 5, 7, 9) and FAO-PPARα cells (lanes 4, 6, 8, 10) treated for 48 h with DMSO control (lanes 3, 4), 100 μM Wy-14,643 (lanes 5, 6), 250 μM MEHP (lanes 7, 8), or 250 μM nafenopin (lanes 9, 10).

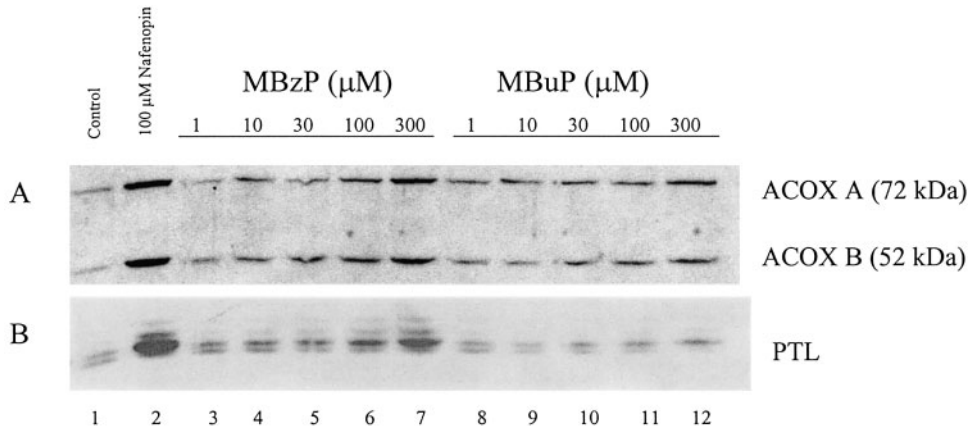

FIG. 6. Induction of fatty acid β-oxidation enzymes in FAO-PPARα cells after phthalate monoester treatment. FAO-mPPARα cells were treated with the indicated concentrations of MBzP or MBuP for 48 h. Total cell extracts were analyzed by Western blotting, as described in Materials and Methods. Data shown are representative of the responses to MBzP and MBuP seen in other independent experiments. Densitometry showed a ~4-fold induction of both the 72-kDa and 52-kDa bands in response to treatment with MBzP, whereas a 2- to 3-fold induction of both bands was observed with MBuP. PTL protein was increased 6-fold with MBzP treatment, whereas minimal to no induction was observed with MBuP.

Further study is required to determine whether the activation of PPARγ is involved in this latter toxicity of MEHP.

MBzP, the primary metabolite of butyl benzyl phthalate, and M(n)BuP, the primary metabolite of dibutyl phthalate, are both teratogenic in animal studies (Ema *et al.*, 1993a,b). MBzP was found to activate PPARα, as did MBuP, both in a *trans*-activation assay and by the induction of the endogenous PPARα target gene proteins ACOX and PTL in FAO rat liver cells that stably express elevated levels of PPARα. Furthermore, MBzP increased mRNA levels of both PTL and PBE, whereas MBuP treatment increased PBE mRNA only. A small ($\leq$2-fold) increase in ACOX mRNA was observed with MBzP or MBuP treatment, but this effect did not reach statistical significance. In the present *in vitro* studies, human PPARα was found to be somewhat less sensitive than mouse PPARα to both MBzP and MBuP. *In vivo* studies will be required to determine whether the lower sensitivity of human PPARα to MBzP and MBuP helps explain the significantly reduced peroxisome proliferation observed in human compared with rodent liver cells. Other factors are likely to include the lower PPARα expression in human compared with rodent liver (Palmer *et al.*, 1998). MBzP activated mPPARα to the same extent as the potent peroxisome proliferator Wy-14,643, although at considerably higher concentrations. Similarly, MBzP activated hPPARα to 70% of the maximal level observed with MEHP. By contrast, the activation of mPPARα by MBuP was substantially lower than the maximal activation observed with either MBzP or Wy-14,643. The potential of MBzP to activate mouse PPARα demonstrated by these experiments is consistent with the potential of the parent compound, butyl benzyl phthalate, to induce hepatic peroxisomal proliferation in rodents (Marsman, 1995; National Toxicology Program, 1997).

This study established a rank order for phthalate activation of mouse and human PPARα: MEHP > MBzP > MBuP > M(n)BuP. Furthermore, only monoester metabolites were capable of activating PPAR; each of the diester phthalates investigated (dimethyl phthalate, diethyl phthalate, and DEHP) was inactive at the highest concentrations tested (typically 300 μM). These findings are in agreement with the relative ability of phthalate esters to induce peroxisome proliferation in rodents, where long-chain esters are more potent than short-chain esters, and branch-chain esters are more potent than straight chains (Barber *et al.*, 1987). Other data suggest that the carboxyl moiety of phthalates is critical for peroxisome proliferation. For example, several DEHP metabolites (MEHP and 2-ethylhexanoic acid) are more potent peroxisome proliferators than another metabolite (2-ethylhexanol; Cornu *et al.*, 1992; Keith *et al.*, 1992). These data agree with earlier findings from this laboratory, where the DEHP metabolites MEHP and 2-ethylhexanoic acid both activated PPARα in a transient transfection assay, whereas no activation was observed with 2-ethylhexanol (Maloney and Waxman, 1999). A better understanding of these structure-activity relationships may help to determine what contribution, if any, PPARα and PPARγ make to phthalate toxicity in response to human environmental or occupational exposure.

PPARγ regulates a broad range of physiological processes, including adipogenesis, fatty acid uptake, cell proliferation, and the formation of atherosclerotic plaques (Rosen and Spiegelman, 2000, 2001). The present PPARγ *trans*-activation assays revealed that mouse and human PPARγ both respond to MBzP, although the sensitivity of PPARγ to MBzP (EC_{50} = 75–100 μM) and the maximal activation, compared with that achieved with the established PPARγ agonist troglitazone, was several-fold lower than seen in the case of MBzP and PPARα. Moreover, MBuP exhibited weak (mouse PPARγ) or no (human PPARγ) activation of PPARγ. Several other phthalate

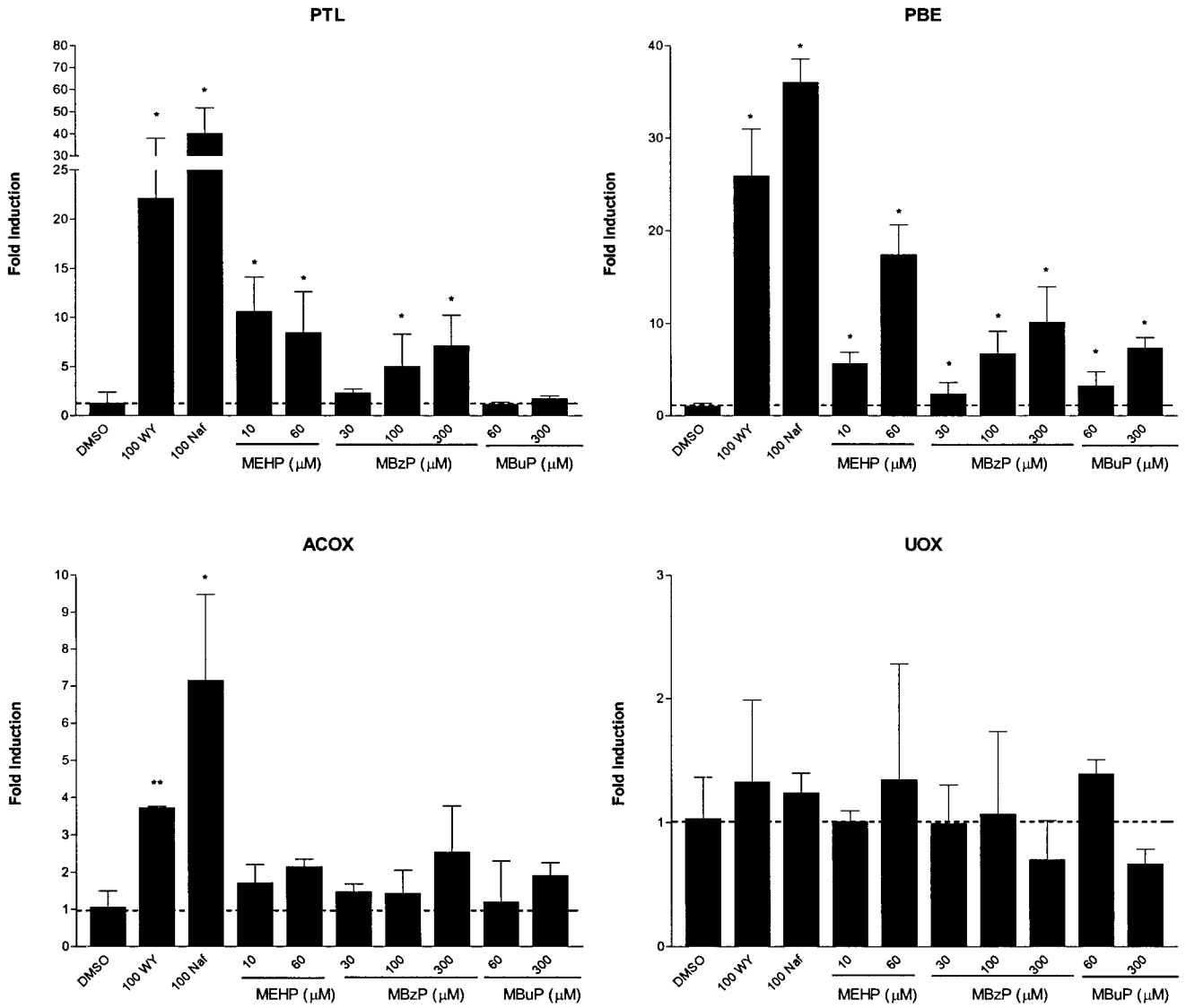

FIG. 7. Induction of fatty acid β-oxidation pathway mRNAs following phthalate monoester treatment of FAO-mPPARα cells. Real-time PCR analysis was carried out as described in Materials and Methods. Data shown are fold induction in mRNA levels, relative to DMSO-treated controls. PTL, ACOX, PBE, and urate oxidase (UOX) mRNA values were normalized to 18s RNA levels from the same cDNA samples to control for differences in the amount of cDNA template. Data shown are the mean ± SD of triplicate PCR analyses of $n = 3$ independent RNA samples isolated from cell cultures. $*p < 0.01$; $**p < 0.05$ vs. DMSO-control by ANOVA. WY, Wy-14,643; Naf, nafenopin, as in Fig. 5. Note Y-axis scale differences between panels.

esters were found to be inactive (monomethyl phthalate, diethyl phthalate, and M(n)BuP). Activation of PPARγ by MBzP was verified using the 3T3-L1 mouse embryo fibroblast cell model, which undergoes adipogenic differentiation when treated with a PPARγ agonist in the presence of a cocktail of hormones. MBzP and MBuP both induced a dose-dependent increase in adipocyte differentiation, with MBuP less active than MBzP, in agreement with the rank order effectiveness for PPARγ activation (MEHP > MBzP > MBuP > M(n)BuP) seen in the COS cell transfection experiments. PPARγ is expressed at high levels in a broad range of human tissues, including heart, skeletal muscle, colon, intestine, kidney, and adipose tissue. Recent studies highlight the critical role of this receptor in adipocyte differentiation, insulin sensitivity, type 2 diabetes, atherosclerosis, and cancer. Consequently, MEHP, MBzP, MBuP, and other environmental chemicals that activate PPARγ may potentially interfere with critical PPARγ-dependent physiological processes, leading to adverse consequences. Other data suggest, however, a potential therapeutic role for PPARγ ligands in the treatment of several cancers, with PPARγ agonists inducing terminal differentiation of human liposarcoma cells (Demetri *et al.*, 1999) and malignant breast cancer cells (Mueller *et al.*, 1998), raising the possibility that PPARγ activation by phthalates may have beneficial effects.

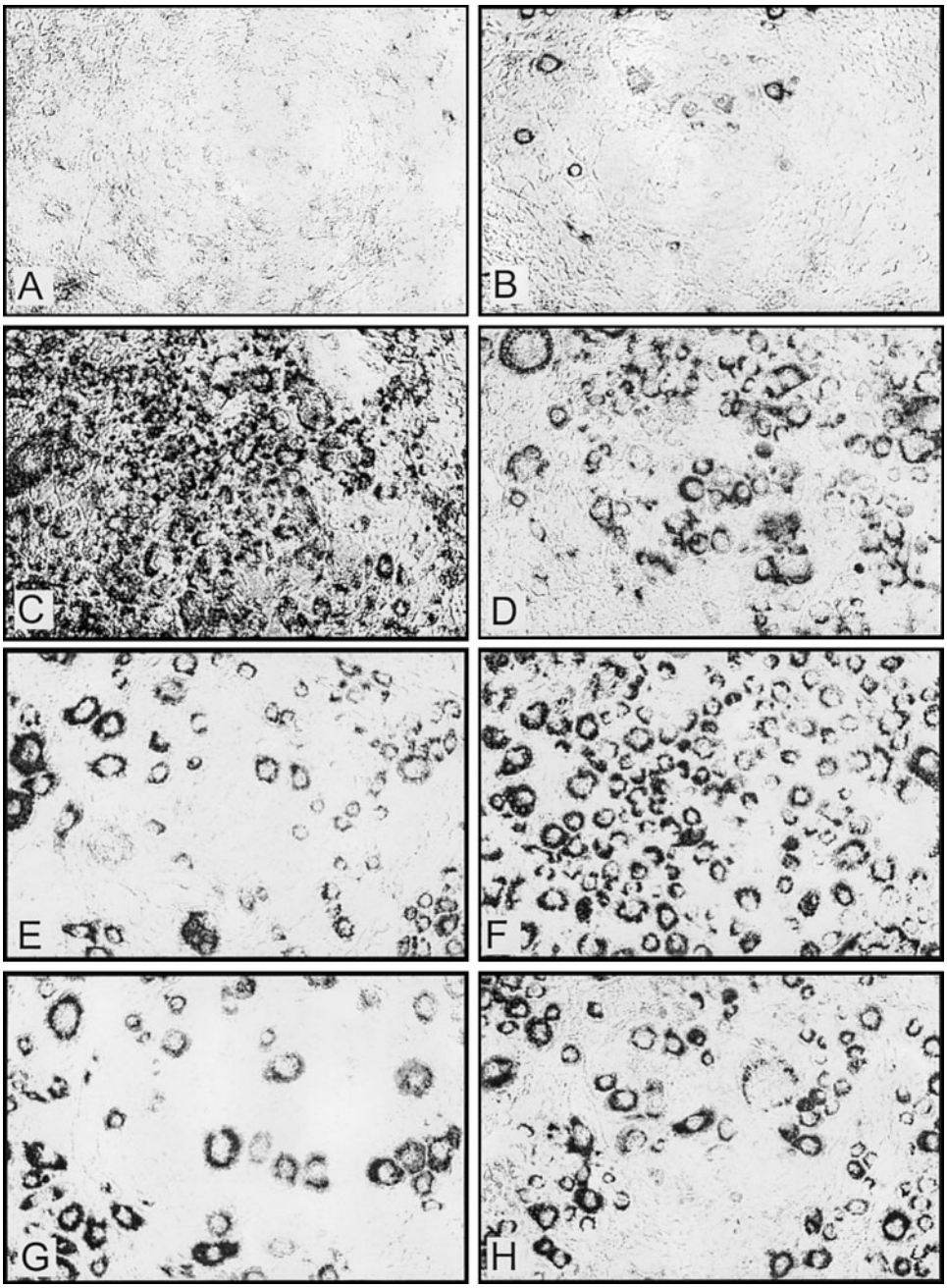

FIG. 8. Phthalate monoester-stimulated adipogenesis in 3T3-L1 cells. 3T3-L1 cells were cultured for 6 days (see Materials and Methods) under the following treatment conditions: (A) DMSO; (B) differentiation cocktail; (C) differentiation cocktail + 10 μM troglitazone; (D) differentiation cocktail + 50 μM MEHP; (E) differentiation cocktail + 100 μM MBzP; (F) differentiation cocktail + 300 μM MBzP; (G) differentiation cocktail + 100 μM MBuP; (H) differentiation cocktail + 300 μM MBuP. Fat cell differentiation is indicated by the accumulation of lipid droplets, whose formation is seen to be dependent on the presence of a PPARγ activator (panels C–H vs. panels A and B). Data shown are representative of 2–3 additional independent experiments. Cells were stained with Oil Red O and photographed, as described in Materials and Methods.

The ineffectiveness of M(n)BP with respect to activation of PPARα and PPARγ is surprising, given the finding that the parent compound, di-*n*-butyl phthalate, can induce expression of at least some PPAR target genes (albeit weakly) in liver and testes (Kobayashi *et al.*, 2003). Conceivably, these responses, as well as the reproductive toxicities associated with di-*n*-butyl phthalate exposure *in vivo* (Foster *et al.*, 2000), may be associated with activation of PPARδ, which plays a role in embryonic development (Barak *et al.*, 2002). Alternatively, these responses may be mediated by a metabolite other than M(n)BuP or perhaps by a PPAR-independent mechanism.

In a study designed to determine human exposure to seven commonly used phthalates, Blount *et al.*, (2000) measured several monoester metabolites in human urine samples. The maximum urinary phthalate monoester concentrations for MBzP and M(n)BP were 1,020 ng/ml (4 μM) and 4,670 ng/ml (21 μM), respectively. Based on these measurements and animal pharmacokinetic data, maximal human daily exposure levels of 29 and 110 μg/kg/day were estimated for MBzP and M(n)BuP, respectively (Kohn *et al.*, 2000). Median exposures calculated in this manner were comparable with those calculated by the National Toxicological Program Center for the

Evaluation of Risks to Human Reproduction (National Toxicology Program, 2000). Because limited human phthalate pharmacokinetic data exist, there are several uncertainties that may affect exposure estimates, e.g., uncertainties in creatinine excretion rates and estimates of total and urinary fractions of the dose eliminated. Taking these uncertainties into account, the above human exposure estimates are likely to be reliable within an order of magnitude (Kohn *et al.*, 2000), with the possibility that some individual exposures may be substantially higher. For example, maximal exposure of di-*n*-butyl phthalate for women aged 20–40 years is five times greater than exposure estimates for the general population (Kohn *et al.*, 2000). Although M(n)BuP, the monoester hydrolysis product of di-*n*-butyl phthalate, did not activate PPARα or PPARγ at the concentrations tested in this study, it is apparent that specific individuals within the population are exposed to levels of the monoester metabolites of di-*n*-butyl phthalate and other environmental phthalate activators of PPAR that far exceed average exposure levels in the overall population (Blount *et al.*, 2000). Further study is required to determine whether these phthalate levels *in vivo* give rise to tissue concentrations sufficient to achieve the activation of PPARα and PPARγ observed in the present cell culture studies.

In conclusion, multiple environmental phthalates were shown to activate PPARα and PPARγ. Although the weight of evidence based on toxicokinetic data suggests that humans are refractory toward PPC-induced, PPARα-dependent hepatic peroxisome proliferation, the toxicological impact of phthalates and other PPCs that activate PPARγ are unknown. PPARγ is much more highly expressed in human tissues than is PPARα (Kliewer *et al.*, 2001) and is thought to play an important role in differentiation, insulin sensitivity, atherosclerosis, and cancer. There is, consequently, great interest in understanding the human health impact of environmental chemicals that interfere with the tightly controlled metabolic and regulatory processes mediated by PPARγ. Further investigation is required to determine relevant phthalate tissue concentrations and whether these compounds activate PPARα or PPARγ in human cells and tissues and to identify highly exposed populations and individuals who may be at risk.

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