Dehydroepiandrosterone 3β-sulphate is an endogenous activator of the peroxisome-proliferation pathway: induction of cytochrome *P*-450 4A and acyl-CoA oxidase mRNAs in primary rat hepatocyte culture and inhibitory effects of Ca^{2+}-channel blockers

Prabha A. RAM and David J. WAXMAN*

Department of Biological Chemistry and Molecular Pharmacology and Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA 02115, U.S.A.

The role of steroids related to the adrenal androgen dehydroepiandrosterone (5-androstene-3β-ol-17-one; DHEA) in regulating the expression of peroxisomal and cytochrome *P*-450 4A (CYP4A) enzymes active in fatty acid metabolism was assessed using a primary rat hepatocyte culture system. Exposure of hepatocytes to the peroxisome proliferator, clofibric acid (10–250 μM), for 48–96 h led to substantial increases in CYP4A protein, CYP4A1, CYP4A2 and CYP4A3 mRNAs, and the mRNAs encoding both forms of peroxisomal acyl-CoA oxidase (ACOX-I and ACOX-II), as judged by Northern-blot analysis using gene-specific oligonucleotide probes. Although DHEA treatment *in vivo* is effective in inducing these mRNAs in rat liver, it had no effect in the cultured hepatocytes. In contrast, treatment of the cells with DHEA 3β-sulphate (DHEA-S; 10–250 μM) stimulated major increases in CYP4A and ACOX mRNA levels. Examination of several analogues indicated a preference for 3β-sulphate over 17β-sulphated steroids and the inactivity of a 3α-hydroxy-17β-sulphate derivative (DHEA-S > 5-androstene-3β,17β-diol 3-sulphate ~ 5α-androstene-3β-ol-17-one 3-sulphate > 5-androstene-3β,17β-diol 17-sulphate ~ 5β-androstane-3α-ol-17-one 3-sulphate ≫ 5α-androstane-3α,17β-diol 17-sulphate). Induction of CYP4A mRNAs by either DHEA-S or clofibric acid was partially blocked by structurally diverse Ca^{2+}-channel antagonists (nicardipine, nifedipine and diltiazem; 50 μM), suggesting that both the steroidal and fibrate classes of CYP4A inducers stimulate peroxisomal-proliferative responses via a Ca^{2+}-dependent pathway. Retinoic acid alone slightly induced CYP4A mRNAs but did not enhance the induction by clofibrate or DHEA-S. As DHEA-S corresponds to a physiologically important major circulating androgen, these findings suggest that it may serve as an endogenous regulator of hepatic peroxisome enzyme levels. They further suggest that Ca^{2+}-channel blockers may be useful pharmacological tools for the further study of the underlying cellular mechanisms whereby endogenous steroids and fibrate drugs induce peroxisome proliferation, and the relationship of these events to activation of the peroxisome proliferator-activated receptor.

INTRODUCTION

Peroxisomes are single membrane-containing cytoplasmic organelles that carry out diverse metabolic functions. They are enriched in catalase and several H_2O_2-producing oxidases, including acyl-CoA oxidase (ACOX), a rate-limiting enzyme of peroxisomal fatty acid β-oxidation. Peroxisomes are not synthesized *de novo*, but rather are propagated from germ cells to the embryo [1]. Peroxisomal proteins, synthesized in the cytoplasm, are imported into pre-existing peroxisomes, which enlarge in size and then divide. Although protein factors required for peroxisomal assembly have been identified [2], the endogenous factors that regulate peroxisome biogenesis during development [3] and in different cell types are poorly understood.

Insight into the physiological factors that regulate peroxisome size and abundance may be gained from the finding that the peroxisome is an inducible organelle in mammalian liver cells. Treatment of rodents with structurally diverse lipophilic chemicals, including clofibrate and other hypolipidaemic drugs, phthalate ester plasticizers, phenoxyacid herbicides and certain fatty acids stimulates a dramatic increase in both the size and number of peroxisomes found in liver cells [4,5]. This induction is associated with transcriptional activation leading to a dramatic increase in expression of the genes encoding peroxisomal and cytochrome *P*-450 4A (CYP4A) enzymes [6] active in liver fatty acid metabolism [7–9]. Recent studies have identified a peroxisome proliferator-activated receptor (PPAR) as playing a key role in this inductive response [10]. PPAR is a liver-enriched transcription factor that belongs to the steroid/thyroid receptor superfamily [11]. Peroxisome proliferators activate PPAR in transient transfection experiments in a manner that parallels the relative effectiveness of these chemicals as peroxisome proliferators in liver cells [10,12–14]. Direct binding of these PPAR activators to PPAR has not yet been demonstrated, however, suggesting that PPAR may be activated by an indirect mechanism, perhaps involving perturbations in the levels of endogenous lipophiles that serve as natural ligands for this receptor.

One possible endogenous PPAR activator is the adrenal androgen, dehydroepiandrosterone (DHEA). This naturally occurring steroid induces a classic peroxisome-proliferation response when given to rodents at pharmacological doses [15–18]. Recent studies have shown that the 17β-reduced metabolite of DHEA, ADIOL, can also induce peroxisome proliferation in rat liver at somewhat lower concentrations than DHEA, whereas several other intermediates of the adrenal-steroid-biosynthetic pathway are inactive [19]. DHEA can be distinguished from

Abbreviations used: ACOX, acyl-CoA oxidase; CYP, cytochrome *P*-450; PPAR, peroxisome proliferator-activated receptor; DHEA, dehydroepiandrosterone (5-androstene-3β-ol-17-one); DHEA-S, DHEA 3β-sulphate; ADIOL-3βS, 5-androstene-3β,17β-diol 3β-sulphate.

* To whom correspondence should be addressed, at: Division of Cell and Molecular Biology, Department of Biology, Boston University, 5 Cummington Street, Boston, MA 02215, U.S.A.

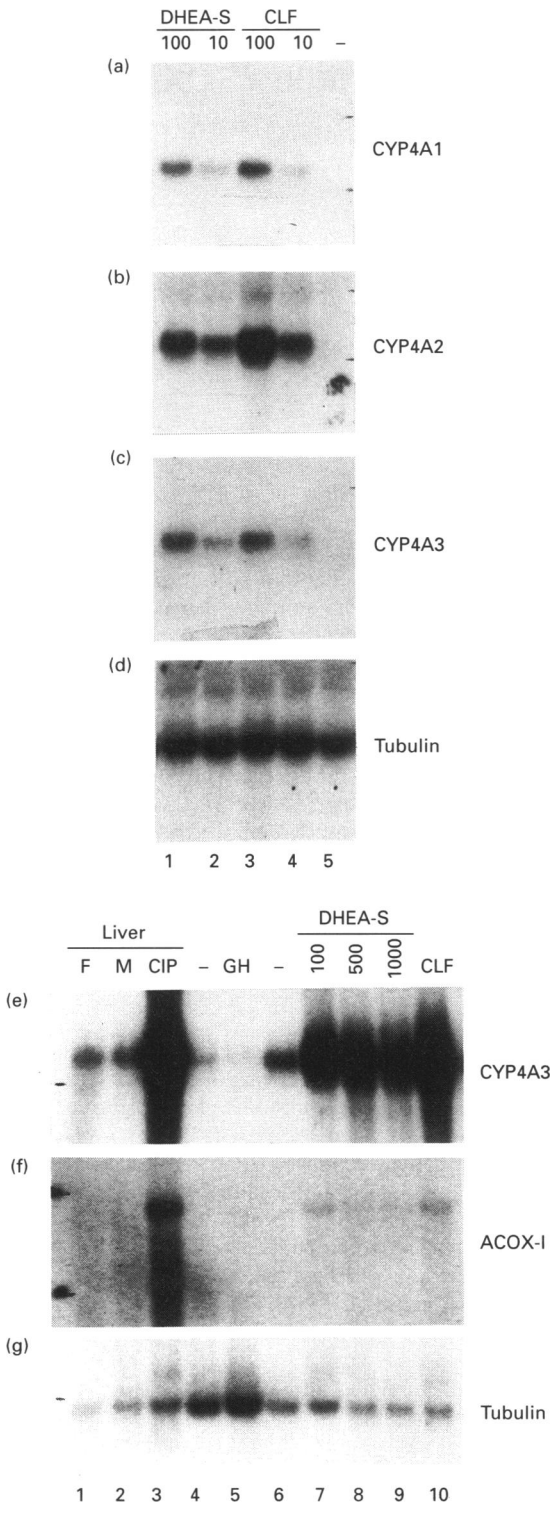

Figure 1 Northern-blot analysis of DHEA-S induction of CYP4A and ACOX-I mRNA levels in primary rat hepatocytes

Primary rat hepatocytes were isolated then cultured for 4 days in media containing DHEA-S (10 μM–1 mM), clofibric acid (CLF; 10 μM–1 mM), growth hormone (GH; 50 ng/ml) or dimethyl sulphoxide vehicle, as indicated for each lane. RNA was then isolated and individual mRNAs analysed by Northern blotting for individual CYP4A, ACOX or tubulin mRNAs, as described in the Materials and methods section. Non-sulphated DHEA and ADIOL had no effect on CYP4A mRNA expression (Figure 3 and results not shown). Hepatocyte RNA samples are shown in lanes 1–5 of (**a**)–(**d**) and lanes 4–10 of (**e**)–(**g**), which are taken from a separate experiment. Induction of CYP4A3 and ACOX mRNAs in livers of intact male rats treated with ciprofibrate (CIP; 2 mg/100 g body weight per day, injected intraperitoneally for 7 days) is shown in lane 3 of (**e**). (**e**)–(**g**), lanes 1 and 2, untreated female (F) and male (M) rat liver RNA. The weak ACOX-I mRNA signals seen in lane 1 of (**f**) may reflect the poor hybridization efficiency of the oligonucleotide probe, rather than low ACOX-I mRNA abundance, as suggested by the comparatively weak liver ACOX-I mRNA signal even in the induced liver (lane 3), which is known to have a high level of ACOX mRNA expression.

foreign chemical peroxisome proliferators by its anticarcinogenic and other therapeutic properties [20–22]. That DHEA itself is not likely to be a direct activator of the peroxisome-proliferation pathway is suggested by the high doses of this steroid required to elicit an inductive effect *in vivo* [15,17,19] and by the inactivity of DHEA with respect to induction of peroxisomal and CYP4A enzymes in primary hepatocytes under conditions in which the cells are responsive to induction by foreign chemical peroxisome proliferators such as clofibrate [23]. The present study was undertaken to evaluate directly the characteristics of DHEA and DHEA-related steroids as inducers of a peroxisome-proliferative response and their effect on CYP4A mRNAs in a primary rat hepatocyte culture system. The results obtained indicate that the hepatic effects of DHEA do not involve a direct action of DHEA on the hepatocyte, but are mediated by sulphate conjugates of DHEA.

MATERIALS AND METHODS

Primary hepatocyte cultures

Hepatocytes were isolated from adult male Fischer 344 rats (150–200 g) by a collagenase perfusion method as previously described [24]. Cell viability was 80–95%, as tested by Trypan Blue exclusion. Freshly isolated cells (4×10^6/plate) were seeded on Vitrogen-precoated 60 mm-diameter culture dishes in 5 ml of modified Chee's medium containing 6.25 μg/ml insulin, 50 μg/ml gentamicin sulphate and 1 μM dexamethasone [24]. After 4 h, the culture medium was changed to medium containing 0.1 μM dexamethasone. Cultures were maintained at 37 °C in a humidified atmosphere and the medium was replaced every 24 h.

Clofibric acid, steroid sulphates, Ca^{2+}-channel blockers and retinoic acid were added to the culture medium 0–48 h after seeding (as specified) to give the final concentrations indicated in each experiment. Fresh media and test compounds were added every 24 h thereafter. Chemicals were dissolved in dimethyl sulphoxide and added to the culture medium so that the final solvent concentration was 0.4% (v/v) in all cases, including the controls. Cells from two to four dishes were harvested with a rubber 'policeman' and washed with PBS. The cell pellet was used for RNA or Western-blot analysis.

RNA preparation and analysis

Total RNA was prepared from the cultured hepatocytes using a guanidinium thiocyanate method [25] and then analysed by Northern blotting as detailed elsewhere [26]. Nylon filters were probed with 5′-end-labelled oligonucleotides complementary to individual *P*-450 and ACOX mRNAs using hybridization and high-stringency washing conditions specific to each oligonucleotide [26]. The oligonucleotides used in the study were ON-29, ON-30, ON-31 and ON-50, for detection of the mRNAs encoding CYP4A1, CYP4A2, CYP4A3 and tubulin respectively [27]. ACOX-I and ACOX-II mRNAs were individually detected using oligonucleotides ON-160 and ON-161, which are respectively complementary to a region of high sequence divergence between the ACOX-I and ACOX-II mRNAs (coding sequence nucleotides 304–325) [28]. ON-160 = 5′-GAA-TAA-ACA-TGG-AGT-AAT-TGA-G-3′ and ON-161 = 5′-GTA-GGA-ACA-TGC-CCA-AGT-GAA-G-3′. Hybridization at 45 °C in buffer

containing no formamide (ON-160) or 10% formamide (ON-161) and high-stringency washings were carried out as described elsewhere [26].

Western immunoblot analysis

Immunoblot analysis of isolated hepatocyte microsomes was carried out as described previously [27] using polyclonal antibodies to rat liver CYP4A1 that are cross-reactive with CYP4A2 and CYP4A3 and were kindly provided by Dr. James P. Hardwick (Rootstown, OH, U.S.A.). Western-blot analysis using polyclonal antibody to CYP2B1 was performed as reported previously [24].

RESULTS

Effects of DHEA-S on CYP4A and ACOX induction in primary rat hepatocytes

Studies carried out in intact rats and in cultured hepatocytes have demonstrated that clofibric acid induces the expression of several cytochromes *P*-450 belonging to family 4A (CYP4A), and this induction may correspond to an early and perhaps obligatory step in the peroxisome-proliferation pathway [9,27,29,30]. As primary rat hepatocytes are known to be responsive to clofibric acid and other peroxisome proliferators [31,32], we utilized this cell system to study the mechanisms whereby DHEA induces a peroxisome-proliferative response. Initial experiments revealed, however, that primary rat hepatocytes cultured in a chemically defined serum-free medium were not responsive to DHEA (25–100 μM) under conditions in which clofibric acid induced a major increase in the mRNAs encoding CYP4A1, CYP4A2 and CYP4A3, as determined by hybridization using gene-specific oligonucleotide probes (10–100 μM clofibric acid for 48–72 h; Figure 1 and results not shown). Similarly, the hepatocytes were not responsive to the 17β-reduced metabolite of DHEA (5-androstene-3β,17β-diol; 25–100 μM), which is at least as active as DHEA in inducing liver peroxisome proliferation in intact rats [19]. This suggested that neither DHEA nor its 17β-reduced metabolite is the active inducer, but rather, that these steroids must first be activated by metabolic reactions that are carried out in intact rats but not in cultured hepatocytes. Since DHEA primarily circulates in the sulphated form [21,22], we examined the effects of the 3β-sulphate of DHEA, DHEA-S, on CYP4A mRNA levels in the hepatocyte cultures. Northern-blot analysis revealed that when hepatocytes are treated with DHEA-S, a substantial induction of all three CYP4A mRNAs is achieved (Figures 1a–1c). These inductions were observed at DHEA-S concentrations as low as 10 μM, which is within the range of physiological circulating levels of this adrenal androgen [21,22].

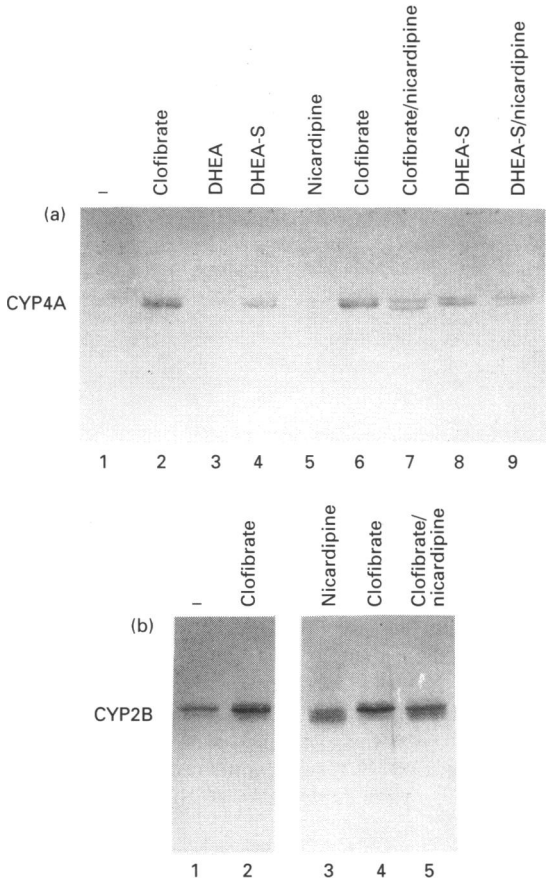

Figure 2 Western-blot analysis of CYP4A and CYP2B induction in primary hepatocytes

Cells were treated from day 3 to day 7 in culture with clofibric acid, DHEA, DHEA-S (250 μM each; media changed fresh daily) or dimethyl sulphoxide vehicle in the presence or absence of nicardipine (50 μM), as indicated. Microsomes were prepared from cells harvested from four plates and then analysed for immunoreactive CYP4A (**a**) and CYP2B (**b**) by Western blotting. Clofibric acid and DHEA-S induced at least two CYP4A proteins (**a**, lanes 2, 4 and 6), and these inductions were partially blocked by nicardipine (lanes 7 and 9). The clofibric acid induction of immunoreactive CYP2B (**b**, lanes 2 and 4) was also inhibited by nicardipine (lane 5). Nicardipine itself induced several unidentified CYP2B-immunoreactive proteins showing higher electrophoretic mobilities than the major clofibric acid-inducible protein (**b**, lane 3).

Western-blot analysis of microsomes isolated from these hepatocytes further established that the induction of CYP4A mRNA by clofibric acid and DHEA-S is reflected by an increase in CYP4A protein accumulation (Figure 2a, lanes 2 and 4). Further analysis revealed that clofibric acid, but not DHEA-S, is a weak inducer of CYP2B protein (Figure 2b, lane 2 compared with lane 1), as well as CYP2B1 mRNA and CYP2B-dependent androstenedione 16β-hydroxylation catalysed by isolated hepatocyte microsomes (results not shown). This latter observation is consistent with our earlier finding that clofibrate induces CYP2B1-specific microsomal activities in rat liver [33] but contrasts with the conclusion of others, based on measurements of benzphetamine metabolism in isolated rat liver microsomes, that clofibrate is not a CYP2B inducer [29,34].

We next examined whether the induction of CYP4A by DHEA-S is also associated with the induction of mRNA encoding the peroxisomal enzyme ACOX, as occurs for true peroxisomal enzyme inducers (e.g. [35]). Previous studies of rat liver ACOX cDNAs have suggested the existence of two ACOX mRNAs, which are predicted to encode proteins that are identical, with the exception of a 54-amino acid region where the sequence identity drops to 50% [28]. The two ACOX mRNAs are derived from a single gene by differential splicing [36]. Since the expression of these two mRNAs has not been previously examined individually, we designed oligonucleotide probes that can readily distinguish the two mRNAs. Northern-blot analysis using these probes revealed that DHEA-S induces the expression of the mRNAs encoding both forms of ACOX, ACOX-I (Figure 1f) and ACOX-II (results not shown), as does clofibric acid. Thus DHEA-S induces the expression of both microsomal and peroxisomal enzymes in primary rat hepatocytes.

Steroid sulphate induction of CYP4A mRNAs: effects of DHEA-S analogues

To characterize more fully the inductive response to DHEA-S,

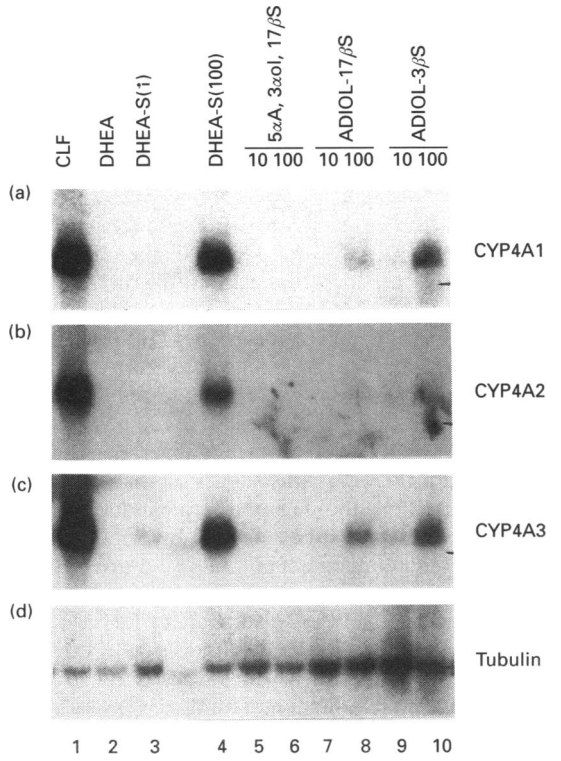

Figure 3 Induction of CYP4A mRNAs by DHEA-S and analogues in primary rat hepatocytes

Cells were induced with clofibric acid (CLF), DHEA (100 μM each), DHEA-S (1 or 100 μM), 5α-androstane-3α,17β-diol 17-sulphate (5αA,3αol,17βS), 5-androstene-3β,17β-diol 17-sulphate (ADIOL-17βS) and 5-androstene-3β,17β-diol 3-sulphate (ADIOL-3βS) for 5 days beginning 24 h after seeding of the hepatocytes. Northern-blot RNA analysis was as described in the Materials and methods section.

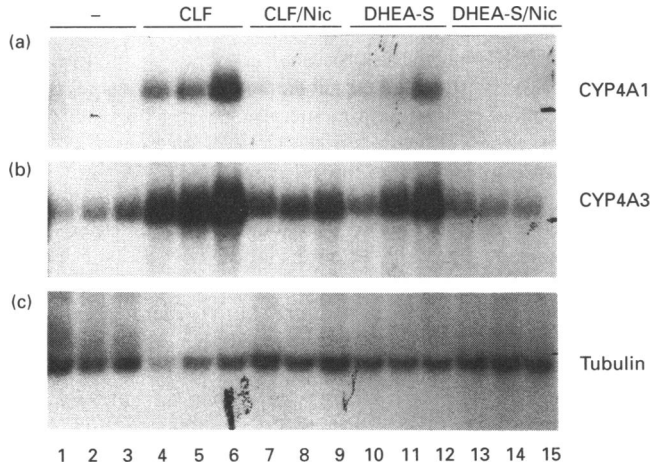

Figure 4 Inhibition of CYP4A induction by the Ca^{2+}-channel blocker nicardipine

Primary rat hepatocytes were induced in cell culture for 2, 3 or 4 days with dimethyl sulphoxide vehicle (lanes 1–3) or with clofibric acid (CLF; 250 μM) or DHEA-S (250 μM) in the presence or absence of nicardipine (Nic; 50 μM), as indicated (lanes 4–15). CYP4A expression was analysed by Northern blotting. Lanes 1, 4, 7, 10 and 13, drug treatment for 2 days in culture; lanes 2, 5, 8, 11 and 14, drug treatment for 3 days; lanes 3, 6, 9, 12 and 15, drug treatment for 4 days. Nicardipine treatment alone had no detectable effect on cell viability or basal CYP4A mRNA expression (see also Figure 5, lanes 2–4).

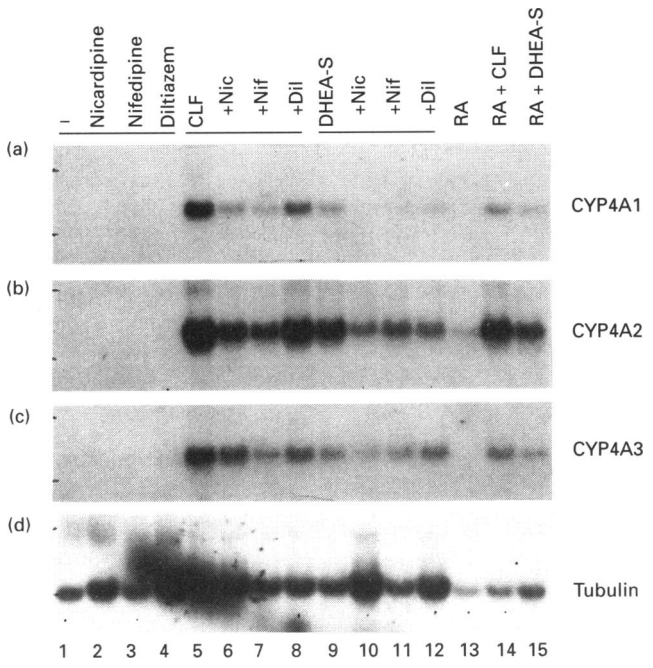

Figure 5 Effect of Ca^{2+}-channel blockers and retinoic acid on CYP4A mRNA induction

Primary hepatocytes were cultured for 4 days in the absence of inducer (lane 1) or in the presence of clofibric acid (100 μM CLF; lanes 5–8), DHEA-S (100 μM; lanes 9–12) or retinoic acid, either alone (1 μM RA; lane 13) or in combination with 100 μM clofibric acid or 100 μM DHEA-S (lanes 14 and 15). Cells were additionally treated with the Ca^{2+}-channel blockers nicardipine (Nic; lanes 2, 6 and 10), nifedipine (Nif, lanes 3, 7 and 11) or diltiazem (Dil; lanes 4, 8 and 12), each at 50 μM, for the 4-day period. Shown is a Northern blot of RNA samples analysed for CYP4A mRNA expression as described in the Materials and methods section. Tubulin RNA levels seen in (**d**) indicate that the RNA samples in lanes 2, 6, 10 and 12 are overloaded by $\sim$ 2-fold compared with the other lanes.

we next examined the effects of several analogues and metabolites of DHEA-S. These studies revealed that the 17β-reduced metabolite ADIOL-3βS was nearly as active as DHEA-S (Figure 3, lanes 9 and 10), a finding that is consistent with the high peroxisome activity *in vivo* of the parent (unsulphated) ADIOL [19]. Somewhat lower activity was observed with the isomeric 17β-sulphate, ADIOL-17βS (Figure 3, lanes 7 and 8) and with 5α-reduced DHEA-S (results not shown). More substantial decreases in activity were obtained with the 5β-reduced, 3α-sulphate analogue of DHEA-S (results not shown), whereas the 5α-reduced, 3α-hydroxy analogue of ADIOL-17βS was inactive (Figure 3, lanes 5 and 6).

Influence of the Ca^{2+}-channel blocker nicardipine on CYP4A induction

The Ca^{2+}-channel blocker nicardipine has been shown to partially block peroxisome proliferative responses induced by fibrate drugs when given chronically to intact rats *in vivo* [37]. We therefore tested nicardipine as an inhibitory probe in primary rat hepatocytes to ascertain whether clofibric acid and DHEA-S may utilize a common pathway in their induction of a peroxisome proliferative response. Our findings revealed that nicardipine (50 μM) is an effective inhibitor of CYP4A induction stimulated by either clofibric acid or DHEA-S (Figure 4). Similar effects were achieved with nifedipine and diltiazem, Ca^{2+}-channel

blockers that act by mechanisms different from that of nicardipine [38], although diltiazem was somewhat less effective as an inhibitor of CYP4A induction than the other two compounds at the concentration tested (50 μM) (Figure 5, lane 5 compared with lanes 6–8, and lane 9 compared with lanes 10–12). Together, these findings suggest that clofibric acid and DHEA-S both activate peroxisome proliferative responses via a common mechanism, and that this mechanism involves activation of a Ca^{2+}-dependent intracellular signalling pathway.

Influence of retinoic acid on CYP4A mRNA induction

Recent studies have shown that retinoic acid can stimulate an increase in the expression of ACOX and other peroxisomal enzymes in primary rat hepatocytes [39], and in addition, retinoic acid-derived metabolites can facilitate the induction of peroxisome-proliferator-inducible genes by binding to the retinoid X-receptor, which in turn heterodimerizes with PPAR, which then binds to 5′-flanking genomic DNA segments and activates transcription of peroxisomal enzymes [40,41]. We therefore examined whether retinoic acid could enhance the induction of CYP4A mRNAs by either clofibric acid or DHEA-S. These experiments, shown in Figure 5, revealed that retinoic acid could induce the expression of CYP4A mRNA to a small extent [induction seen most clearly for CYP4A2 in this experiment (Figure 5, lane 13)]. However, retinoic acid did not further stimulate the induction of these mRNAs when given in combination with clofibric acid or DHEA-S (Figure 5, lane 14 compared with lane 5, and lane 15 compared with lane 9).

DISCUSSION

In the present study, we used a rat hepatocyte culture system to study the role of DHEA and related steroids in inducing peroxisome proliferation. Exposure of cultured rat hepatocytes to DHEA-S or ADIOL-S led to substantial increases in CYP4A protein and CYP4A1, CYP4A2 and CYP4A3 mRNAs, as well as the mRNAs encoding both forms of peroxisomal ACOX (I and II). The unsulphated steroids DHEA and ADIOL were inactive in this cellular system, in contrast with their high peroxisome proliferation activity *in vivo* [19]. This suggests that DHEA sulphation, which occurs both in liver and at the site of steroid synthesis in the adrenal [42], not only serves to facilitate systemic transport of the steroid, but also activates the steroid to a form that can stimulate the peroxisome-proliferation pathway. Whether DHEA-S itself is the ultimate activator of CYP4A expression, or whether DHEA-S requires further metabolism for conversion into an active form, cannot yet be determined. However, our conclusion that DHEA-S is an activated form of DHEA is consistent with our unpublished *in vivo* studies which demonstrate that DHEA-S is a good inducer of CYP4A mRNAs in intact rat liver, and that its effects correlate well with those of DHEA; moreover, DHEA-S was seen to be more potent than DHEA with respect to CYP4A3 induction when given to rats at low doses (10 mg/kg) (P. A. Ram and D. J. Waxman, unpublished work). The inactivity of DHEA in our hepatocyte cultures and in those of others [23] suggests that DHEA sulphotransferase activity is low in these cells, although it may be high under other culture conditions, as suggested by a recent study that reported effective peroxisome-proliferative responses elicited by both DHEA and DHEA-S in primary rat hepatocytes [43].

Analysis of several analogues of DHEA-S for their ability to induce CYP4A expression revealed a preference for 3β-sulphate over 17β-sulphated steroids, and the inactivity of certain 3α-substituted derivatives. As DHEA-S is a major circulating steroid [44], these findings suggest that DHEA-S is an important mediator of the effects of DHEA on liver enzyme expression, and consequently, that DHEA-S may play a key physiological role in regulation of liver peroxisomal enzyme levels *in vivo*. As sulphation of DHEA and related steroids is actively catalysed by liver sulphotransferase enzymes [45], DHEA, ADIOL and perhaps other endogenous steroids could regulate liver peroxisome proliferation *via* intracrine mechanisms. Steroid sulphation/desulphation could also provide a mechanism for regulation of liver CYP4A and peroxisomal enzyme synthesis independent of the actions of fatty acids, which comprise a second, structurally distinct, class of endogenous peroxisome proliferators [5,46].

The recently discovered orphan receptor PPAR has been shown to mediate the effects of foreign chemicals, such as clofibric acid, on CYP4A and peroxisomal enzyme gene expression [14,47,48]. Although PPAR belongs to the steroid/thyroid receptor superfamily, whose members correspond to intracellular ligand-activatable transcription factors [11,49,50], it is unclear in the case of PPAR whether receptor activation results from a direct interaction between PPAR and its foreign chemical activators. This uncertainty derives from the inability to detect direct binding of foreign chemical peroxisome proliferators to the receptor in cases where it has been examined [10], and from the low apparent ligand-specificity requirements for PPAR activation, which contrasts with the high ligand specificity that is characteristic of other members of the steroid receptor superfamily [50]. In contrast, it is apparent from structure–activity data for peroxisome proliferation induced by DHEA-S and related steroid sulphates reported in the present study that small changes in steroid structure (e.g. replacement of 3β-sulphate by 17β-sulphate, or replacement of 3β-OH by 3α-OH) can greatly reduce CYP4A induction activity. This suggests a higher degree of structural specificity for the steroid sulphates than other chemical classes of peroxisome proliferators, and this in turn raises the possibility that DHEA-S could correspond to an endogenous activating ligand of PPAR that acts by a direct binding mechanism, as occurs in the case of the classic steroid hormones and steroid hormone receptors [51]. On the other hand, the inability of DHEA-S to activate a cloned glucocorticoid receptor–PPAR chimaera when tested in a transient *trans*-activation assay [13] suggests that receptor activation may be mediated by a metabolite of DHEA-S that is formed in hepatocytes but not in the heterologous cell systems used in the transient receptor/reporter transfection experiments. Alternatively, DHEA-S may activate a PPAR-related receptor rather than PPAR itself. Multiple PPARs have been characterized in both *Xenopus* [12] and humans [52,53]; multiple PPARs appear to be present in rodents as well, as suggested by the tissue specificity of mouse PPAR mRNA [10], as compared with that of a 3.5 kb mRNA detected on Northern blots probed with a cDNA encoding a human PPAR-related receptor [52]. Whether PPAR itself is activated by DHEA-S, or whether the effects of steroid sulphates on CYP4A and ACOX gene transcription reported in this study are mediated by a PPAR-related receptor protein, has yet to be established.

In view of the fact that direct receptor-binding mechanisms have been difficult to demonstrate, peroxisome proliferation induced by fibrate drugs, fatty acids and perhaps also steroid sulphates might indeed proceed through indirect binding interactions. One lead that may prove useful in the identification of possible intracellular metabolic or signalling pathways that are stimulated by both fibrate and steroid sulphate peroxisome proliferators is provided by the present demonstration that the

Ca^{2+}-channel blocker nicardipine can inhibit a peroxisome proliferation response not only in an intact rat model [37], but in rat hepatocytes in cell culture as well. Moreover, this inhibition was achieved using several structurally distinct Ca^{2+}-channel blockers and was observed using both DHEA-S and clofibric acid as inducing agents. These findings suggest several possible working models, including (*a*) Ca^{2+}-dependent processes that are modulated by peroxisome proliferators and which lead to PPAR activation in the absence of direct binding to PPAR of PPAR activators or alternatively (*b*) models involving direct binding to PPAR of PPAR activators, followed by the activation of Ca^{2+}-dependent enzymes that influence the ability of ligand-bound PPAR to increase transcription of target genes. Support for our proposed role of Ca^{2+}-dependent processes in the activation of PPAR-dependent gene transcription is provided by the finding that the peroxisome proliferator and PPAR activator ciprofibrate can inhibit the endoplasmic reticulum Ca^{2+}-ATPase in hepatocytes, leading to an elevation of intracellular Ca^{2+} levels [54,55]. Independent of whether such an increase in Ca^{2+} turns out to be sufficient to activate PPAR (i.e. even in the absence of a peroxisome proliferator), the effects of nicardipine and the other Ca^{2+} antagonists used in the present study point to the importance of Ca^{2+} for PPAR activity. As receptor phosphorylation has been shown to modulate the transcriptional activity of several steroid receptors [56], the action of nicardipine could be at the level of one or more Ca^{2+} or Ca^{2+}–calmodulin-dependent phosphoprotein phosphatases (e.g. calcineurin) or protein kinases (e.g. protein kinase C) that modulate the level of PPAR phosphorylation. The finding that protein kinase activators and phosphoprotein phosphatase inhibitors can activate the progesterone receptor in a hormone-independent manner [57] provides a precedent for any ligand-independent and phosphorylation-dependent PPAR activation models that might emerge from further studies of the role of Ca^{2+} in steroid- and fibrate-induced peroxisome-proliferation events.

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