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Peroxisome Proliferator-Activated Receptor α Required for Gene Induction by Dehydroepiandrosterone-3β-sulfate

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SUMMARY

Peroxisome proliferator-activated receptor α (PPARα) mediates the effects of foreign chemical peroxisome proliferators on liver and kidney, including the induction of peroxisomal, mitochondrial, and microsomal enzymes involved in β-oxidation of fatty acids. However, the role of this receptor in the peroxisome proliferative effects of the endogenous steroid dehydroepiandrosterone (DHEA) is not known. DHEA-3β-sulfate (DHEA-S) is shown to induce a liver peroxisome proliferative response in rats *in vivo* at a dose at which DHEA is much less active, which is consistent with cultured hepatocyte studies indicating a requirement for sulfation for the activation of DHEA. Transient transfection experiments demonstrated that in contrast to the prototypic foreign chemical peroxisome proliferator pirinixic acid, DHEA-S and its 17β-reduced metabolite, 5-androstene-3β,17β-diol-3β-sulfate, are inactive in mediating *trans*-activation by PPARα in COS-1 cells. Two other mammalian PPAR isoforms, γ and δ/Nuc1, were also inactive with respect to DHEA-S *trans*-activation. To test whether PPARα mediates peroxisomal gene induction by DHEA-S in intact animals, we administered DHEA-S or clofibrate to mice lacking a functional PPARα gene. Both peroxisome proliferators markedly increased hepatic expression of two microsomal cytochrome P450 4A proteins as well as six mRNAs known to be associated with the peroxisomal proliferative response in wild-type mice. In contrast, neither DHEA-S nor clofibrate induced these hepatic proteins and mRNAs in PPARα-deficient mice. Clofibrate-induced expression of kidney CYP4A mRNAs was also blocked in the PPARα gene knockout mice. Thus, despite its unresponsiveness to steroidal peroxisome proliferators in transfection assays, PPARα is obligatory for DHEA-S-stimulated hepatic peroxisomal gene induction. DHEA-S, or one of its metabolites, may thus serve as an important endogenous regulator of liver peroxisomal enzyme expression via a PPARα-mediated pathway.

The adrenal steroid DHEA is a naturally occurring precursor of both androgens and estrogens that is distinguished from other classes of endogenous steroids by its striking anticarcinogenic activities and its other chemoprotective and therapeutic properties (1–5). DHEA can also stimulate a dramatic increase in both the size and the number of peroxisomes present in liver and kidney when administered to rodents at high doses. This proliferative response is accompanied by a substantial increase in peroxisomal β-oxidation and fatty acid-metabolizing CYP4A enzymes (6–10) and under conditions of chronic administration can lead to hepatocarcinogenesis (11).

Although it is clear that DHEA and its 17β-reduced metabolite ADIOL can induce a classic peroxisome proliferator-activated response via a mechanism involving activation of gene transcription (10), the cellular mechanisms underlying this effect are poorly understood (for a review, see Ref. 12). In primary rat hepatocytes, DHEA and ADIOL are both inactive as peroxisome proliferators unless first metabolized by steroid sulfotransferase to the corresponding 3β-sulfates (13, 14). Peroxisome proliferation activated by endogenous fatty acids, as well as by structurally diverse fibrate drugs and other foreign chemicals, is mediated by PPARα, an intracellular receptor belonging to the steroid receptor superfamily (15, 16). Based on results from *in vitro* transient transfection/*trans*-activation assays with intact or chimeric PPAR receptors, it was suggested that DHEA and DHEA-S may not function through the PPARα receptor because neither of these steroids was able to induce an increase in reporter gene activity, which is in contrast to the activation of this receptor by xenobiotic peroxisome proliferators such as Wy-14,643, clofibrate, and fatty acid analogues (15, 17, 18).

Despite these observations, however, it is still possible that DHEA or DHEA-S mediates its effects through other related

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ABBREVIATIONS: DHEA, dehydroepiandrosterone; DHEA-S, dehydroepiandrosterone-3β-sulfate; ADIOL-S, 5-androstene-3β,17β-diol-3β-sulfate; PPAR, peroxisome proliferator-activated receptor; RXR, retinoid X receptor; Wy-14,643, pirinixic acid; CYP, cytochrome P450.

receptors, specifically, PPARγ (19, 20) or PPARδ/Nuc1 (21). Alternatively, the *in vitro* transfection systems used to test for DHEA and/or DHEA-S activation of PPARα may be insufficiently sensitive to detect weak activation by the steroid or may lack metabolic capacity or other key factors present only in the intact animal. We examine several of these possibilities in the present study. We also evaluate the role of PPARα in DHEA-S-induced peroxisome proliferation *in vivo* using a mouse line that lacks the PPARα receptor and its associated pleiotropic response to foreign chemical peroxisome proliferators (22). These studies establish that despite its apparent inactivity *in vitro*, PPARα is required for (and may mediate) the *in vivo* effects of DHEA-S on hepatic peroxisomal proliferation.

Materials and Methods

Plasmids. Reporter plasmid pLuc-4A6–880, containing 880 nt of 5′-flanking DNA of the rabbit *CYP4A6* gene cloned into p19Luc, and the mouse PPARα expression plasmids pCMV-PPARα and pCMV-PPARα-G (23) were kindly provided by Dr. E. Johnson (The Scripps Research Institute, La Jolla, CA). A plasmid expressing full-length human PPARδ/Nuc1 cloned into pJ3ω (21) was kindly provided by Dr. A. Schmidt (Merck Research Labs, West Point, PA). Mouse PPARγ cloned into pSV-Sport1 (19) was provided by Dr. J. Reddy (Northwestern University Medical School, Chicago, IL), and mouse RXRα expression plasmid pCMX-mRXRα (24) was provided by Dr. R. Evans (Salk Institute, San Diego, CA). β-Galactosidase expression plasmid (pSV-β-gal) was purchased from Promega.

Transfection studies. Transfection of COS-1 cells grown onto 12-well tissue culture plates was carried out according to a calcium phosphate precipitation method (25). At 9 hr after addition of DNA, cells were washed and then incubated in Dulbecco's modified Eagle's medium (GIBCO) containing 10% charcoal-stripped, delipidated bovine calf serum (catalog No. C-1696, Sigma Chemical Co., St. Louis, MO). Transfections were performed with the following amounts of plasmid DNAs/well (3.83 cm^2) of a 12-well tissue culture plate: 0.4 μg of reporter construct (pLuc-4A6–880), either 8 ng of mouse PPARα expression plasmid (pCMV-mPPARα or pCMV-mPPARα-G), 0.6 μg of mouse PPARγ expression plasmid or 0.6 μg of human PPARδ/Nuc1 expression plasmid, and 0.16 μg of β-galactosidase expression plasmid as an internal control. pCMX-mRXRα was included at 80 ng/well where indicated. The total amount of DNA was adjusted to 0.96 μg/well through the addition of pBluescript DNA as required (Stratagene). The PPAR test activators Wy-14,643 (Wyeth-Ayerst, Princeton, NJ), DHEA-S, ADIOL-S, and DHEA (Sigma; ≥99% purity by thin layer chromatography), 7-keto-DHEA (Steraloids, Wilton, NH; ≥98% purity by thin layer chromatography), and LY171883 (Biomol Research Labs, Plymouth Meeting, PA) were obtained from the sources indicated. PPAR test activators were each diluted from a 1000-fold stock in dimethylsulfoxide and were added to the cells in fresh media at the concentrations indicated 24 hr after cells were washed to remove the DNA precipitates. Control cells received an equal amount of dimethylsulfoxide. At 24 hr after the addition of the PPAR test activators, cells were washed twice with cold phosphate-buffered saline and then extracted in lysis solution (100 mM KPi, pH 7.8, 0.2% Triton X-100, with 1 mM dithiothreitol added before use; 80 μl/well) for 15 min at 4°. The cell extract was then scraped and transferred to a centrifuge tube for removal of insoluble cell debris in an Eppendorf centrifuge (model 5415C; 4 min at 14,000 rpm). Luciferase activity was measured with the use of an assay kit and a Monolight 2010 Luminometer instrument (Analytical Luminescence Laboratory, Ann Arbor, MI). β-Galactosidase activity was determined with a Galacto-light chemiluminescent reporter kit (Tropix, Bedford, MA) scaled down to 6.7 μl of extract, 67 μl of reagent A, and 100 μl of reagent B. Luciferase activity values were normalized for transfection efficiency with the use of β-galactosidase activity values determined from the same preparation of cell lysate.

DHEA-S and DHEA induction of rat liver CYP4A. Adult male Fischer 344 rats (Taconic, Germantown, NY) were given daily intraperitoneal injections of DHEA or DHEA-S at either 1 mg/100 g body weight or 6 mg/100 g body weight for 4 consecutive days. DHEA-induced peroxisome proliferative responses are minimally detectable at the lower dosage (10), in which case it may be possible to detect differences between the effectiveness of DHEA and DHEA-S that are not apparent at higher doses. Animals were killed 24 hr after the last injection. Rat liver RNA was prepared from tissue frozen at −80° and then analyzed for *CYP4A1* and *CYP4A3* mRNA through Northern blotting with gene-specific oligonucleotide probes as described previously (26).

PPAR gene knockout mice. Male PPARα (−/−) mice or (+/+) (F_3 homozygotes or wild-type; hybrids of C57BL/6N × Sv/129 genetic background; 10–12 weeks of age) (22) were housed in plastic cages in a temperature- and light-controlled environment. Groups of mice (three mice/group) were injected with either DHEA-S or clofibrate (Sigma Chemical Co.) at 15 mg/100 g body weight or corn oil (vehicle control) for 4 consecutive days by intraperitoneal injection. This saturating dose of DHEA-S was chosen to allow detection of even a low level peroxisome proliferative response in the PPARα-deficient mice. Body weights were measured daily. Twenty-four hours after the final injection, mice were killed through carbon dioxide asphyxiation, and the liver and kidneys were removed and rapidly frozen in liquid nitrogen. Tissues were stored at −80° until they were used for isolation of microsomes or RNA.

Analysis of microsomal CYP4A protein expression. Liver and kidney microsomes were prepared from frozen mouse tissues through differential centrifugation and then analyzed through Western blotting with polyclonal anti-rat CYP4A antibody raised to a di(2-ethylhexyl)phthalate-inducible rat liver CYP4A protein related to CYP4A1. This antibody has been characterized as being cross-reactive with multiple rodent CYP4A proteins (27) and was kindly provided by Dr. R. T. Okita (Washington State University, Pullman, WA).

Mouse liver and kidney mRNA analysis. Total RNA samples were obtained after Polytron sonic disruption of frozen tissue in guanidine thiocyanate and subsequent centrifugation through cesium trifluoroacetate. Samples were quantified through the use of standard spectrophotometric analysis. Samples (10 μg) of total RNA from each individual animal were electrophoresed on a 1.0% agarose gel containing 2.2 M formaldehyde and transferred onto a Genescreen Plus (DuPont) nylon membrane in 20× standard saline citrate (3 M NaCl, 0.15 M sodium citrate, pH 7.0). RNA was fixed to the membrane by being baked at 80° for 2 hr in a vacuum oven. Membranes were then prehybridized in a hybridization buffer containing 50% formamide, 5× standard saline/phosphate/EDTA buffer (1× SSPE = 0.18 M NaCl, 10 mM NaH_2PO_4, 1 mM EDTA, pH 7.7), 5× Denhardt's solution, 200 μg of salmon sperm DNA/ml, 0.1% sodium dodecyl sulfate, and 10% dextran sulfate for ≥2 hr. Hybridization probes were labeled with [^{32}P]dCTP through random priming (Pharmacia, Piscataway, NJ). Seven cDNA probes, described previously (22), were used for sequential Northern blot analysis. These cDNA probes encode rat peroxisomal acyl-CoA oxidase, rat peroxisomal bifunctional enzyme, rat peroxisomal 3-ketoacyl-CoA thiolase, rat *CYP4A1*, rat *CYP4A3*, mouse liver fatty acid-binding protein, and mouse β-actin as a control. The sizes of these probes were 1037, 1094, 1017, 2200, 1800, 450, and 1150 bp, respectively. After overnight hybridization at 42°, the nylon membranes were washed three times for 20 min with 2× standard saline citrate with 0.5% sodium dodecyl sulfate and subjected to autoradiography through exposure of membranes to X-ray film for periods ranging from 2 to 24 hr depending on the signal strength.

Statistical analysis. Differences in mean liver and body weights were assessed through the use of two-way analysis of variance (Statview II ver. 1.03; Abacus Concepts).

Results

DHEA-S-induced *CYP4A* induction *in vivo*. The finding that DHEA is an active peroxisome proliferator when administered to intact animals but cannot activate peroxisomal or *CYP4A* gene expression in cultured hepatocytes (13) suggests that DHEA undergoes metabolism to a more active metabolite, which in turn mediates the proliferative response. Because, unlike DHEA, DHEA-S is an active inducer of *CYP4A* and peroxisomal enzyme expression in rat hepatocyte cultures, we examined whether a preferential activity of the sulfate is also evident *in vivo*. Fig. 1 shows that when DHEA and DHEA-S are administered to rats at a comparatively low dose [10 mg/kg/day for 4 days (10)], DHEA-S is a substantially more effective inducer of liver *CYP4A3* expression than is DHEA (compare *lanes 10–12* and *3–5* with controls in *lanes 1 and 2*). In contrast, DHEA and DHEA-S are equally effective as *CYP4A* inducers when administered at a 6-fold higher doses. The effect of DHEA at the higher dose is presumably due to its sulfation by liver sulfotransferases.

***trans*-Activation of PPAR by DHEA-S and related steroids.** To investigate the role of PPARα in DHEA-S-activated peroxisome proliferation, we cotransfected COS-1 cells with the PPARα expression plasmid pCMV-mPPARα and the peroxisome proliferator response element-containing reporter plasmid pLuc-4A6–880. Treatment of the cells with the prototypic peroxisome proliferator Wy-14,643 (20 μM) resulted in a ≤15-fold increase in luciferase reporter activity measured 24 hr later. In contrast, no reproducible activation of reporter gene expression was observed after the addition of DHEA, 7-keto-DHEA, DHEA-S, or ADIOL-S when tested at concentrations of 100 μM (Fig. 2A).

PPARα can form heterodimers with RXR, leading to increased binding of PPAR to peroxisome proliferator response elements and enhanced transcriptional activation (28). To investigate whether RXR is required for DHEA-S-induced PPAR activation, we cotransfected a mouse RXRα expression plasmid with pCMV-PPARα. As shown in Fig. 2A, basal luciferase reporter activity was increased 2–3-fold in cells transfected with RXRα; however, no further increase in activity was detected after treatment of the cells with DHEA, 7-keto-DHEA, DHEA-S, or ADIOL-S.

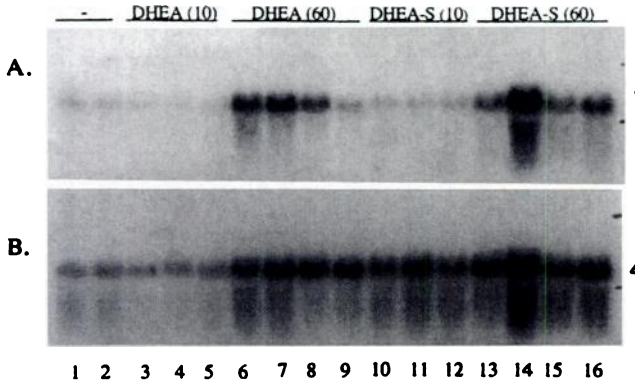

Fig. 1. Induction of liver *CYP4A* mRNAs by DHEA and DHEA-S administered to adult male rats. Shown is a Northern blot of total liver RNA samples prepared from individual rats that were untreated (*lanes 1 and 2*) or were induced with DHEA (*lanes 3–9*) or DHEA-S (*lanes 10–16*) (intraperitoneal injections at 10 or 60 mg/kg/day for 4 days, as indicated). Blot was probed sequentially with oligonucleotide probes specific for *CYP4A1* (A) and *CYP4A3* (B) (26). DHEA-S is seen to be much more effective than DHEA with regard to induction of *CYP4A3* (and, to a lesser extent, *CYP4A1*) at the 10 mg/kg dose (compare *lanes 10–12* with *lanes 3–5*).

The basal luciferase reporter activity seen in these experiments (i.e., activity in the absence of exogenous PPAR activator) was dependent on the presence of transfected PPARα plasmid DNA, as seen by comparing the −PPARα sample with the +PPARα/vehicle control in Fig. 2B. Conceivably, this endogenous activator activity of the vehicle control sample could mask a low level activation of PPAR by DHEA-S. Muerhoff *et al.* (23) and Hsu *et al.* (29) reported that the activity of this basal (endogenous) PPAR activator is greatly reduced, however, when using a PPARα mutant, designated PPARα-G, that has a Glu282-to-glycine substitution. Fig. 2B shows that transfection of COS-1 cells with PPARα-G does indeed result in a significantly lower basal PPAR activity compared with transfections using wild-type PPARα cloned into the same expression plasmid. Consequently, use of PPARα-G resulted in a 6-fold greater net increase in the fold-induction by Wy-14,643 of luciferase reporter activity, giving a 30-fold overall increase above vehicle control level in the experiment shown. However, despite this greater sensitivity of the PPAR-G-transfected cells for detection of PPAR activation, no reproducible increase in reporter gene activity could be detected in cells treated with DHEA, 7-keto-DHEA, DHEA-S, or ADIOL-S in either the absence (Fig. 2B and data not shown) or the presence of cotransfected RXR (data not shown).

To address the possibility that other PPAR subtypes may be involved in DHEA-S-dependent peroxisome proliferator responses, cotransfection experiments were carried out with PPARγ and PPARδ/Nuc1 expression plasmids in the presence of cotransfected RXRα. PPARγ and PPARδ/Nuc1 were weakly activated by Wy-14,643 (100 μM) (Fig. 3), which is in agreement with earlier results (30). Furthermore, the PPARγ activator and leukotriene D4/E4 antagonist LY171883 (20) selectively activated PPARγ. However, DHEA, DHEA-S, and ADIOL-S did not induce significant responses from PPARγ or PPARδ/Nuc1 (Fig. 3 and data not shown).

Influence of PPARα gene knockout on DHEA-S-induced peroxisome proliferation in liver and kidney. PPARα gene knockout and wild-type mice were tested for their responsiveness to DHEA-S-induced peroxisome proliferation. Relative liver weights were significantly higher in wild-type mice (+/+) treated with clofibrate or DHEA-S than in vehicle-injected controls. Similar increases have been reported for DHEA-treated wild-type mice (31). However, this increase in mean liver mass was not observed in PPARα gene knockout mice (−/−) treated with either peroxisome proliferator (Fig. 4). No differences were observed in initial or final body weights of animals injected with clofibrate or DHEA-S compared with control mice. Also, there was no difference in body weight between (+/+) or (−/−) mice, independent of treatment (data not shown).

Northern blot analysis of liver RNA prepared from these same mice revealed strong increases in the hepatic mRNAs encoding acyl-CoA oxidase, bifunctional enzyme, and 3-ketoacyl-CoA thiolase in (+/+) mice injected with either clofibrate or DHEA-S compared with control mice of the same

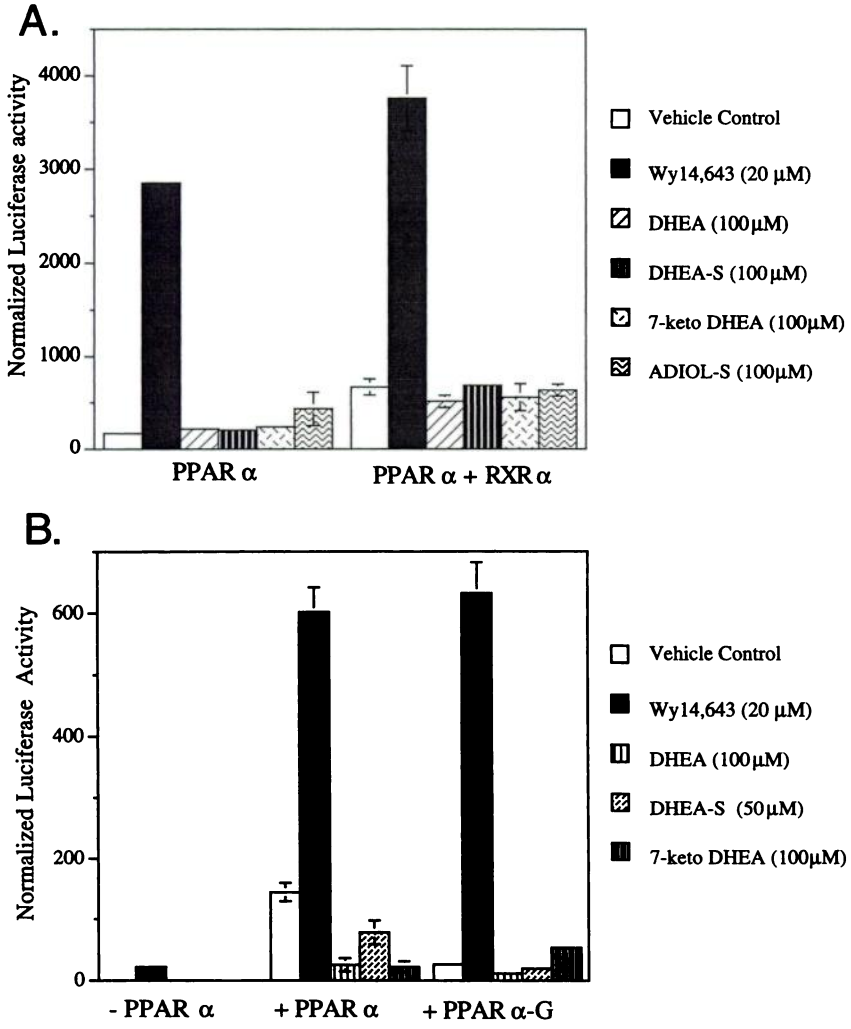

Fig. 2. Activation of PPARα by peroxisome proliferators: transient *trans*-activation assay. A, Comparison between PPARα alone and PPARα cotransfected with RXRα. B, Comparison of the activation of PPARα with that of PPARα-G. Cotransfection of PPARα or PPARα-G expression plasmids with P4504A6 promoter-luciferase reporter construct in the presence or absence of an RXRα expression plasmid was carried out in COS-1 monkey kidney cells with a calcium phosphate precipitation method. Cells were treated with the indicated PPAR activators for a 24-hr period beginning 24 hr after washing of the cells to remove the calcium phosphate DNA precipitates. Luciferase reporter activity of cell extracts was then determined, and the data were normalized to a β-galactosidase reporter (pSV-β-gal) as an internal standard. Data shown are mean ± range for duplicate determinations and are representative of three or more independent experiments. Error bars not visible for several of the treatment groups are too small to be seen. COS-1 cells exhibited a low level of endogenous receptor activity (−PPARα, first bar in B) but a comparatively high level of endogenous PPARα activator (+PPARα, vehicle control). This endogenous activator activity was greatly reduced when the mouse PPARα-G mutant was used (B). Both receptors were strongly activated by Wy-14,643 but not by DHEA-S, DHEA, 7-keto-DHEA, or ADIOL-3β-sulfate (not shown). Normalized luciferase activities were induced by Wy-14,643 (20 μM) 5–15-fold in different experiments. Concentrations of steroids up to 250 μM were tested and also found to be inactive. Solubility problems or, in some cases, inhibition of β-galactosidase internal standard activity precluded studies at higher steroid concentrations.

genetic background (Fig. 5A, compare *lanes 4–9* with *lanes 1–3*). Similarly, mRNAs for liver fatty acid-binding protein and two CYP4A forms were also present at higher levels compared with controls in the livers of the peroxisome proliferator-treated (+/+) mice. In contrast, none of these liver mRNAs were induced by either clofibrate or DHEA-S when administered (−/−) mice as assessed with the use of Northern blot analysis (Fig. 5A, *lanes 13–18*). The signals obtained from the β-actin probe were similar (<2-fold difference) for all groups. These findings were confirmed at the protein level in the case of CYP4A: two CYP4A proteins were highly inducible in livers of (+/+) but not (−/−) mice after treatment with either clofibrate or DHEA-S (Fig. 5B, compare *lanes 4–9* with *lanes 13–17*, *bands A* and *B*). A constitutively expressed

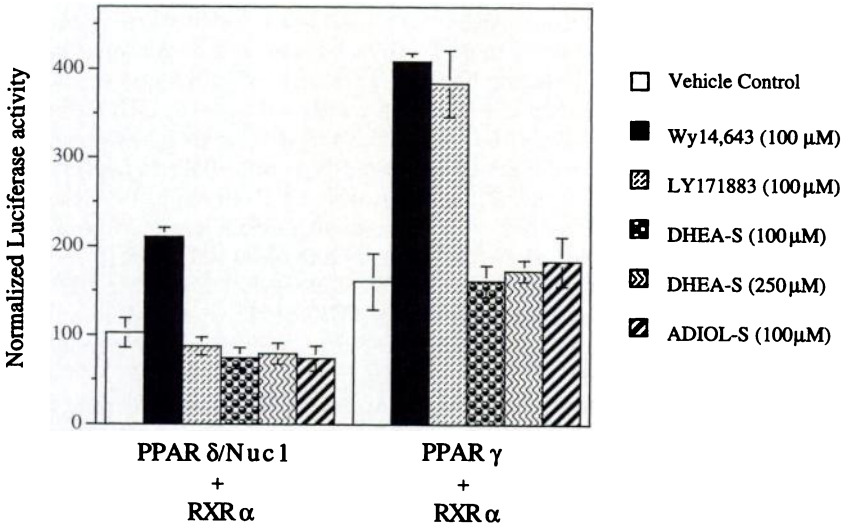

Fig. 3. DHEA-S does not activate PPARγ or PPARδ/Nuc1 in *trans*-activation assays. COS-1 cells were cotransfected with PPARγ or PPARδ/Nuc 1 expression plasmids in the presence of RXRα expression plasmid and luciferase reporter plasmid and then treated with the indicated PPAR activator test compounds and analyzed as described in legend to Fig. 2.

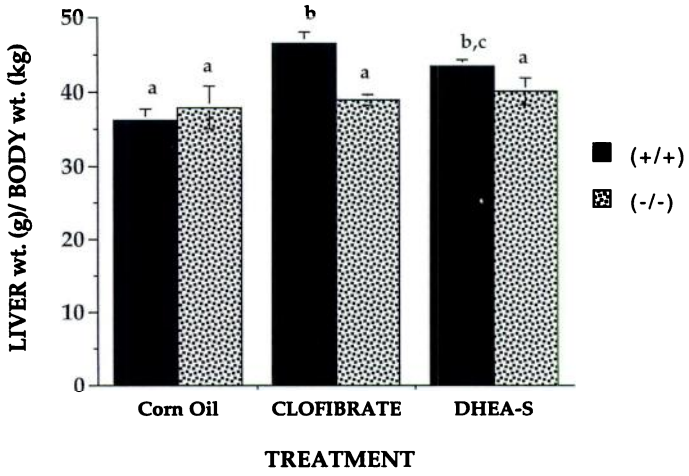

Fig. 4. Influence of clofibrate and DHEA-S on relative liver weights. Shown are liver-to-body weight ratios determined after four daily intraperitoneal injections of either corn oil, clofibrate, or DHEA-S given to PPARα (+/+) mice or PPARα (−/−) mice. Values represent mean ± standard error for three mice per group. Differences in mean values were assessed with two-way analysis of variance. Bars marked with different letters are significantly different at $p \leq 0.05$. Body weights did not differ significantly between the groups.

CYP4A-immunoreactive protein of slightly lower apparent molecular weight was also detected (Fig. 5B, *band C*), but its level was unaffected by either peroxisome proliferator or by the PPARα knockout phenotype. The precise identifies of these CYP4A-immunoreactive bands could not be established.

In contrast to the inductive effects observed in hepatic tissue samples from (+/+) mice, in the kidney, neither clofibrate nor DHEA-S induced acyl-CoA oxidase, bifunctional enzyme, or 3-ketoacyl-CoA thiolase mRNAs compared with vehicle-treated controls (Fig. 6A). Basal levels of all three mRNAs were similar between (+/+) and (−/−) mice, independent of treatment. Furthermore, constitutive levels of these mRNAs were higher in kidney than in liver (compare Fig. 6A with Fig. 5A). Furthermore, although clofibrate did induce two kidney *CYP4A* mRNAs in (+/+) mice compared with controls, DHEA-S was inactive in this regard. The increase in *CYP4A* expression was not observed, however, in kidney RNA samples from clofibrate-treated (−/−) mice (Fig. 6A, *lanes 13–15*). Western blot analysis of kidney microsomes prepared from the same animals revealed a single CYP4A-immunoreactive protein; however, no induction was observed, even for clofibrate-treated wild-type mice (Fig. 6B), suggesting that this protein is distinct from those encoded by the *CYP4A1* and *CYP4A3* cross-hybridizing mRNAs detected in Fig. 6A.

Discussion

The finding from the current study that DHEA-S is more potent *in vivo* with respect to peroxisome proliferation than DHEA is consistent with earlier findings that sulfation is required to activate the peroxisome proliferative potential of DHEA in primary rat hepatocytes (13, 14). Despite this, DHEA-S was inactive with respect to PPAR activation in transient *trans*-activation assays carried out in cultured COS-1 cells. DHEA, its 7-keto metabolite, and ADIOL-S were also inactive in this transfection assay, both when using the full-length, naturally occurring mouse PPARα receptors and when using PPARα-G, a receptor mutant identified previously (23, 29), which in principle could allow for the detection of low DHEA-S activity due to its low background activity in the absence of exogenous PPAR activators. DHEA-S was also ineffective with respect to activation of two other PPAR isoforms, PPARγ and PPARδ/Nuc1, suggesting that these receptors also may not be involved in the DHEA response. Experiments carried out in PPARα gene knockout mice demonstrated, however, that mice lacking PPARα not only lack the hepatic peroxisome proliferative response to clofibrate (22) but also do not respond to DHEA-S. This finding establishes that although PPARα is unresponsive to DHEA-S in transfection studies, this receptor is nevertheless required for the induction of a hepatic peroxisome proliferation response by DHEA-S. Accordingly, the major peroxisome proliferative responses of liver to both DHEA and to foreign chemicals such as clofibrate are obligatorily dependent on PPARα. Although it seems likely that PPARα itself is a mediator of the peroxisome proliferative effects of DHEA, it is also possible that the PPARα requirement identified in the currect study is an indirect one. The current studies also indicate that the peroxisome proliferative responses of DHEA cannot be mediated by two other PPAR forms, PPARγ and PPARδ/Nuc1, despite the presence of the latter receptor at a significant level in liver tissue (20, 32, 33). Thus, the contribution to hepatic peroxisome proliferation made by these, and probably other receptors, such as FFAR, a novel fatty acid-activated receptor that is 88% similar to PPARδ/Nuc1 (34), is probably negligible.

Several mechanisms could account for the discrepancy between the clear requirement for PPARα to mediate DHEA-S activation *in vivo* and the apparent unresponsiveness of this steroid-like receptor to DHEA-S in transient transfection assays under conditions where it is responsive to Wy-14,643 and other, structurally diverse foreign chemical peroxisome proliferators. First, one or more factors that may be necessary for DHEA-S induction of the peroxisome proliferation response *in vivo* may be absent in the *in vitro trans*-activation system. These factors could include a requirement for heterodimerization partners other than RXR (28), which was ineffective with respect to restoration of DHEA-S-stimulated reporter activity (Fig. 2A), or a need for other accessory protein factors that might modulate PPAR activity, such as HNF-4 (35) or COUP-TF (36). Alternatively, the steroid might act in liver or other tissues to stimulate production of another endogenous chemical that serves as a more proximal PPAR activator. A third possibility is that the entry of DHEA-S into cells may require a specific plasma membrane transporter that is present in hepatocytes (37) but may be absent in other cell types. Indeed, if such a transporter is lacking in kidney cells, this could explain the absence of a significant DHEA-S response in kidney under conditions where clofibrate does stimulate gene induction (see Fig. 6A). Fourth, DHEA-S may require further metabolism that occurs in hepatocytes but not in the cultured cell lines used in the *trans*-activation assays. This metabolism could involve cytochrome P450-dependent hydroxylation reactions (38, 39) or may involve further conjugation of DHEA-S to yield DHEA-sulfatide, a diglyceride ester of DHEA-S that can be a dominant form of the steroid in plasma (40). Finally, DHEA-S may need to undergo metabolism to fatty acid ester deriva-

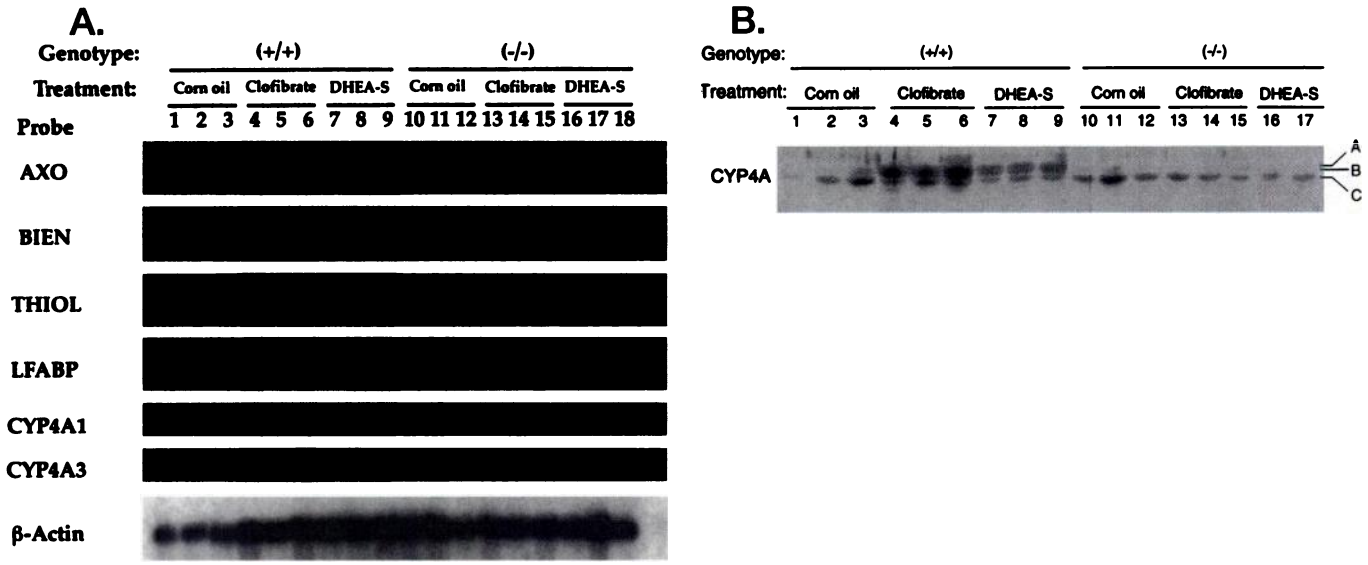

Fig. 5. Evaluation of DHEA-S induction of a liver peroxisome proliferator response in PPARα (+/+) and PPARα (−/−) mice. A, Northern blot analysis of wild-type (+/+) and PPARα (−/−) mice treated with corn oil (*lanes 1–3* and *10–12*), clofibrate (*lanes 4–6* and *13–15*), or DHEA-S (*lanes 7–9* and *16–18*) as described in Materials and Methods. Total RNA (10 μg) isolated from individual livers was electrophoresed on a 1% formaldehyde-agarose gel and then transferred to a nylon membrane before probing with each of the seven cDNA probes indicated. *AXO*, acyl-CoA oxidase; *BIEN*, bifunctional enzyme; *THIOL*, 3-ketoacyl-CoA thiolase; *LFABP*, liver fatty acid-binding protein. B, Liver microsomes (10 μg of protein in *lane 1*; 15 μg of protein in all other lanes) were prepared from tissue samples corresponding to the samples shown in A and then analyzed for immunoreactive P450 4A proteins by Western blotting. P450 4A-immunoreactive proteins marked *A* and *B* are induced by clofibrate and DHEA-S but only in PPARα (+/+) mice, whereas band *C* corresponds to a constitutively expressed protein that is unaffected by the inducers or the gene knockout.

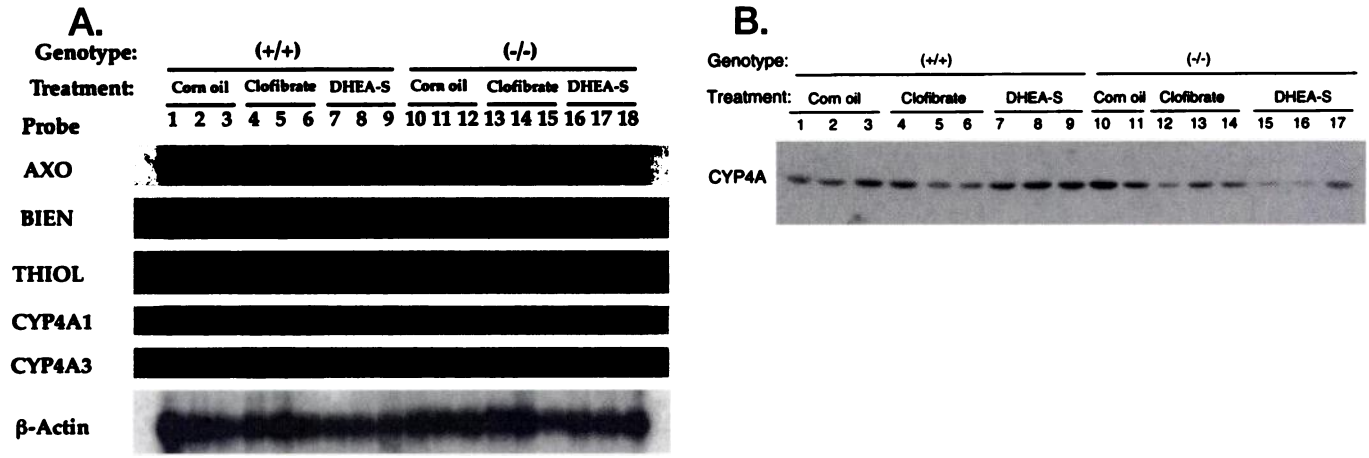

Fig. 6. Influence of PPARα gene knockout on induction of kidney mRNAs and proteins. A, Northern blot analysis of wild-type (+/+) and PPARα (−/−) mice treated with corn oil (*lanes 1–3* and *10–12*), clofibrate (*lanes 4–6* and *13–15*), or DHEA-S (*lanes 7–9* and *16–18*) was carried out as described in legend to Fig. 5A for liver samples from the same animals. Probe designations were as detailed in legend to Fig. 5. B, Kidney microsomes prepared from the same tissues analyzed in A were analyzed for immunoreactive CYP4A proteins by Western blotting. A single constitutively expressed kidney CYP4A protein that was not inducible by clofibrate or DHEA-S was revealed through these analyses.

tives, which readily form in plasma from serum lipoproteins (41, 42), and this metabolism might, in turn, provide a mechanism for transport into the cell of fatty acids that could serve as the more proximal peroxisome proliferators.

Compared with liver, the kidney is known to be less responsive to peroxisome proliferators (8–10, 43). Indeed, only a mild induction of peroxisomal bifunctional enzyme as measured with the use of Western blot analysis has been reported to occur after exposure to a high dose of DHEA (300 mg/kg), whereas several other enzyme activities involved in the peroxisome proliferator-activated response in liver are relatively unaffected in kidney (8, 9). Similarly, in our study, clofibrate and DHEA-S treatment did not result in a noticeable increase in kidney mRNAs associated with the peroxisome proliferator-activated response, although *CYP4A1*- and *CYP4A3*-related mRNAs were both induced by clofibrate treatment. Because this latter induction was absent in the PPARα-deficient mice, it is apparent that PPARα also mediates the clofibrate induction response in kidney. The absence of a substantial DHEA-S response in kidney, even in wild-type (+/+) mice, suggests that the liver, but not the kidney, is an important target tissue for DHEA-S-regulated peroxisome proliferation. DHEA-S transport into kidney cells may be inefficient, as suggested above, or perhaps enzymes nec-

essary for conversion of DHEA-S to more active metabolites are limited to the liver or present in the kidney at levels too low to allow for peroxisomal or CYP enzyme induction.

In conclusion, our data establish that PPARα is required for the hepatic gene induction effects of DHEA-S, an endogenous regulator of hepatic peroxisomal and CYP4A microsomal enzymes involved in fatty acid β-oxidation and ω-hydroxylation reactions. DHEA-S thus may correspond to an endogenous steroidal PPAR activator that is structurally distinct from the fatty acid- and eicosanoid-derived endogenous PPAR activators identified recently (44–46). It is presently unknown whether these two classes of endogenous PPAR activators use the same or distinct cellular activation pathways and molecular activation mechanisms. DHEA-S and its metabolites may activate PPARα via a direct binding mechanism, such as has been demonstrated in the case of an antidiabetic thiazolidinedione activator of PPARγ (30), or DHEA-S may activate PPARα via an indirect mechanism, perhaps one that is Ca^{+2} dependent (13, 47). Further investigation will be necessary to determine whether this potential of DHEA-S and its metabolites to serve as physiological modulators of liver fatty acid metabolism and peroxisomal enzyme expression contributes to the anticarcinogenic and other beneficial chemoprotective properties of this intriguing class of endogenous steroids.

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