

## Differential Activation of Peroxisome Proliferator-activated Receptors by Eicosanoids\*

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**Peroxisome proliferator-activated receptors (PPARs) are nuclear hormone receptors that regulate gene transcription in response to peroxisome proliferators and fatty acids. PPARs also play an important role in the regulation of adipocyte differentiation. It is unclear, however, what naturally occurring compounds activate each of the PPAR subtypes. To address this issue, a screening assay was established using heterologous fusions of the bacterial tetracycline repressor to several members of the peroxisome proliferator-activated receptor (PPAR) family. This assay was employed to compare the activation of PPAR family members by known PPAR activators including peroxisome proliferators and fatty acids. Interestingly, the activation of PPARs by fatty acids was partially inhibited by the cyclooxygenase inhibitor indomethacin, which prevents prostaglandin synthesis. Indeed, prostaglandins PGA1 and 2, PGD1 and 2, and PGJ2-activated PPARs, while a number of other prostaglandins had no effect. We also screened a variety of hydroxyeicosatetraenoic acids (HETEs) for the ability to activate PPARs. 8(S)-HETE, but not other (S)-HETEs, was a strong activator of PPAR $\alpha$ . Remarkably, PPAR activation by 8(S)-HETE was stereoselective. In addition, 8(S)-HETE was able to induce differentiation of 3T3-L1 preadipocytes. These results indicate that PPARs are differentially activated by naturally occurring eicosanoids and related molecules.**

The cloning and characterization of nuclear receptors has greatly enhanced our understanding of gene regulation by lipophilic hormones such as steroids, vitamin D, thyroxine, and retinoids. These receptors comprise a superfamily of transcription factors containing highly related DNA-binding domains (1, 2). This family includes multiple subtypes of receptors for thyroxine and retinoids, encoded by distinct genes which are regulated quite differently during development and in the adult. There is evidence that retinoid receptor subtypes differentially bind retinoids (3, 4). In addition, there is a large number of "orphan" receptors that have important roles in the develop-

ment of species as diverse as invertebrates and mammals (5). It is not known whether all of the orphan receptors will prove to be activated directly by small lipophilic molecules. However, at least one class of nuclear receptor, the retinoid X receptor, was initially an orphan member of the family (6) but later proved to bind and activate transcription in response to a naturally occurring retinoid, 9-*cis*-retinoic acid (7, 8).

Peroxisome proliferator-activated receptors (PPAR)<sup>1</sup> were initially cloned as orphan receptors and were subsequently found to be activated by peroxisome proliferators. These include compounds such as clofibrate and Wy-14,643 which have been used clinically to treat hyperlipidemia, as well as by plasticizers which may be carcinogenic for mammals (9). There are multiple subtypes of PPAR, called  $\alpha$ ,  $\delta$  (or NUC-I), and  $\gamma$  in mammals. Studies from several investigators have suggested that these subtypes are differentially activated by various agents (10–14). PPAR $\alpha$  is most abundant in liver, while the tissue distribution of PPAR $\delta$  is more widespread. In contrast, expression of PPAR $\gamma$  is limited to adipose tissue (15, 16) and, indeed, activators of PPAR can suffice to induce adipose conversion of preadipocyte cell lines (17, 18). Moreover, ectopic expression of PPAR $\gamma$  causes fibroblast cell lines to differentiate into adipocytes in the presence of PPAR activators (19). The role of PPARs in adipocyte differentiation is likely to be complex, since other PPARs are induced during adipocyte differentiation (18, 20).

Because none of the PPAR activating compounds have been demonstrated to bind directly to PPAR, a number of groups have searched for an endogenous ligand. These studies revealed fatty acids to be activators of PPAR at high micromolar concentrations (21, 22). It remains unclear, however, whether fatty acids are physiological activators of one or more PPAR subtypes. Therefore, we devised a screen for PPAR activators and applied it to known and potential activating compounds. PPAR $\alpha$ ,  $\delta$ , and  $\gamma$  had highly divergent properties with respect to activation by peroxisome proliferators and fatty acids. In addition, we found that prostaglandins A, D, and J differentially activated PPAR subtypes. Moreover, the naturally occurring, 12-*O*-tetradecanoylphorbol-13-acetate-inducible eicosanoid 8(S)-hydroxyeicosatetraenoic acid (8(S)-HETE) activated PPAR $\alpha$  with greater effectiveness than other known compounds. Activation by 8-HETE was stereoselective, and

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<sup>1</sup> The abbreviations used are: PPAR, peroxisome proliferator-activated receptor; Wy-14,643, pirinixic acid; ETYA, 5,8,11,14-eicosatetraenoic acid; LA, linoleic acid; DHA, docosahexaenoic acid; NDGA, nordihydroguaiaretic acid; HETE, hydroxyeicosatetraenoic acid; PG, prostaglandin; HPLC, high performance liquid chromatography; PCR, polymerase chain reaction; TetR, tetracycline repressor; TetO, tetracycline operon;  $\beta$ -gal,  $\beta$ -galactosidase; DMEM, Dulbecco's modified Eagle's medium; LBD, ligand-binding domain.

other (S)-HETEs were ineffective. In addition to activating PPAR-mediated transcription, 8(S)-HETE induced adipogenic differentiation of 3T3-L1 preadipocytes. Together, our results confirm that PPAR subtypes are pharmacologically distinct and suggest that certain naturally occurring eicosanoids are PPAR activators.

#### MATERIALS AND METHODS

**Chemicals**—Wy-14,643 (pirinixic acid; 4-chloro-6-(2,3-xylylidino)-2-pyrimidinylthioacetic acid) was obtained from Chemsyn Science Laboratories (Lenexa, KS) or from Wyeth-Ayerst (Radnor, PA). Clofibrate, ETYA (5,8,11,14-eicosatetraenoic acid), linoleic acid (LA), docosahexaenoic acid (DHA), indomethacin, and nordihydroguaiaretic acid (NDGA) were purchased from Sigma. Concentrated stocks of these compounds were prepared in Me<sub>2</sub>SO or EtOH. The final concentrations used for cells were made by dilutions with culture media. ME<sub>2</sub>SO or EtOH concentrations in final cell media were ≤0.1–0.2%. All prostaglandins, the (S)-hydroxyeicosatetraenoic acids (HETE) HPLC mixture, the (±)-HETE HPLC mixture, and the pure (S)- and (R)-HETE compounds were purchased from Cayman Chemical Company (Ann Arbor, MI). The prostaglandins and (S)-HETE pure compounds were supplied as concentrated solutions in EtOH. Final concentrations were similarly made by dilutions in culture media. For 3T3-L1 preadipocyte differentiation experiments, the HETE stocks were concentrated prior to addition to media. The (S)-HETE HPLC mixture (100 μg/ml) contains 20 μg/ml each of the following: 5(S)-HETE, 8(S)-HETE, 11(S)-HETE, 12(S)-HETE, and 15(S)-HETE. The (±)-HETE HPLC mixture (100 μg/ml) contains 20 μg/ml each of the following: 5(±)-HETE, 8(±)-HETE, 11(±)-HETE, 12(±)-HETE, and 15(±)-HETE. These HPLC stock mixtures were diluted 200-fold to give the final concentration (~0.3 μM of each compound) tested on cells.

**Plasmids**—The reporter gene plasmid pTetO-Luc was kindly provided by Dr. H. Bujard (University of Heidelberg, Germany) (23). It contains the firefly luciferase gene placed downstream from the operator sequence of the bacterial tetracycline operon (TetO sequence). A minimal TATA element from cytomegalovirus was inserted between the TetO and the luciferase gene. The pSG5TetR-ER, pSG5TetR-PPAR $\alpha$ , pSG5TetR-PPAR $\delta$  (hNuc-1), and pSG5TetR-PPAR $\gamma$  fusion receptor expression constructs all contain the bacterial tetracycline repressor (TetR) (serves as a TetO-specific DNA-binding domain) in-frame fused to a full-length or to the ligand-binding domain of these receptors (Fig. 1). The parental vector pSG5TetR-PL (provided by Dr. W. Kaelin, Dana Farber Cancer Institute, Boston, MA) contains the TetR domain generated by polymerase chain reaction (PCR) using PUHD15-1 (24) as a template. The 3' PCR primer included extra polylinker sites (*Bam*HI/*Sma*II/*Eco*RI) to facilitate construction of TetR fusions. To construct pSG5TetR-ER(FL), the full-length human ER cDNA was isolated by digestion of pMA-ER (25, 26) with *Eco*RI, purified, and inserted into the *Eco*RI site of pSG5TetR-PL. The human PPAR $\alpha$  cDNA was obtained from Dr. F. Gonzalez (National Cancer Institute, Bethesda, MD) (27). To construct pSG5TetR-PPAR $\alpha$  full-length and the ligand-binding domain fusions, PCR was performed using the following primers with added *Bam*HI site on both 5' and 3' primers. For PPAR $\alpha$  full-length, 5' primer (GGA TCC ATG GTG GAC ACG) and 3' primer (GGA TCC TCA GTA CAT GTC). For PPAR $\alpha$  ligand-binding domain, 5' primer (GGA TCC TCA CAC AAC GCG ATT CGT) and the same 3' primer used for hPPAR $\alpha$  full-length PCR. The PCR products were first cloned into pCRII using a TA cloning kit (Invitrogen). The *Bam*HI fragments containing the PCR products were then excised from pCRII, purified, and inserted into the *Bam*HI site of pSG5TetR-PL. The human PPAR $\delta$  (NUCI) cDNA was provided by Dr. A. Schmidt (Merck Research Laboratories, West Point, PA) (11). The mouse PPAR $\gamma$ 1 cDNA was provided by Dr. B. O'Malley (Baylor College of Medicine, Houston, TX) (10). PCR of the full-length PPAR $\delta$  were carried out using 5' primer (CC GGA TCC ATG GAG CAG CCA) and 3' primer (GGA TCC TTA GTA CAT GTC). PCR of the ligand-binding domain of PPAR $\gamma$ 1 was performed with 5' primer (GGA TCC TCT CAC AAT GCC ATC AGG) and 3' primer (GGA TCC TCC TGC TAA TAC AAG TCC). The PCR products were similarly cloned into pCRII by TA cloning kit. These were then excised from pCRII as *Bam*HI fragments, purified, and inserted into the *Bam*HI site of pSG5TetR-PL (Fig. 1).

**Screening for PPAR Activators Using Transiently Transfected Fusion Proteins**—The human osteosarcoma line U2OS (ATCC HTB96) cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (BioWhittaker, Walkersville, MD) at 37 °C, 5% CO<sub>2</sub>. They were typically split 1:5 and 1:10 every 3–4 days. Transfection of U2OS cells with pTetO-Luc and various

fusion receptor expression vectors was performed by electroporation as follows. Cells were split (1:2) in 15-cm culture dishes 24 h prior to transfection experiments. For each transfection, cells were collected by gentle trypsinization followed by low speed centrifugation. Collected cells were resuspended in 1 × phosphate-buffered saline (-Mg<sup>2+</sup>, -Ca<sup>2+</sup>) at ~20 × 10<sup>6</sup> cells/ml. Approximately 8–10 × 10<sup>6</sup> cells (0.45 ml) were mixed with DNAs in a volume of 20 μl or less (20 μg of pTetO-Luc, with or without 0.5–3.0 μg of individual pTetR-receptor fusion construct DNAs prepared in 0.1 × TE) and allowed to incubate on ice for 5 min. The cell-DNA mixtures were then transferred into a cuvette (0.4-cm gap distance/0.8-ml volume capacity) and electroporated (0.3 kV/500 microfarads) using a Bio-Rad Gene Pulser. Electroporated cells were immediately resuspended in media containing 10% fetal bovine serum which was treated with dextran-coated charcoal (DCC-fetal bovine serum) (28) at a density of 150,000 cells/ml. Cells were then plated in 96-well microtiter plates (Microlite<sup>®</sup> from Dynatech Laboratories) at ~20,000 cells/150 μl/well using an eight-channel multistep pipetter. 50 μl of control or test compounds (see below) diluted fresh in media were then added to cells. Each control (Me<sub>2</sub>SO or EtOH) and various test treatments with chemicals were carried out using 8–12 repeated wells. The presented results are the average values of the repeated wells.

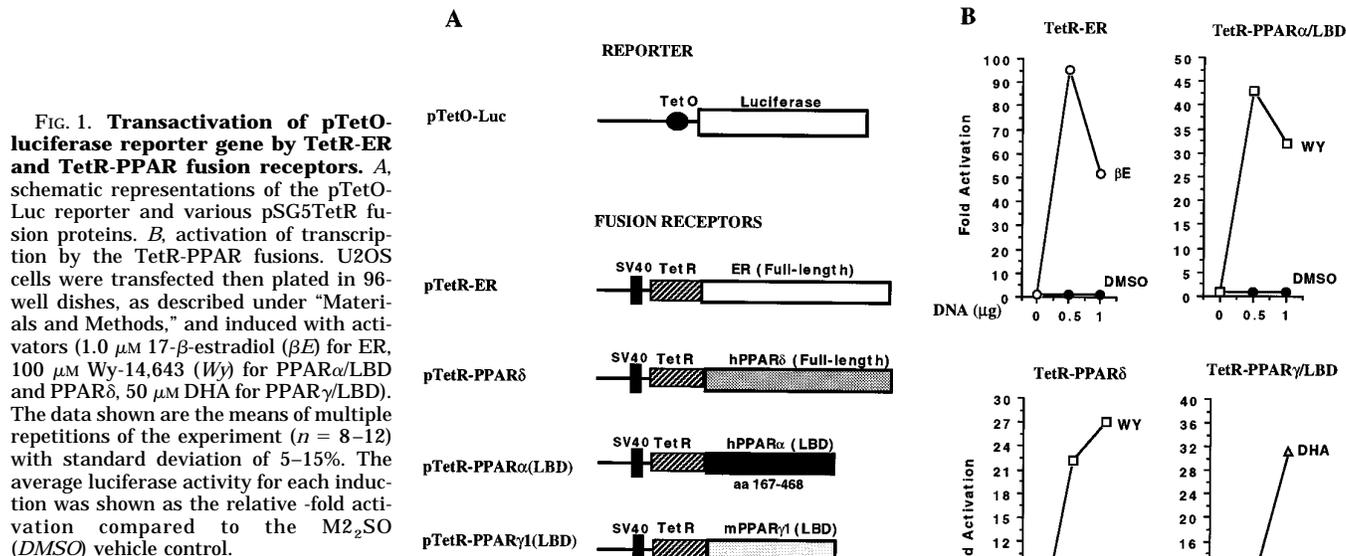
48 h after transfection and addition of activators, media was removed from cells. The 96-well plates were washed two times with 100 μl/well 1 × phosphate-buffered saline (-Mg<sup>2+</sup>, -Ca<sup>2+</sup>). Cells were then lysed by 20 μl of 1 × luciferase lysis reagent (25 mM Tris-phosphate, pH 7.8, 2 mM dithiothreitol, 2 mM 1,2-diaminocyclohexane-*N,N,N,N*-tetraacetic acid, 10% glycerol, 1% Triton X-100) for 15 min at room temperature using an orbital shaker. A 96-well format luminometer (ML3000, Dynatech Laboratories) was used for reading the luciferase assay results. Immediately after addition of 100 μl/well of luciferase assay reagent (25 mM glycylglycine, 15 mM Mg(OAc)<sub>2</sub>, 0.75 mM ATP, 20 mM dithiothreitol, 0.1 mM EDTA, 0.8 mM luciferin, 0.24 mM acetyl-CoA), the plates were read in the appropriate mode (*e.g.* cycle mode). The mean value and its associated standard deviation for each set of repeated wells were obtained using BioLink software that was supplied with the luminometer. The effects of various compounds on activation of PPARs were presented as "fold-activation" relative to the vehicle (Me<sub>2</sub>SO or EtOH) control values. Transfection efficiency was not a variable in these experiments because cells were simultaneously transfected by electroporation prior to plating and addition of compounds.

**Transient Transfection of Wild-type PPARs**—JEG-3 human choriocarcinoma cells were maintained and transfected in DMEM low glucose, 10% calf serum. Cells were switched to DMEM low glucose with 10% charcoal and hexane-stripped calf serum 1 h prior to transfection. 60-mm dishes were transfected by the calcium phosphate precipitation method as described previously (29) using 5 μg of PPAR $\alpha$  expression vector (gift of Stephen Green), 1 μg of PPAR-responsive luciferase reporter, and 0.5 μg of  $\beta$ -galactosidase ( $\beta$ -gal) expression vector. The PPAR-responsive reporter gene was created by inserting an oligonucleotide (GATCTAATGTAGGTAATAGTTCATAGGTCA) containing the PPAR-response element from the hydratase dehydrogenase (bifunctional enzyme) gene (30, 31) into the *Bg*III site upstream of the thymidine kinase promoter in pTK-luc (kindly provided by David Moore). EtOH, or compounds in EtOH, was added 16 h after transfection and cells were harvested 24 h later. Cells were lysed in Triton X-100 buffer, and both  $\beta$ -gal and luciferase assays were performed using standard protocols (32). Luciferase activity was measured in relative light units, normalized to  $\beta$ -gal activity which served as internal control for transfection efficiency, and expressed as fold-activation relative to control (no PPAR, no activator).

**Culture and Differentiation of 3T3-L1 Cells**—3T3-L1 cells (ATCC) were cultured in growth medium containing DMEM and 10% bovine calf serum (Hyclone), with a change of media every 2 days. The cells were cultured in growth medium until confluent, then switched to growth medium supplemented with 0.51 mM WY-14,643 (kindly supplied by Wyeth-Ayerst), or 50 μM 8(S)-HETE or 8(R)-HETE, all in ethanol. Control cells were treated with the same volume of ethanol alone. Medium was changed after 2 days, with fresh compound added when appropriate, then the cells were cultured for an additional 5 days prior to harvest for RNA. RNA preparation and Northern analysis were performed as described previously (18).

#### RESULTS

**Activation of TetR-PPAR Fusion Receptors**—Wild-type PPARs by themselves activated transcription of target genes containing PPAR-response elements 4–5 fold, limiting the sensitivity and specificity of this assay when applied to novel



compounds (data not shown). In order to establish a reliable and highly sensitive system to screen and study activators of PPARs, we constructed expression vectors producing proteins consisting of either full-length receptor or receptor ligand-binding domain (LBD) fused to the bacterial TetR (Fig. 1A), which provided the DNA-binding domain and did not activate transcription on its own (data not shown). Transactivation by the fusion proteins was specifically detected using a luciferase (luc) reporter gene containing the operator sequence of the bacterial TetO. This heterologous fusion system provided a sensitive assay with low background activity.

The system was first tested using the human estrogen receptor (hER). The ER full-length receptor and LBD sequences were each fused to the TetR and cotransfected with pTetO-Luc reporter plasmid into U2OS cells, plated with or without 1  $\mu\text{M}$  17- $\beta$ -estradiol (E2). Fig. 1B shows that E2 induced transcription by the full-length receptor 50–100-fold (Fig. 1B). The ER LBD fusion produced very similar results (data not shown). We next constructed and tested TetR-PPAR $\alpha$  (full-length and LBD), TetR-PPAR $\delta$  (full-length), and TetR-PPAR $\gamma$  (LBD) fusion constructs (Fig. 1B). The TetR-PPAR $\alpha$  (LBD) and full-length TetR-PPAR $\delta$  were both activated ~20–40-fold by 100  $\mu\text{M}$  Wy-14,643, a known peroxisome proliferator and activator of PPAR. The PPAR $\alpha$  full-length and LBD constructs gave very similar results except that the full-length PPAR $\alpha$  fusion had a relatively higher background luciferase activity in the absence of added activator (data not shown). The TetR-PPAR $\gamma$  (LBD) fusion was only weakly activated by Wy-14,643 but was activated up to 30-fold by 50  $\mu\text{M}$  DHA (Fig. 1B and see below).

**PPAR $\alpha$ ,  $\delta$ , and  $\gamma$  Are Differentially Activated by Peroxisome Proliferators, Fatty Acids, and ETYA**—We first compared the activation patterns of these fusion receptors using previously described activators for mammalian and *Xenopus* PPAR $\alpha$ . The results in Fig. 2 showed that TetR-hPPAR $\alpha$ /LBD was activated by all five compounds up to 40–60-fold relative to the Me<sub>2</sub>SO control (Fig. 2, upper panel). These data for hPPAR $\alpha$  are consistent with previous studies of  $\alpha$  PPAR subtypes from mouse and *Xenopus*. The TetR-hPPAR $\delta$  was significantly activated by Wy-14,643 (20-fold) and by LA and DHA (10–15-fold). However, we did not observe activation of TetR-PPAR $\delta$  by clofibrate or ETYA (Fig. 2, middle panel). In agreement with our results, Schmidt *et al.* (11) reported PPAR $\delta$  activation by Wy-14,643

but not by clofibrate. For TetR-PPAR $\gamma$ /LBD, we observed relatively weak activation by Wy-14,643 (3-fold) and clofibrate (6-fold) and no activation by ETYA or LA. However, DHA activated the PPAR $\gamma$  fusion by 35-fold (Fig. 2, lower panel). Our observations of poor activation of PPAR $\gamma$  by Wy-14,643 and LA are consistent with the recent studies using wild-type PPAR $\gamma$  by Kliewer *et al.* (13). However, Tontonoz *et al.* (15) have found that LA and ETYA can activate PPAR $\gamma$ 2 when cotransfected with RXR $\alpha$  into NIH3T3 cells. The explanation for this apparent discrepancy is likely to be a combination of the different transfection strategies, reporter genes, and cell lines used in these studies. Nevertheless, our comparison of the TetR-PPAR fusion receptors in a single cell system clearly demonstrated that PPAR subtypes are pharmacologically quite distinct.

**Indomethacin Partially Blocks Activation by PPAR**—We next employed these receptor fusions to screen for novel PPAR activators. To test the possibility that fatty acid metabolism might be needed for PPAR activation, we examined the effects of the cyclooxygenase inhibitor indomethacin as well as the lipooxygenase inhibitor NDGA on the activation of PPAR $\alpha$  and PPAR $\delta$  by Wy-14,643, LA, and DHA, and the activation of PPAR $\gamma$  by DHA (Table I). Neither of these inhibitors affected estradiol-induced transcription by human estrogen receptor. However, 10  $\mu\text{M}$  indomethacin inhibited the Wy-14,643 and DHA activation of PPAR $\delta$  by 58 and 61%, respectively. The activation of PPAR $\delta$  by LA was inhibited by 75%. In contrast, indomethacin only subtly inhibited PPAR $\alpha$  activation by Wy-14,643 (20% inhibition) or LA (30% inhibition) and had no detectable inhibitory effect on PPAR $\gamma$  activation by DHA (Table I). Unlike indomethacin, 10  $\mu\text{M}$  NDGA had little effect on PPAR activation by LA or DHA (data not shown). The results in Table I, however, suggested a potential role of cyclooxygenase in some cellular events that lead to PPAR activation.

**Activation of PPARs by a Subset of Prostaglandins**—Since cyclooxygenase is required for prostaglandin synthesis, we next screened naturally occurring prostaglandins A, B, D, E, F, I, and J for the ability to activate PPARs. Fig. 3 shows that of 15 prostaglandins tested, PGA1 and 2, PGD1 and 2, and PGJ2 activated TetR-PPAR fusions with overall higher efficacy than dietary fatty acids. For PPAR $\alpha$  (Fig. 3, upper panel), PGD2 (15-fold) and PGD1 (10-fold) were most active, followed by

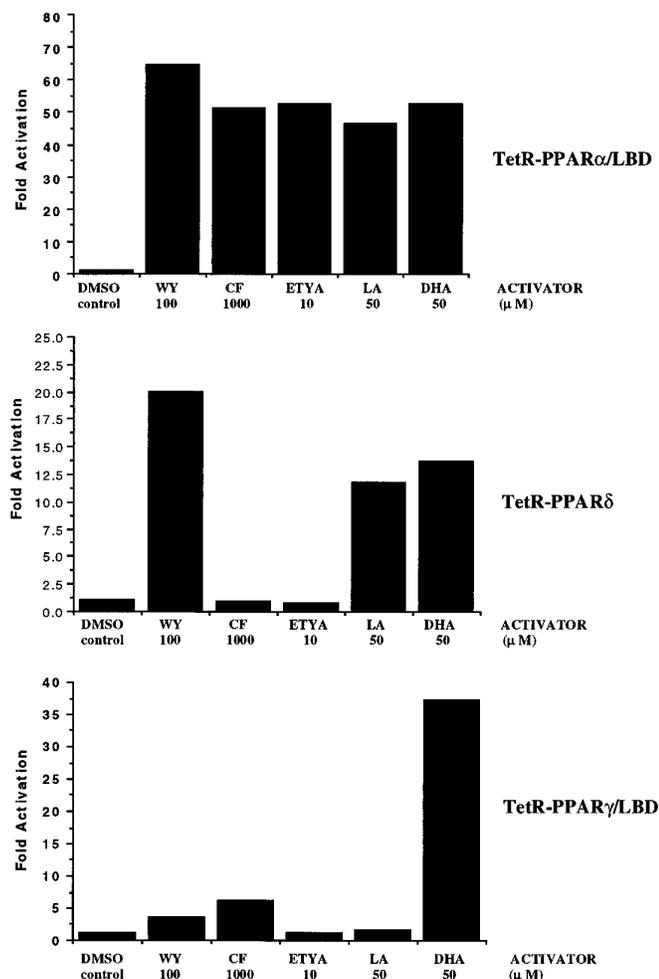


FIG. 2. Differential activation of PPAR $\alpha$ , PPAR $\delta$ , and PPAR $\gamma$  by peroxisome proliferators, fatty acids, and ETYA. U2OS cells were transfected then plated in 96-well dishes as described under "Materials and Methods" then treated with the following concentrations of the indicated PPAR activators: 100  $\mu$ M Wy-14,643, 1.0 mM clofibrate, 10  $\mu$ M ETYA, 50  $\mu$ M LA, and 50  $\mu$ M DHA. Each treatment was carried out in repeated wells ( $n = 8-12$ ). The results for induction were expressed as -fold activation relative to Me<sub>2</sub>SO (DMSO) vehicle controls. WY, Wy-14,643; CF, clofibrate. This experiment was repeated at least three to four times with qualitatively and quantitatively similar results.

PGA1, PGA2, and PGJ2 (each 4–5-fold). For PPAR $\delta$ , PGA1 showed highest activity and was comparable to 100  $\mu$ M Wy-14,643 (Fig. 3, middle panel), followed by PGD2 (10-fold), PGD1 (6-fold), and PGA2 (4-fold). For PPAR $\gamma$ , high levels of activation (70–80-fold) were noted with PGD1 and PGD2 (Fig. 3, lower panel). PGA1 (35-fold), PGA2, and PGJ2 (each ~20-fold) were also significantly active. Although these prostaglandins activated all three PPAR subtypes, there may be some selectivity in this activation. For example, while PGD2 had similar activity for all three receptors, PGA1 appeared to be more selective for PPAR $\delta$  (also see below).

**Potent and Enantioselective Activation of PPAR $\alpha$  by 8(S)-HETE**—Prostaglandins are eicosanoids, a term which broadly refers to metabolites of arachadonic acid. We next tested a less well characterized class of eicosanoids, hydroxyeicosatetraenoic acids, or HETEs. These are intracellular hydroxy fatty acids derived from the oxygenation of arachidonic acid, mainly by lipoxygenases and monooxygenases, many of which have been implicated in a variety of cellular functions (33–35). We initially screened two HPLC mixtures, an (S)-HETE mixture containing 0.3  $\mu$ M each of 5(S)-HETE, 8(S)-HETE, 11(S)-

TABLE I  
Effect of indomethacin on PPAR activation

U2OS cells were transiently cotransfected with pTetO-Luc and each of the indicated TetR fusion receptors, plated in 96-well dishes in the presence of the indicated compounds (100  $\mu$ M Wy-14,643, 50  $\mu$ M linoleic acid, or docosahexaenoic acid, 1  $\mu$ M 17- $\beta$ -estradiol) with or without 10  $\mu$ M indomethacin. Cells were lysed 48 h after treatment for luciferase reporter assay. Results shown are the mean  $\pm$  S.D. of three independent experiments, in which each condition was tested eight times. The indomethacin effect was calculated as percent of the average luciferase activity in the absence of indomethacin.

Receptor	Activator	Indomethacin effect
		% of control
PPAR $\alpha$	Wy-14,643	80 $\pm$ 3 <sup>a</sup>
	Linoleic acid	70 $\pm$ 2 <sup>a</sup>
	Docosahexaenoic acid	80 $\pm$ 20
PPAR $\delta$	Wy-14,643	42 $\pm$ 2 <sup>b</sup>
	Linoleic acid	25 $\pm$ 6 <sup>a</sup>
	Docosahexaenoic acid	39 $\pm$ 7 <sup>a</sup>
PPAR $\gamma$	Docosahexaenoic acid	120 $\pm$ 15
ER	17- $\beta$ -estradiol	95 $\pm$ 2

<sup>a</sup>  $p < 0.01$ .

<sup>b</sup>  $p < 0.001$ . Other values not significantly different than in absence of indomethacin by paired  $t$  test.

HETE, 12(S)-HETE, and 15(S)-HETE, as well as a ( $\pm$ )-HETE mixture containing both (S)- and (R)-stereoisomers of the same five HETEs (the sum of the concentrations of (S) and (R) isoforms was 0.3  $\mu$ M). Fig. 4A shows that the (S)-HETE mixture activated PPAR $\alpha$  ~9-fold, but did not significantly activate PPAR $\delta$  or  $\gamma$ . The ( $\pm$ )-HETE mixture also selectively activated PPAR $\alpha$ , although the magnitude of this effect was only about half that of the (S)-HETE mixture (4–5 fold), consistent with the activation by one or more of the (S)-HETEs (present at half the concentration in the (S) mixture) and suggesting that the (R)-HETEs were inactive.

We next tested the effect of each of one of the five pure (S)-HETEs that were contained in the (S)-HETE mixture. Fig. 4B shows that 8(S)-HETE was responsible for almost all of the activity from the HPLC mixture. 9(S)-HETE, as well as its 9(R)-stereoisomer, were also inactive (data not shown). 1.3  $\mu$ M 8(S)-HETE was as active as 100  $\mu$ M of Wy-14,643 (Fig. 4 and below). As suggested by the results using the (S)-HETE mixture, none of the pure (S)-HETEs activated PPAR $\delta$  or PPAR $\gamma$  (data not shown). To test the stereospecificity of PPAR $\alpha$  activation by 8(S)-HETE, we directly compared the activities of 8(S)-HETE and 8(R)-HETE. The results in Fig. 4C show that while the 8(S)-enantiomer was a strong activator, 8(R)-HETE showed very little activity. These findings indicated that activation of PPAR $\alpha$  by 8(S)-HETE was stereoselective. The ability of only the 8(S)-enantiomer to activate PPAR $\alpha$  but not other PPARs was confirmed using wild type PPARs along with a naturally occurring PPAR-response element (data not shown).

To confirm that these results were not an artifact related to the use of fusion proteins or the TetO element, wild type PPAR $\alpha$  was transfected into JEG3 human choriocarcinoma cells along with a luciferase reporter containing a naturally occurring PPAR-response element from the hydratase-dehydrogenase (bifunctional enzyme) gene (30, 31). As mentioned earlier, PPAR $\alpha$  activated this reporter gene approximately 5-fold in the absence of exogenous activator. Fig. 4D shows that this level of activation was doubled by 8(S)-HETE, but not by 8(R)-HETE. Indeed, the magnitude of activation of PPAR $\alpha$  by 8(S)-HETE was about the same as that induced by maximal concentrations of Wy-14,643 and ETYA.

**Induction of Adipocyte Differentiation by 8(S)-HETE**—The ability of PPAR activators to induce adipocyte differentiation of 3T3-L1 preadipocytes provides another means of assaying for their activity. We therefore studied the effects of the 8-HETEs

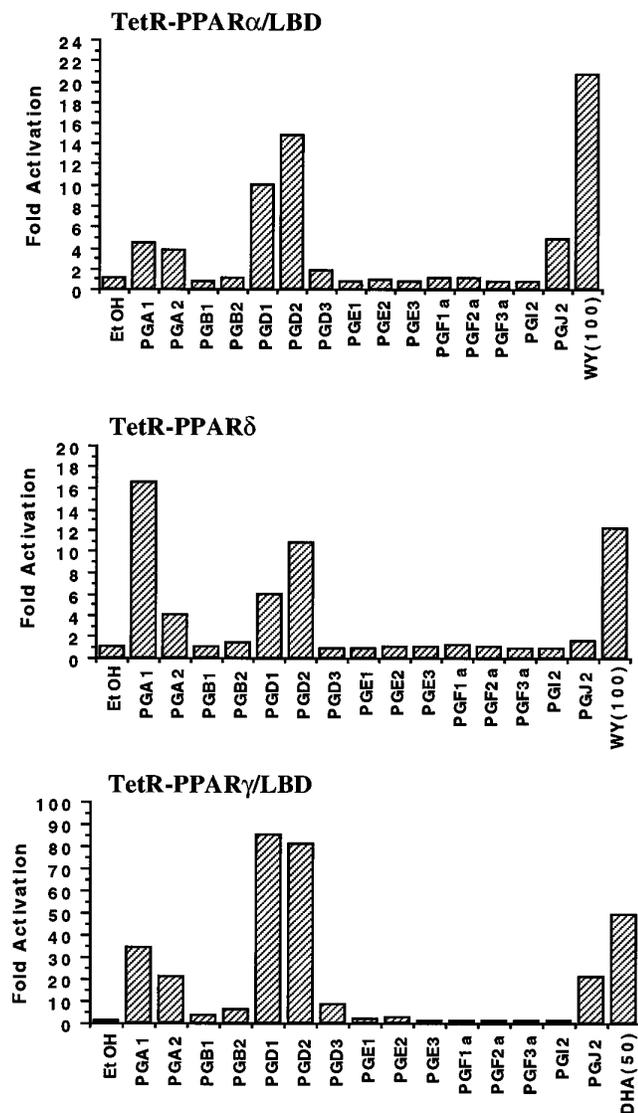


FIG. 3. **Selective prostaglandins can activate PPARs.** Transfected U2OS cells were treated with  $10 \mu\text{M}$  each of the indicated prostaglandins in repeated wells ( $n = 4$ ).  $100 \mu\text{M}$  Wy-14,643 (Wy) and  $50 \mu\text{M}$  DHA were used as positive controls. The effects of each prostaglandin were determined from the repeated wells. PGA, prostaglandin A; PGB, prostaglandin B; PGD, prostaglandin D; PGE, prostaglandin E; PGF, prostaglandin F; PGJ, prostaglandin J.

in this tissue culture model system. Fig. 5A shows that treatment of preadipocytes with  $50 \mu\text{M}$  8(S)-HETE resulted in conversion of  $\sim 10\%$  of the cells into adipocytes. Fig. 5B shows that at the molecular level 8(S)-HETE treatment induced the expression of the adipocyte-specific gene aP2. For comparison, the PPAR activator Wy-14,643 caused the cells to differentiate with an efficiency of about 50% (Fig. 5A), with similarly increased induction of aP2 (Fig. 5B). In contrast, less than 1% of cells treated with ethanol alone underwent adipose conversion. Treatment of cells with  $50 \mu\text{M}$  8(R)-HETE also resulted in adipogenesis, but at a much lower rate ( $\sim 1\%$ ) than the 8(S)-enantiomer. One such example is shown in Fig. 5A, although many fields contained no adipocytes. The molecular changes induced by 8(R)-HETE were also much less than those of 8(S)-HETE (Fig. 5B), again reflecting the low overall rate of adipose conversion. The low degree of adipose conversion induced by 8(R)-HETE was also delayed relative to adipocyte differentiation in response to 8(S)-HETE (data not shown). Thus, it is possible that this small effect of 8(R)-HETE was due to isomerization to 8(S)-HETE during the 7–8 days in which these cells

were cultured. In fact, HETEs are enzymatically oxidized to ketoisocaproic acids (KETEs) (36), and cellular reduction of KETEs to mixtures of (S)- and (R)-stereoisomers of HETEs has been described (37).

**Comparative Activities of Naturally Occurring and Synthetic PPAR Activators**—The activities of the naturally occurring eicosanoids were quantitatively compared with activation by synthetic compounds for each of the three PPAR subtypes (Fig. 6). For PPAR $\alpha$ , 8(S)-HETE was as effective an activator as the synthetic ETYA, and the dose-response curves for these compounds were similar, with both being active at  $0.1 \mu\text{M}$  and maximally active at  $1.0 \mu\text{M}$  (Fig. 6, top right panel). 8(S)-HETE was maximally active at lower concentrations than required for similar activation by PGD2, another naturally occurring activator of PPAR $\alpha$ . However, when maximally active doses of 8(S)-HETE and PGD2 were added simultaneously, no additivity was reproducibly noted (data not shown). At  $10^{-4} \text{M}$ , PGD2 caused cell death and hence an apparent reduction in reporter gene expression for all PPAR subtypes.

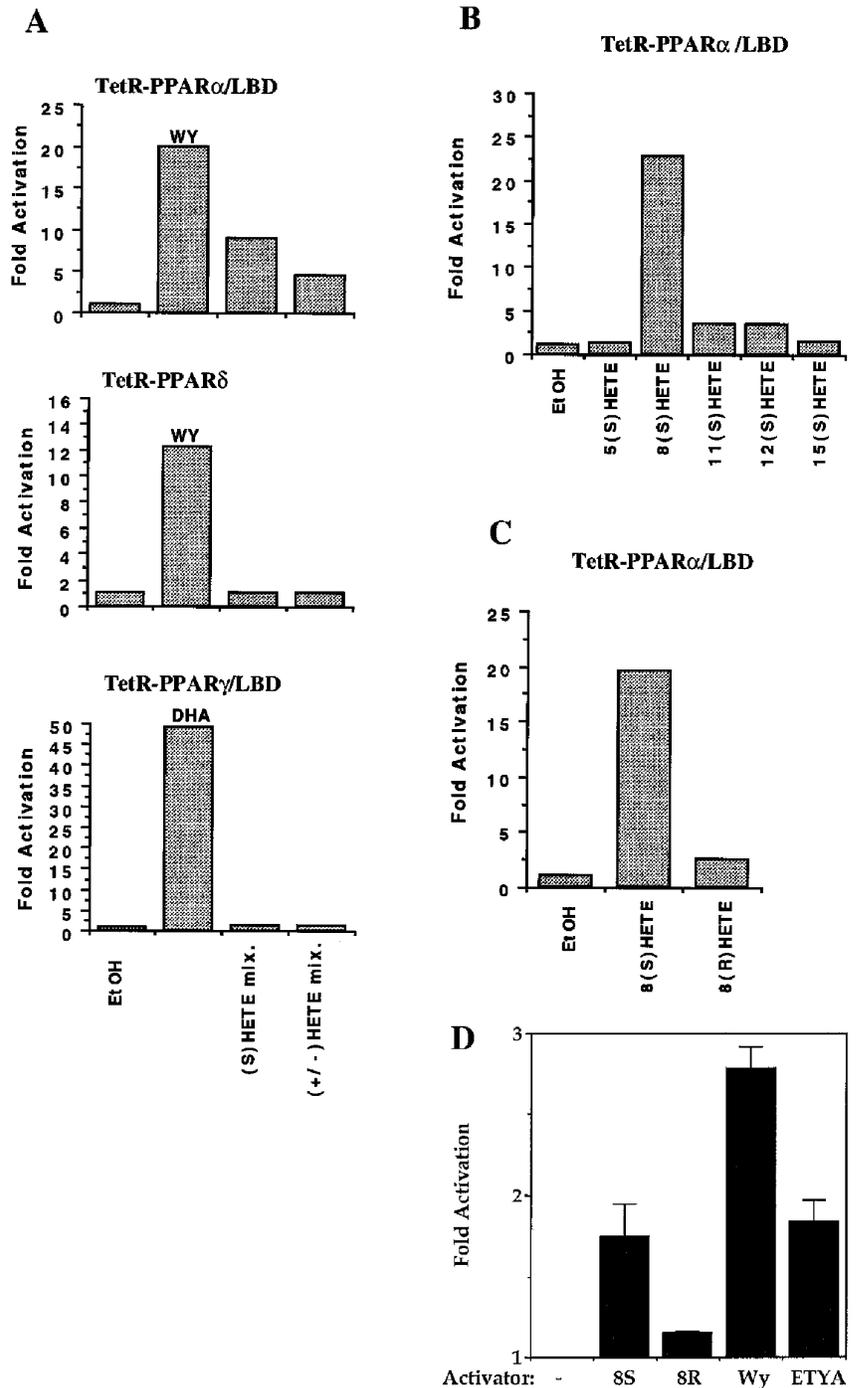
The relative abilities to activate PPAR $\delta$  were  $\text{PGA1} > \text{PGD2} > \text{DHA} > \text{LA}$  and Wy-14,643 (Fig. 6, middle panels). Significantly, the highest concentrations of PGA1 ( $50 \mu\text{M}$ ) and DHA ( $100 \mu\text{M}$ ) caused a greater fold activation of PPAR $\delta$  than did  $100 \mu\text{M}$  Wy-14,643 (Fig. 6, middle panels; note differences in scale). Studies of PPAR $\gamma$  indicated relative activities of  $\text{PGD2} > \text{PGA1}$ ,  $\text{DHA} > \text{clofibrate}$ , Wy-14,643, and LA (Fig. 6, lower panels). From the analyses conducted here, DHA, PGD2, and PGD1 (Figs. 3 and 6) appear to be the best activators of PPAR $\gamma$ . The effects of Wy-14,643 and clofibrate on PPAR $\gamma$  were much less than on PPAR $\alpha$ . LA was also a very poor activator for PPAR $\gamma$  relative to PPAR $\alpha$  and PPAR $\delta$ .

#### DISCUSSION

We have established a screen for PPAR activators which has the advantage of greatly reduced background when compared to assays involving transient transfection of wild type PPARs. The use of the TetR/TetO system also allows direct comparison of the magnitude of transactivation between nuclear hormone receptors, such as the ER and PPAR, as shown here. The use of chimeric PPARs involving fusion to a heterologous DNA-binding domain could lead to differences resulting from altered DNA binding and/or heterodimerization with RXR. However, parallel studies with wild type receptors and natural PPAR-response elements confirmed the validity of the screen. It is also possible that we would have obtained somewhat different results had we studied different cell types. Nevertheless, application of this assay to known PPAR activators confirmed the suggestion by others that different PPAR subtypes are differentially regulated by peroxisome proliferators and fatty acids. Furthermore, use of this assay as a screen led to the finding of differential PPAR activation by naturally occurring eicosanoids. The differential activation of PPARs is consistent with the fact that the C-terminal ligand-binding domains are less highly conserved among PPAR subtypes than among thyroid hormone receptor and retinoic acid receptor subtypes (10, 13, 14).

Prostaglandins are lipid regulators of a number of important cellular processes. Much of the prostaglandin literature has focused on the role of cell surface receptors in mediating the pleiotropic effects of these compounds (38, 39). However, given their circulating concentrations, low molecular weights, and lipophilicity, it seems plausible that a subset of prostaglandins could activate nuclear receptors directly or indirectly after diffusion into (or production within) target cells. Indeed, certain prostaglandins such as PGA1, PGD2, and PGJ2 have anti-tumor effects on human cancer cells, including those derived from melanoma (40), leukemia (41), and ovarian carci-

**FIG. 4. Enantioselective activation of PPAR $\alpha$  by 8(S)-HETE.** *A*, U2OS cells transfected with pTetO-Luc and PPAR fusion receptors were screened for receptor activation with HPLC mixtures. The (S)-HETE mixture contained 0.3  $\mu$ M each of 5(S)-HETE, 8(S)-HETE, 11(S)-HETE, 12(S)-HETE and 15(S)-HETE. The ( $\pm$ )-HETE mixture contained 0.3  $\mu$ M each of 5( $\pm$ )-HETE, 8( $\pm$ )-HETE, 11( $\pm$ )-HETE, 12( $\pm$ )-HETE, and 15( $\pm$ )-HETE. Each treatment was performed in repeated wells ( $n = 4$ ). WY-14,643 (WY) and DHA were used as positive control activators. Luciferase assays were performed 48 h later as described under "Materials and Methods." Note that the (S)-HETE mixture gave a 9-fold activation, and the ( $\pm$ )-HETE mixture showed about half of the activity (4–5-fold). *B*, stimulation of pTetO-Luc by TetR-PPAR $\alpha$  was tested in the presence of 1.3  $\mu$ M each of the five pure HETEs: 5(S)-HETE, 8(S)-HETE, 11(S)-HETE, 12(S)-HETE, and 15(S)-HETE. *C*, activation of PPAR $\alpha$  activation by 8-HETE is stereoselective. Cells were induced with 1.0  $\mu$ M each of the two stereoisomers, 8(S)- and 8(R)-HETE. Luciferase activities determined 48 h after induction were shown relative to the EtOH vehicle control. *D*, stimulation of PPAR $\alpha$ -induced transcriptional activation of the bifunctional enzyme PPAR response element-TK-luciferase reporter gene. The concentrations of 8(S)-HETE, 8(R)-HETE, Wy-14,643, and ETYA were 1, 5, 10, and 10  $\mu$ M, respectively. Mean and range of duplicate points are shown, normalized to the luciferase activity from the reporter in the presence of PPAR $\alpha$  and absence of activator, which was four to five times the activity of the reporter in the absence of transfected PPAR $\alpha$ . The results shown are representative of two separate experiments.



noma (42). The ability of these agents to regulate cell proliferation and apoptosis at least partially involves nuclear mechanisms (43–45). It is therefore of particular interest that these prostaglandins are the same subset which activated PPARs. Peroxisome proliferators are hepatocarcinogens in rodents, but the relationship between the anti-tumor effects of prostaglandins described above and hepatic tumorigenicity is not clear. The above-mentioned prostaglandins were generally equal or more effective PPAR activators than the fatty acids and peroxisome proliferators. Not all prostaglandins tested activated PPARs, and the failure of PGE and PGF to activate was consistent with earlier studies of *Xenopus* PPARs by Keller *et al.* (22). However, that group also reported that xPPAR $\alpha$  was not activated by PGD2 while we found that it did activate the mammalian PPAR $\alpha$  used in the present study. PGD2 and the

other prostaglandins that had activity in our system activated all three PPAR subtypes. Since the structures of inactive and active prostaglandins are not extremely different, analysis of common features of the active prostaglandins may provide clues to the structural requirements for activation of PPARs, whether direct or indirect.

The ability of indomethacin to inhibit some of the effects of peroxisome proliferators and fatty acids suggested that these agents could act by a mechanism which is convergent with that of the prostaglandins. Indeed, inhibitors of fatty acid oxidation can also activate PPARs (46). It should be cautioned, however, that although metabolism of LA is consistent with a potential involvement of cyclooxygenase pathways, DHA is an  $\omega$ -3 fatty acid not traditionally considered to be a precursor of arachidonic acid or prostaglandins. To explain this, we speculate that

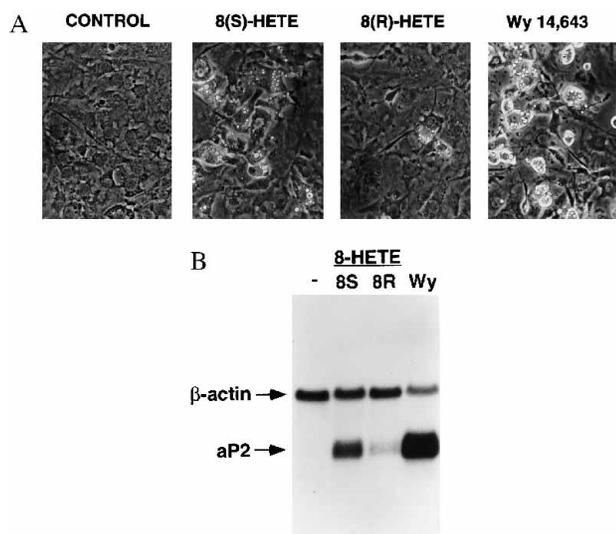


FIG. 5. **8(S)-HETE induces adipocyte differentiation.** *A*, morphological differentiation. Confluent 3T3-L1 preadipocytes were treated with 50  $\mu$ M 8(S)-HETE, 50  $\mu$ M 8(R)-HETE, 0.51 mM Wy-14,643 (Wy), or EtOH alone for 7 days. Phase contrast microscopy is shown. *B*, induction of aP2, a molecular marker of adipocyte differentiation. Northern analysis of 3  $\mu$ g of total RNA prepared from cells treated as in *A*. - indicates control (EtOH) treatment. Northern analysis of  $\beta$ -actin mRNA expression is shown for comparison.

either the effects of indomethacin were not due entirely to inhibition of cyclooxygenase or, alternatively, that DHA indirectly influenced prostaglandin synthesis or metabolism. However, consistent with its function as a cyclooxygenase inhibitor, indomethacin did not inhibit activation of any of the PPAR subtypes by PGD<sub>2</sub> (data not shown). The ability of both ETYA, an inhibitor of arachidonate metabolism, as well as eicosanoids, which are arachidonic acid metabolites, to activate PPARs also appears paradoxical, but suggests that either ETYA has other cellular effects or that certain arachidonic acid metabolites exert indirect effects which mimic those of ETYA. Furthermore, the mechanism of the interaction between Wy-14,643 and cyclooxygenase pathway is not presently clear.

The ability of a HETE compound to activate PPAR is of significance. HETEs are lipoxygenation products of arachidonate, whose synthesis is cyclooxygenase-independent. In this regard, it is noteworthy that indomethacin was least effective in inhibiting the activation of PPAR $\alpha$ , the PPAR that was activated by 8(S)-HETE. The best studied HETEs, 5-, and 12-, and 15-hydroxyeicosatetraenoic acids, are involved in a variety of biological processes including inflammation, blood pressure regulation, renal function, and respiratory airway smooth muscle tone (33–35). There is evidence that these compounds function in part through cell surface receptors, but it seems possible that they could exert a subset of their effects via nuclear receptors. However, these compounds did not activate PPARs in our experiments. 8(S)-HETE, in contrast, was a strong activator of transcription by PPAR $\alpha$ . To our knowledge, 8(S)-HETE is the first example of a compound which stereoselectively activates PPAR.

8(S)-HETE has not been as thoroughly studied as other HETEs. It is a naturally occurring compound, and a 8(S)-lipoxygenase activity involved in 8(S)-HETE biosynthesis has been shown to be present in mouse epidermis (47, 48). The tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate induces this enzymatic activity and causes a large increase in 8(S)-HETE (but not its 8(R)-enantiomer) in skin (47, 48). The normal function of 8(S)-HETE in skin is unknown. Since PPAR $\alpha$  is the primary mediator of peroxisome proliferator action in liver

(49), and peroxisome proliferators cause liver tumors in rodents (50), it is of interest to consider whether 8(S)-HETE has similar effects in liver, where PPAR $\alpha$ -inducible cytochrome p450 enzymes provide an alternative pathway for eicosanoid biosynthesis (51).

Little is also known about the mechanism of 8(S)-HETE action. In our experiments 8(S)-HETE selectively activated PPAR $\alpha$  as well or better than any natural or synthetic compound tested to date. Using a protease protection assay that has been used to detect ligand binding by other nuclear receptors (52), we have been unable to demonstrate direct binding of 8(S)-HETE to PPAR $\alpha$ .<sup>2</sup> However, a negative result in this assay may be due to failure of binding to induce a conformational change which alters protease sensitivity, rather than actual failure to bind to PPAR $\alpha$ . Nevertheless, the stereoselectivity of 8(S)-HETE raises the possibility that this compound, or a closely related metabolite, binds directly to PPAR $\alpha$ . Interestingly, the hydroperoxyeicosatetraenoic acid related to 8(S)-HETE (8(S)-HPETE) did not activate xPPAR $\alpha$  in a previous study in which the 8-HETEs were not evaluated (22).

In the present studies we found that 8(S)-HETE, which appeared PPAR $\alpha$ -specific in transient transfection experiments, induced endogenous aP2 gene expression and adipocyte differentiation of cultured 3T3-L1 cells. In this regard it is interesting that epidermal expression of a dominant negative RAR which also blocked PPAR action led to loss of multilamellar lipid structures from the stratum corneum of mouse skin (53, 54). The requirement for higher concentration of 8(S)-HETE for adipocyte differentiation than for transactivation of PPAR $\alpha$  may be due to the very low expression of PPAR $\alpha$  in 3T3-L1 preadipocytes (18). Another possibility is that 8(S)-HETE is metabolically inactivated during 3T3-L1 cell culture. Similar discrepancies between the ED<sub>50</sub> for induction of adipocyte differentiation and PPAR activation have been observed for WY-14,643 and ETYA (18). It should also be noted that although WY-14,643 caused a greater extent of adipocyte differentiation than 8(S)-HETE in the present studies, the concentration of Wy-14,643 was 10 times higher than the highest concentration of 8(S)-HETE.

The predominant PPAR in adipocytes is PPAR $\gamma$  whose expression is itself highly specific for adipocytes (15, 16). The potential role of activated PPAR $\gamma$  as a primary determinant of adipocyte differentiation is underscored by the observation that ectopic expression of PPAR $\gamma$  is sufficient for adipose conversion of fibroblast cell lines (19). Furthermore, while this paper was under review, antidiabetic thiazolidinediones which induce adipocyte differentiation (55, 56) were found to specifically activate PPAR $\gamma$  (57).<sup>3</sup> It is possible that 8(S)-HETE, which was PPAR $\alpha$ -specific in other cell lines, or its metabolite(s) induce adipocyte differentiation by activating PPAR $\gamma$  in 3T3-L1 cells. However, it is conceivable that PPARs other than PPAR $\gamma$  may also play a role in adipocyte differentiation. Compounds such as WY-14,643 and ETYA also induced adipocyte differentiation yet were poor activators of PPAR $\gamma$ , and there is a significant time lag between commitment to adipocyte differentiation after exposure to PPAR activators and the induction of PPAR $\gamma$  (15, 16). Both PPAR $\alpha$  and PPAR $\delta$  are also induced during adipocyte differentiation (18). PPAR $\delta$  is present in preadipocytes and activates transcription of the adipocyte-specific aP2 gene, leading to the suggestion that PPAR $\delta$  may be important for the earliest events in induction of adipose differentiation by PPAR activators (20). PPAR $\alpha$  has also been shown to induce aP2 gene expression (19). The present results support

<sup>2</sup> E. Schwarz and M. A. Lazar, unpublished observations.

<sup>3</sup> K. Yu and M. A. Lazar, unpublished observations.

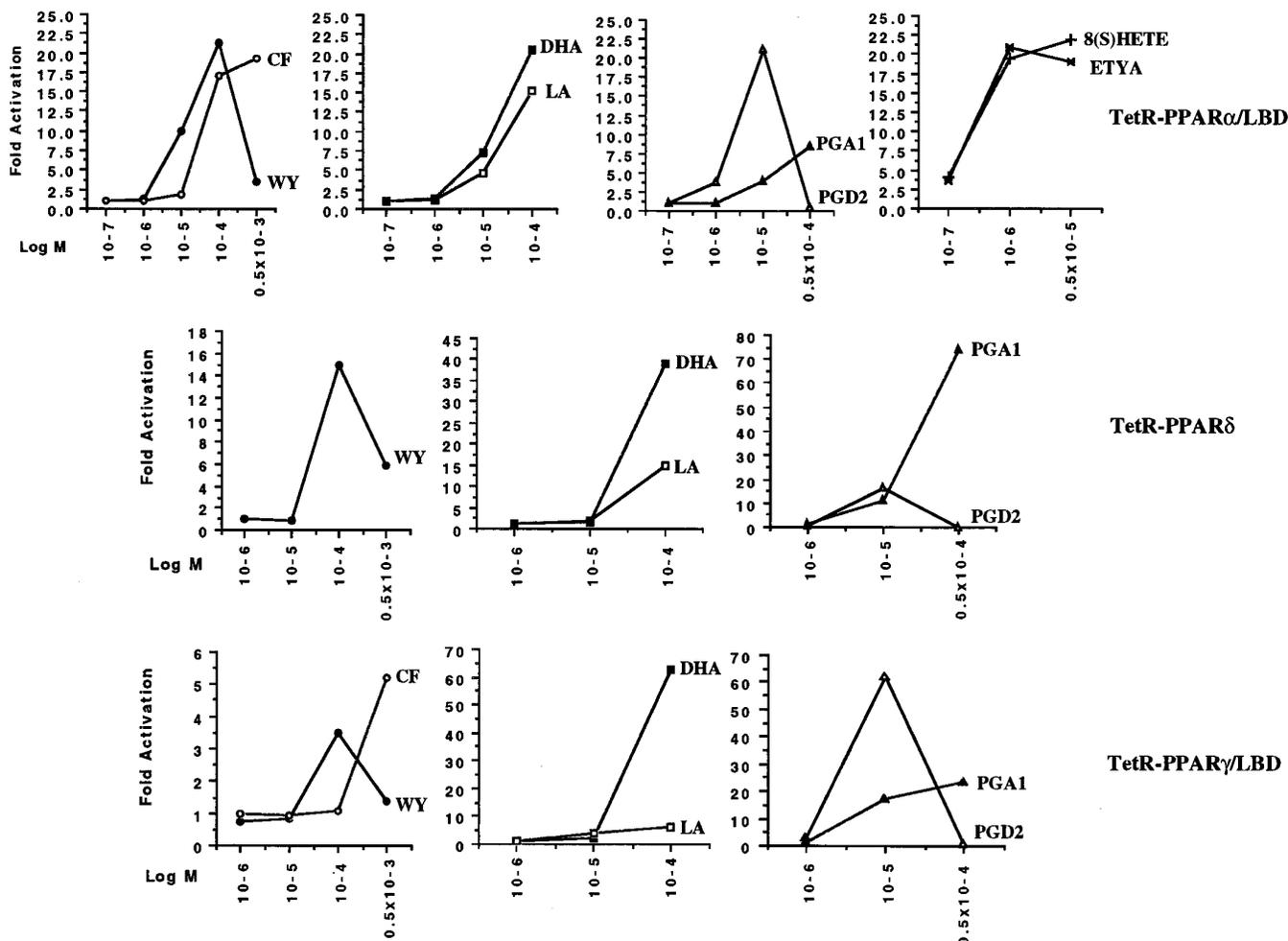


FIG. 6. Comparative activities of naturally occurring and synthetic PPAR activators. U2OS cells transfected with each PPAR fusion were tested with various concentrations of activators. The upper panels, activators of PPAR $\alpha$ ; middle panels, activators of PPAR $\delta$ ; and the lower panels, activators of PPAR $\gamma$ . Each treatment was performed in repeated wells ( $n = 8$ ), and the means are shown as relative fold activation compared to the Me<sub>2</sub>SO or EtOH vehicle control values. This experiment was performed twice with similar results. CF, clofibrate.

the notion that induction and maintenance of adipocyte differentiation by activation of PPARs is likely to be a complex process involving multiple PPAR subtypes. The availability of subtype-specific PPAR activators may allow further dissection of the mechanism of this and other important biological processes regulated by peroxisome proliferators, fatty acids, and eicosanoids.

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