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Negative Regulation by Dexamethasone of Fluvastatin-Inducible CYP2B Expression in Primary Cultures of Rat Hepatocytes: Role of CYP3A

Thomas A. Kocarek and Anita B. Reddy*
INSTITUTE OF CHEMICAL TOXICOLOGY, WAYNE STATE UNIVERSITY, DETROIT, MI 48201, U.S.A.

ABSTRACT. Fluvastatin (Fluva), a synthetic inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase, induces CYP2B1/2 in rat liver and primary cultured rat hepatocytes. However, the overall profile of CYP induction, which includes induction of CYP4A, suggests that Fluva is not a typical "phenobarbital (PB)-like" inducer. Several treatments affecting diverse cell signaling pathways have been reported to modify PB-inducible CYP2B expression in primary cultured rat hepatocytes. We examined the effects of selected treatments on the ability of Fluva to induce CYP2B1/2 mRNA. Only dexamethasone (Dex) produced effects on Fluva-inducible CYP2B1/2 mRNA expression that differed from those produced on PB-inducible CYP2B1/2 mRNA expression. Dex concentrations up to 10^{-7} M of potentiated PB (10^{-4} M)-mediated CYP2B1/2 mRNA induction, while higher Dex concentrations produced a progressive reduction in PB-induced CYP2B1/2 mRNA levels. By contrast, Dex concentrations up to 10^{-8} M had no effect on Fluva (3×10^{-5} M)-induced CYP2B1/2 mRNA levels, while Dex concentrations of 10^{-7} M and higher markedly suppressed Fluva-mediated CYP2B1/2 mRNA induction. The concentrations of several glucocorticoids that produced suppression of Fluva-induced CYP2B1/2 mRNA levels were the same concentrations that induced CYP3A mRNA. Treatment with pregnenolone 16α-carbonitrile also produced a concentration-dependent suppression of Fluva-induced CYP2B1/2 mRNA levels. Dex-mediated suppression of Fluva-induced CYP2B1/2 mRNA was concentration-dependently reversed when hepatocytes were cotreated with troleandomycin, a selective CYP3A inhibitor. The amounts of Fluva detected in culture medium and cells were reduced significantly when hepatocytes were incubated with Dex. However, Dex-mediated suppression of Fluva-induced CYP2B1/2 mRNA expression was not overcome when hepatocytes were incubated with Fluva concentrations greater than 3×10^{-5} M, suggesting that mechanisms other than CYP3A-catalyzed metabolism may contribute to Dex-mediated suppression of Fluva-induced CYP2B1/2 expression. BIOCHEM PHARMACOL **55**;9:1435–1443, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. cytochrome P450; CYP; fluvastatin; phenobarbital; dexamethasone; glucocorticoid; hepatocyte

Fluva† is a synthetic inhibitor of HMG-CoA reductase that is in widespread clinical use for the treatment of hypercholesterolemia. We recently reported that Fluva is an effective inducer of CYP2B1/2 mRNA and immunoreactive protein in rat liver and in primary cultures of rat hepatocytes [1]. Though the ability of Fluva to induce CYP2B1/2 might suggest that Fluva is a typical "PB-like" CYP inducer, the profile of CYPs that was induced following Fluva treatment differed from that induced by PB treatment. Thus, although both PB and Fluva induced primarily CYP2B1/2, with lesser inductions of CYP3A, treatment with Fluva, but not PB, also produced a marked induction of CYP4A, a response characteristic of peroxisome proliferators [1]. An important issue, therefore, is whether PB and Fluva produce their effects on CYP2B expression through the same, or through different, mechanisms. This issue is complicated by the fact that the cellular mechanism(s) through which PB produces its inductive effects on CYP2B expression has not been established. Despite this limitation, several experimental manipulations have been reported to modify the extent of CYP2B induction that occurs when primary cultured rat hepatocytes are treated with PB. These manipulations include altering the composition of the culture medium [2] or of the extracellular matrix [3], inhibiting protein synthesis [4], and activating signal transduction pathways used by growth hormone [5] or cyclic AMP [6]. In addition, supplementation of culture medium with a glucocorticoid hormone has been found to alter the ability of PB to induce CYP2B [2, 7–9]. These findings suggest that multiple cell signaling systems are essential to the PB induction process.

In this study, we have examined the effects of several of the aforementioned manipulations on the abilities of PB and Fluva to induce CYP2B in primary cultured rat hepatocytes. We report that, with one exception, each of the

* Corresponding author: Thomas A. Kocarek, Ph.D., Institute of Chemical Toxicology, Wayne State University, 2727 Second Avenue, Room 4000, Detroit, MI 48201.

† *Abbreviations:* CYP, cytochrome P450; Dex, dexamethasone; Fluva, fluvastatin; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; PB, phenobarbital; PCN, pregnenolone 16α-carbonitrile; and TAO, troleandomycin.

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treatments modified in parallel the CYP2B induction that was produced by either PB or Fluva. However, concentrations of DEX that potentiated PB induction were highly suppressive of Fluva induction. These suppressive effects appeared to be at least partially attributable to increased CYP3A activity, and consequent Fluva biotransformation.

MATERIALS AND METHODS

Materials

Fluva was a gift from the Sandoz Research Institute. Cycloheximide, Dex, dibutyryl cyclic AMP, glucagon, growth hormone (porcine), PB, triamcinolone, triamcinolone acetonide, and TAO were purchased from the Sigma Chemical Co. Prednisolone and 6β-hydroxyprednisolone were purchased from Steraloids. HPLC solvents were purchased from Burdick & Jackson. Vitrogen (95–98% type I collagen with remainder type III collagen) was purchased from Celtrix. Other supplies and reagents were purchased from the sources previously described [1]. Plasmids containing cDNA inserts to CYP2B1 (pSR-p450), CYP3A1 (pDex12), and 7S RNA (pA6) were gifts from Dr. Milton Adesnik (New York University), Dr. Philip Guzelian (University of Colorado), and Dr. Allan Balmain (Beatson Institute), respectively. A 389-bp cDNA probe to rat HMG-CoA reductase was prepared using the reverse transcriptase–polymerase chain reaction technique, as described previously [1].

Hepatocyte Culture

Hepatocytes were isolated from the livers of adult male Sprague–Dawley rats (220–280 g) by collagenase perfusion, and plated onto 60-mm tissue culture dishes that were precoated with 1.5 mg Matrigel, unless otherwise indicated, as previously described [1]. When dishes were coated with Vitrogen, the covalent attachment method described by Waxman *et al.* [2] was employed. Cultured hepatocytes were incubated routinely with serum-free Williams' Medium E supplemented with 0.25 units/mL of insulin, 100 units/mL of penicillin, 100 μg/mL of streptomycin, and 15 mM of HEPES. Drug incubations were performed as described in the individual figure legends. Drugs were added to the cultures as concentrated stock solutions in water (PB and Fluva) or DMSO (glucocorticoids and TAO). The final DMSO concentration in the culture medium was 0.1% unless otherwise indicated.

Northern and Slot Blot Analyses

Total RNA was prepared from two or three pooled dishes of cultured hepatocytes and analyzed by northern blot hybridization as previously described [1]. Alternatively, RNA was prepared individually from triplicate dishes of hepatocytes, and samples (5 μg) were analyzed by slot blot hybridization. After autoradiography, filters were incubated briefly at 90° in 1% SDS to remove hybridized probes. Blots were then rehybridized using 7S cDNA insert to control for RNA loading and transfer. Band intensities were quantified using a scanning laser densitometer (Molecular Dynamics) equipped with ImageQuant software (version 3.3). To ensure that band intensities were quantified within the linear capacity of the film, several serial dilutions of a standard RNA sample were loaded onto each slot blot. For each blot, multiple film exposures were prepared, and only those experimental samples on a given film that fell within a linear range of standard dilutions were quantified.

Analysis of Fluva Levels in Culture Medium and Hepatocytes

The amounts of Fluva remaining in culture medium or hepatocytes were measured following a 24-hr incubation with 3×10^{-5} M of Fluva in the presence or absence of Dex. After incubation, 1-mL aliquots of culture medium were transferred to glass culture tubes. Remaining medium was aspirated, and attached cells were washed one time with ice-cold phosphate-buffered saline. Hepatocytes were then scraped into 1 mL of 0.1 M of ice-cold potassium phosphate buffer, pH 7.4, and sonicated for 30 sec, and lysates were transferred to glass culture tubes. Pravastatin (10^{-8} mol) was added to medium and lysate samples as an internal standard. Four milliliters of acetonitrile were added to each tube of medium or cell lysate, and tubes were vortexed and centrifuged for 10 min to precipitate proteins. Three milliliters of supernatant were transferred to fresh tubes, and the liquids were evaporated to dryness under nitrogen gas. Residues were dissolved in 30 μL of HPLC initial condition mobile phase (30% acetonitrile in water, 5 mM of formic acid), and 20 μL was injected onto an HPLC system consisting of two Waters model 510 pumps and model 680 gradient controller. The HPLC separation method employed was that previously described for resolving lovastatin and simvastatin metabolites [10, 11]. Separations were achieved using a 3.9 × 150 mm C18 column (Waters Nova-Pak) and a binary mobile phase consisting of 5 mM of formic acid (solvent A) and 5 mM of formic acid in acetonitrile (solvent B). The solvent gradient consisted of 70% A for 3 min, 70 to 10% A over 20 min and 10% A for 5 min. Flow rate was 1 mL/min. Ultraviolet absorption of the eluate was monitored at 238 nm. Under these conditions, pravastatin and Fluva eluted with retention times of 4.9 and 13.6 min, respectively. Peak areas were determined using a Waters model 746 data module. Amounts of Fluva present in cells or culture medium were calculated from a Fluva standard curve following correction for recovery of internal standard.

Statistical Analysis

Data were analyzed using one-way ANOVA followed by the Tukey–Kramer test, with the level of significance set at 0.05.

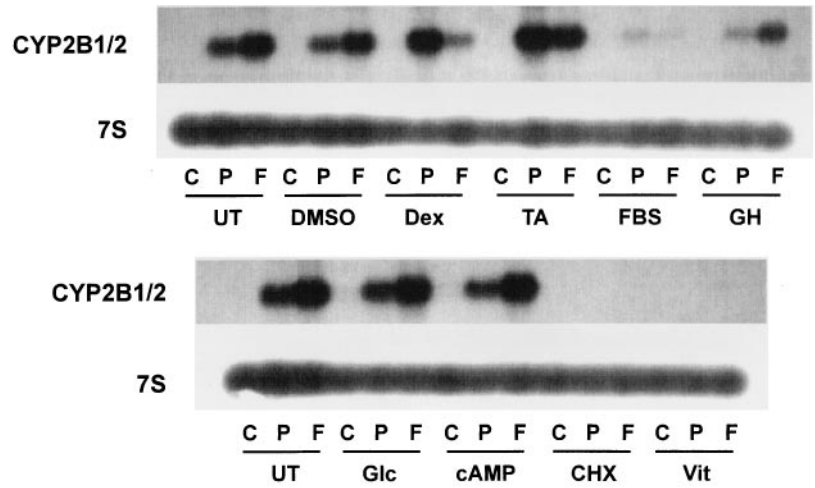

FIG. 1. Effects of treatments that modulate PB-inducible CYP2B1/2 expression on Fluva-inducible CYP2B1/2 mRNA levels in primary cultures of rat hepatocytes. Beginning 24 hr after plating, rat hepatocyte cultures maintained on a Matrigel substratum (9 dishes/treatment group) were treated with one of the following: DMSO (0.1% final volume), 10^{-7} M of Dex, 10^{-7} M of triamcinolone acetonide (TA), 10% fetal bovine serum (FBS), 1 m units/mL of porcine growth hormone (GH), 10^{-7} M of glucagon (Glc) or 10^{-5} M of dibutyryl cyclic AMP (cAMP). Beginning 48 hr after plating, one group of cultures on Matrigel (9 dishes) was treated with 10 μg/mL of cycloheximide (CHX). One group of cultures (9 dishes) was maintained in standard medium on dishes coated with Matrigel (UT), and one group was maintained on dishes coated with Vitrogen (Vit). Beginning 48 hr after plating, 3 dishes from each treatment group described above were treated with 10^{-4} M of PB (P), 3 dishes were treated with 3×10^{-5} M of Fluva (F), and 3 dishes served as controls (C). At 72 hr, the 3 dishes of hepatocytes comprising each treatment group were pooled for preparation of total RNA, and levels of CYP2B1/2 mRNA were analyzed by Northern blot hybridization. Also shown are autoradiographs of the blots following rehybridization with a cDNA to 7S RNA.

RESULTS

The primary mechanism through which PB or any other agent induces CYP2B1/2 is not known. However, manipulations that modulate the ability of PB to induce CYP2B in primary hepatocyte culture may provide insight into whether PB and Fluva induce CYP2B through a common mechanism. We therefore examined the effects of several of these manipulations on the levels of CYP2B1/2* RNA induction produced in primary cultured rat hepatocytes treated for 24 hr (beginning 48 hr postplating) with PB or Fluva, at concentrations (10^{-4} M and 3×10^{-5} M, respectively) that were demonstrated previously to be optimal for CYP2B1/2 induction [1, 12]. Incubations with agents (except cycloheximide) reported to modulate CYP2B1/2 mRNA induction were begun 24 hr prior to PB treatment, and were continued for 48 hr to maximize the ability of these treatments to evoke changes in inducible CYP2B1/2 mRNA expression. Cycloheximide was added to the culture medium simultaneously with PB or Fluva, because incubations with this protein synthesis inhibitor exceeding 24 hr proved to be grossly toxic to the cultured hepatocytes (data not shown). Although we routinely detect a low level of CYP2B1/2 mRNA in 3-day-old untreated cultures of rat hepatocytes maintained under our standard conditions, consistent with an earlier report [13], these low levels of CYP2B1/2 mRNA are not readily apparent in the present study because the experimental conditions (e.g. amounts of RNA that were analyzed on northern and slot blots and autoradiographic exposure times) were selected to focus on the considerably higher levels of CYP2B1/2 mRNA occurring in the PB- or Fluva-induced cultures. CYP2B1/2 mRNA induction by PB or Fluva was suppressed markedly or abolished when hepatocyte cultures were incubated in medium containing fetal bovine serum, growth hormone, or cycloheximide, or when hepatocytes were maintained on a Vitrogen substratum (Fig. 1). Under the treatment conditions used in these studies, treatment of hepatocytes with glucagon or dibutyryl cyclic AMP had little effect on the CYP2B1/2 mRNA induction produced by either PB or Fluva (a likely reason for this lack of effect is discussed below). Thus, none of the above treatments produced effects that discriminated between the induction of CYP2B1/2 that was produced by PB and the induction produced by Fluva. Incubation of hepatocytes in the presence of a 10^{-7} M concentration of the potent glucocorticoid Dex produced a marked potentiation of the PB-induced level of CYP2B1/2 mRNA, as previously reported [2, 7–9], but suppressed Fluva-induced CYP2B1/2 mRNA expression. By comparison, incubating hepatocytes with a 10^{-7} M concentration of another potent glucocorticoid, triamcinolone acetonide, also potentiated PB-mediated CYP2B1/2 mRNA induction, but did not suppress Fluva-induced CYP2B1/2 mRNA expression. Concentration–response analysis revealed that incubation of hepatocytes with concentrations of Dex up to 10^{-7} M potentiated PB-mediated CYP2B1/2 mRNA induction, but that higher

* Because the CYP cDNA probes used in these studies detect multiple related mRNA species, the specific bands detected on the northern or slot blots with the CYP2B1 and CYP3A1 probes are referred to as CYP2B1/2 and CYP3A, respectively.

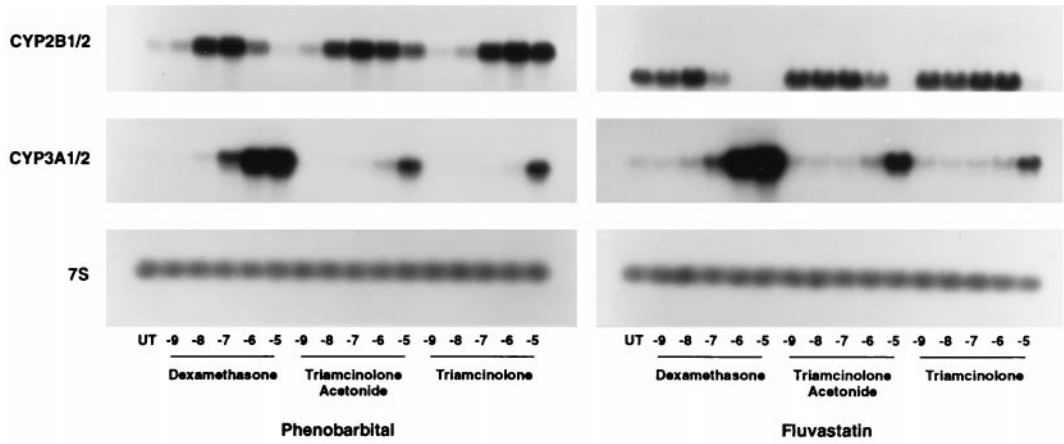

FIG. 2. Concentration-dependent effects of glucocorticoid treatments on PB- or Fluva-induced CYP2B1/2 and CYP3A mRNA levels in primary cultures of rat hepatocytes. Beginning 24 hr after plating, rat hepatocyte cultures (6 dishes/treatment group) were incubated in medium containing 0.1% DMSO vehicle, or containing Dex, triamcinolone acetonide, or triamcinolone, each at concentrations ranging from 10^{-9} to 10^{-5} M. Beginning 48 hr after plating, 3 dishes from each treatment group were treated with 10^{-4} M of PB and 3 dishes were treated with 3×10^{-5} M of Fluva. Drug concentrations are indicated as the logs of the molar concentrations added to the medium. At 72 hr, the 3 dishes of hepatocytes comprising each treatment group were pooled for preparation of total RNA, and levels of CYP2B1/2 and CYP3A mRNA were analyzed by northern blot hybridization. Also shown are autoradiographs of blots following rehybridization with a cDNA to 7S RNA.

Dex concentrations resulted in a progressive reversal back to the CYP2B1/2 mRNA levels seen in the absence of glucocorticoid (Fig. 2). In general, similar concentration-dependent effects on PB-induced CYP2B1/2 mRNA levels were seen when hepatocytes were incubated with triamcinolone acetonide, or with higher concentrations of the less potent glucocorticoid triamcinolone. By contrast, Dex concentrations up to 10^{-8} M produced little or no potentiation of Fluva-induced CYP2B1/2 mRNA levels, while Dex concentrations of 10^{-7} M or higher markedly suppressed the Fluva-mediated CYP2B1/2 mRNA induction. Again, in general, similar concentration-dependent effects on Fluva-induced CYP2B1/2 mRNA expression were seen when hepatocytes were incubated with the potent glucocorticoid triamcinolone acetonide, with the notable difference that suppression of Fluva-mediated CYP2B1/2 mRNA induction did not occur until hepatocytes were incubated with 10^{-6} M of triamcinolone acetonide. Suppression of Fluva-induced CYP2B1/2 mRNA expression occurred at the same concentrations of glucocorticoid at which CYP3A mRNA levels became readily detectable, namely 10^{-7} M for Dex, 10^{-6} M for triamcinolone acetonide, and 10^{-5} M for triamcinolone (Fig. 2). These findings suggested that CYP3A induction following glucocorticoid treatments may be responsible for suppressing Fluva-mediated CYP2B1/2 mRNA induction.

To test the possible relationship between CYP3A induction and suppression of Fluva-induced CYP2B1/2 expression, we examined the effects of a nonglucocorticoid steroidal inducer of CYP3A, namely PCN, on this process. In these experiments, cultured hepatocytes were treated with Dex or PCN for 24 hr, simultaneously with 3×10^{-5} M of Fluva. Consistent with the results obtained for the three glucocorticoids, PCN treatment produced a concentration-dependent suppression of Fluva-induced CYP2B1/2 expression that mirrored the ability of this agent to induce CYP3A mRNA (Fig. 3). By contrast, neither Dex nor PCN treatment had a suppressive effect on the Fluva-induced level of HMG-CoA reductase mRNA.

Evidence that induced CYP3A enzymatic activity was integral to the suppression of CYP2B1/2 mRNA levels was provided by examining the effects of cotreatments with TAO, a selective inhibitor of CYP3A, on Dex-mediated suppression of Fluva-induced CYP2B1/2 mRNA expression (Fig. 4). Treatment of hepatocyte cultures with TAO alone at 3×10^{-5} M, a concentration that was determined in preliminary experiments to be nontoxic to the hepatocytes, had no effect on basal CYP2B1/2 mRNA levels. Treatment with DMSO vehicle alone had a modest suppressive effect on the amount of Fluva-induced CYP2B1/2 mRNA, consistent with data demonstrating that this solvent produces an elevation of CYP3A protein, but not mRNA, levels [14]. This suppression was concentration-dependently reversed when hepatocytes were treated simultaneously with 10^{-5} to 3×10^{-5} M of TAO. As demonstrated in Fig. 2, treatment of hepatocyte cultures with 10^{-7} to 10^{-6} M of Dex produced a concentration-dependent suppression of Fluva-induced CYP2B1/2 mRNA levels. The partial suppression of Fluva-induced CYP2B1/2 mRNA expression that was produced by 10^{-7} M of Dex treatment was reversed almost completely when the cultures were cotreated with 3×10^{-5} M of TAO, while the almost complete suppression of Fluva-induced CYP2B1/2 mRNA levels that was produced by 10^{-6} M of Dex was partially reversed when cultures were cotreated with TAO.

The simplest explanation for these findings would be that induction of CYP3A increased the metabolism of Fluva to species that were inactive as CYP2B1/2 inducers.

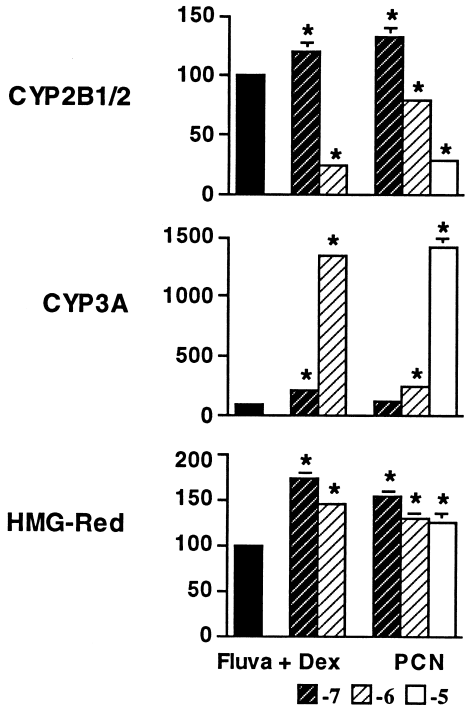

FIG. 3. Effects of CYP3A inducer treatments on Fluva-induced CYP and HMG-CoA reductase mRNA levels in primary cultures of rat hepatocytes. Forty-eight-hour-old rat hepatocyte cultures (3 dishes/treatment group) were treated for 24 hr with medium containing 3×10^{-5} M of Fluva plus 0.1% DMSO, Dex (10^{-7} or 10^{-6} M), or PCN (10^{-7} to 10^{-5} M). Drug concentrations are indicated as the logs of the molar concentrations added to the medium. After treatment, hepatocytes were harvested for preparation of total RNA (individual dishes were processed separately), and levels of CYP2B1/2, CYP3A, and HMG-CoA reductase mRNA were analyzed by slot blot hybridization. Following autoradiography, radioactive probes were washed from the blots, and the blots were rehybridized with 7S probe. Autoradiographic bands were quantified by scanning laser densitometry, and intensities of the CYP or HMG-CoA reductase bands relative to the intensities of the corresponding 7S bands were calculated. Normalized data are shown plotted as percentages of the values obtained for the Fluva plus DMSO treatments. All values are presented as means ± SD ($N = 3$). Error bars that are not visible are contained within the boundaries of the bars. *Significantly different from the Fluva plus DMSO group, $P < 0.05$.

To examine the effect of Dex treatment on the amount of Fluva that was present in the cultured hepatocytes, cultures were incubated with Fluva for 24 hr in the presence or absence of Dex, and the amounts of Fluva in the culture medium and in cells were measured by HPLC. The amounts of Fluva detected in the culture medium and in cells were reduced significantly when hepatocytes were incubated with Dex (Table 1). The reductions in the amount of Fluva were dependent on both the concentration and duration of Dex treatment, and the percentage reductions were almost identical whether Fluva levels were measured in medium or cells. Thus, the amount of Fluva detected in medium or in cells was ~60% of the control (i.e. no Dex) level when hepatocytes were incubated for 24 hr with 10^{-7} M of Dex, and was ~10% of the control level when hepatocytes were incubated for 48 hr with 10^{-7} M of Dex or for 24 hr with 10^{-6} M of Dex. These findings suggested that Dex-induced Fluva metabolism was the major mechanism responsible for the Dex-mediated suppression of Fluva-induced CYP expression. However, if this were the only mechanism at work, it should have been possible to overcome the Dex-mediated suppression simply by incubating the hepatocytes with higher concentrations of Fluva. We found that the suppression of Fluva-mediated CYP2B1/2 mRNA induction that occurred in the presence of 10^{-6} M of Dex was not overcome when hepatocytes were incubated with Fluva concentrations greater than 3×10^{-5} M (Fig. 5). As noted above, the presence of Dex did not appear to affect the ability of Fluva to induce HMG-CoA reductase mRNA levels.

CYP3A catalyzes the hydroxylation of certain steroids, including glucocorticoids, at the 6β position [15, 16], and it has been suggested that 6β-hydroxylated glucocorticoids have biological activities that are, at least in some ways, distinct from those of the parent molecules [17]. To consider the possibility that CYP3A induction following glucocorticoid treatment could result in conversion of a glucocorticoid to a 6β-hydroxylated metabolite, which then exerted a suppressive effect on CYP2B expression, we compared the effects on Fluva-induced CYP2B1/2 mRNA expression of treatment with prednisolone (Dex is 9α-fluoro-16α-methylprednisolone) or 6β-hydroxyprednisolone, both of which are available commercially. Incubation of Fluva-treated hepatocyte cultures with increasing concentrations of prednisolone had no effect on CYP3A mRNA levels at concentrations lower than 10^{-5} M, but significantly increased CYP3A mRNA levels at a concentration of 10^{-5} M, which was also the only concentration of prednisolone that produced suppression of CYP2B1/2 mRNA levels (Fig. 6). By contrast, incubation of hepatocytes with 6β-hydroxyprednisolone had no effect on CYP2B1/2 or CYP3A mRNA levels at any concentration, suggesting that 6β-hydroxylation of glucocorticoid by CYP3A was not likely to contribute to the suppression of CYP2B expression occurring in glucocorticoid-treated hepatocyte cultures.

DISCUSSION

Treatment of primary cultured rat hepatocytes with a variety of agents previously reported to modify PB-inducible CYP2B expression generally produced the expected results. Thus, incubation of cultured rat hepatocytes with fetal bovine serum, growth hormone, or cycloheximide suppressed PB-mediated CYP2B induction, as previously reported [4, 5]. Also, maintenance of hepatocytes on a Vitrogen substratum in glucocorticoid-deficient medium did not support PB-inducible CYP2B1/2 mRNA induction, in agreement with previous findings [3]. We have found that when hepatocytes maintained on Vitrogen are incubated in glucocorticoid-containing medium, PB treatment produces readily detectable increases in CYP2B1/2 mRNA

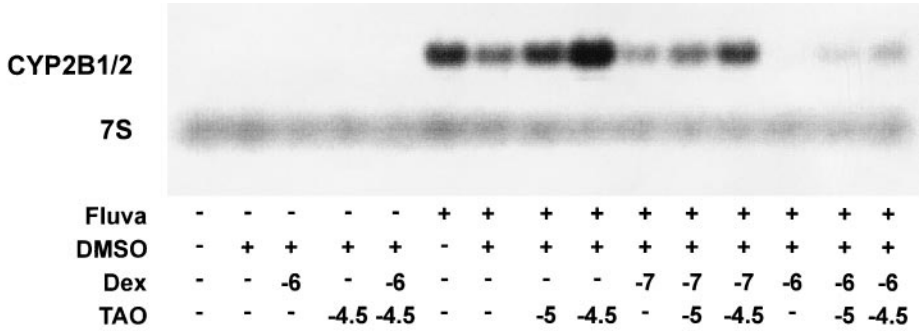

FIG. 4. Effects of Dex and TAO treatments on Fluva-induced CYP2B1/2 mRNA levels in primary cultures of rat hepatocytes. Forty-eight-hour-old hepatocyte cultures (2 dishes/treatment group) were incubated for 24 hr in medium containing Fluva (3×10^{-5} M), Dex (10^{-7} or 10^{-6} M), or TAO (10^{-5} M or 3×10^{-5} M) alone, or with combinations of these drugs, as indicated under the northern blot autoradiograph. The DMSO concentration in the culture medium was balanced to 0.2% in each treatment group for which DMSO addition is indicated. Drug concentrations are indicated as the logs of the molar concentrations added to the medium. After treatment, the 2 dishes of hepatocytes comprising each treatment group were pooled for preparation of total RNA, and levels of CYP2B1/2 mRNA were analyzed by northern blot hybridization. Also shown is the autoradiograph obtained after the blot was rehybridized with a 7S probe.

levels (unpublished observations), consistent with several reports [2, 7, 18]. An apparent exception to our ability to reproduce previous findings was that treatment with glucagon or dibutyryl cyclic AMP had little effect on PB-induced CYP2B mRNA expression under our experimental conditions. Although our culture conditions were similar to those used by Sidhu and Omiecinski [6], there were differences in the manner of presentation of Matrigel to the hepatocytes (i.e. as a substratum or as an overlay) and in the omission or inclusion of glucocorticoid in the culture medium. Upon further examination, we found that when a glucocorticoid was included in the culture medium, treatment with dibutyryl cyclic AMP evoked the same striking concentration-dependent suppression of PB-inducible CYP2B expression that was reported previously (unpublished observations) [6], suggesting that a glucocorticoid-mediated event is linked to the cyclic AMP-mediated regulatory process.

Of the different treatments affecting diverse cellular signaling systems, only treatment with Dex produced differential effects on the abilities of PB and Fluva to induce CYP2B1/2 in primary cultured rat hepatocytes. Dex concentrations of 10^{-9} to 10^{-7} M potentiated the CYP2B1/2 mRNA induction produced by PB treatment, consistent with several previous reports [2, 7–9]. Although the mechanistic basis for this effect is not known, a functional glucocorticoid response element has been identified in the 5′-flanking region of the *CYP2B2* gene, and may play a role [19]. By contrast, Dex concentrations of 10^{-9} to 10^{-8} M had little or no effect on Fluva-induced CYP2B1/2 mRNA levels, while concentrations of 10^{-7} M and greater suppressed Fluva-mediated CYP2B1/2 induction. Dex is often included as a standard supplement in hepatocyte culture medium, usually at concentrations ranging from 10^{-8} to 10^{-7} M. These concentrations are sufficient to activate classical glucocorticoid receptor-mediated effects maximally, but are at the low end of the concentration–response relationship for induction of CYP3A [20]. Nevertheless, our data suggest that a large component of the suppression of Fluva-induced CYP2B1/2 expression that occurred in the presence of Dex concentrations as low as 10^{-7} M was attributable to CYP3A induction and metabolic inactivation of Fluva. We also noted that the CYP2B1/2 mRNA

TABLE 1. Effects of Dex treatments on the amount of fluvastatin in primary cultures of rat hepatocytes

	Amount of fluvastatin† (nmol/dish)	
Treatment*	**Medium**	**Cells**
Untreated	14.4 ± 1.2 (100)	6.06 ± 0.40 (100)
Dex 10^{-7} M 48–72 hr	9.04 ± 0.85‡ (62.8)	3.52 ± 0.28‡ (58.1)
Dex 10^{-7} M 24–72 hr	1.88 ± 0.35‡ (13.1)	0.74 ± 0.06‡ (12.2)
Dex 10^{-6} M 48–72 hr	1.56 ± 0.35‡ (10.8)	0.55 ± 0.08‡ (9.0)

*Primary cultured rat hepatocytes were treated with dexamethasone (Dex) for either 0 hr (designated untreated), 24 hr (10^{-7} or 10^{-6} M beginning 48 hr after plating, designated 48–72 hr) or 48 hr (10^{-7} M beginning 24 hr after plating, designated 24–72 hr), and were cotreated with 3×10^{-5} M of fluvastatin for 24 hr (beginning 48 hr after plating). At 72 hr, amounts of fluvastatin present in culture medium and cells were analyzed by HPLC.

†Results are presented as means ± SD ($N = 4$ dishes). Percentages of untreated controls are shown in parentheses.

‡Significantly different from untreated control ($P < 0.05$).

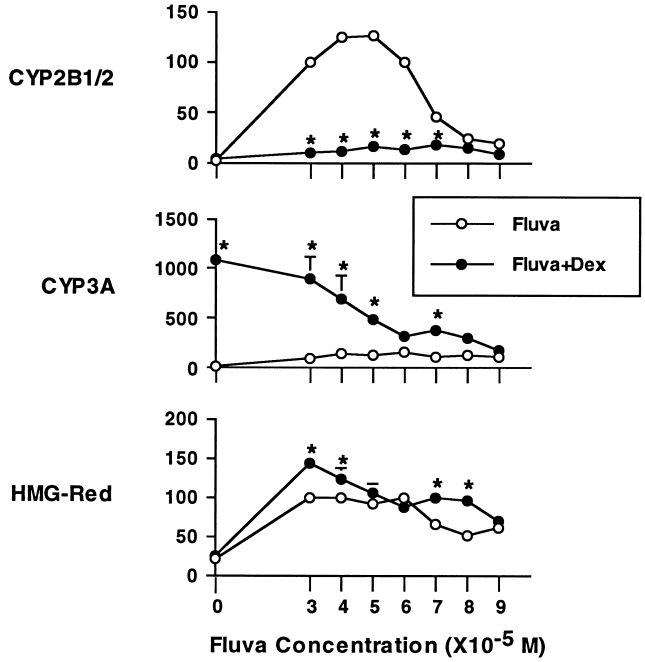

FIG. 5. Effects of Dex treatment on the Fluva concentration-dependence of CYP and HMG-CoA reductase mRNA expression in primary cultures of rat hepatocytes. Forty-eight-hour-old hepatocyte cultures were treated for 24 hr with Fluva at concentrations ranging from 3×10^{-5} to 9×10^{-5} M, either alone or in combination with 10^{-6} M of Dex. After treatment, hepatocytes were harvested for preparation of total RNA (individual dishes were processed separately), and levels of CYP2B1/2, CYP3A, and HMG-CoA reductase mRNA were analyzed by slot blot hybridization, as described in the legend to Fig. 3. Normalized data are shown plotted as percentages of the values obtained for the 3×10^{-5} M of Fluva alone treatment. All values are presented as means ± SD (N = 3). Error bars that are not visible are contained within the boundaries of the data points. *Significantly different from corresponding Fluva alone group, $P < 0.05$.

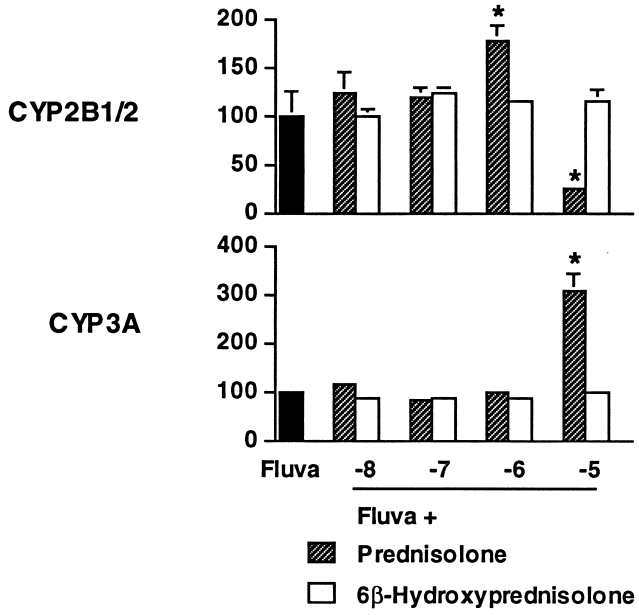

FIG. 6. Effects of prednisolone or 6β-hydroxyprednisolone treatments on the levels of CYP2B1/2 and CYP3A mRNA in Fluva-treated primary cultures of rat hepatocytes. Forty-eight-hour-old hepatocyte cultures were treated for 24 hr with 3×10^{-5} M of Fluva in combination with 0.1% DMSO (vehicle control) or with prednisolone or 6β-hydroxyprednisolone, each at concentrations ranging from 10^{-8} to 10^{-5} M. After treatment, hepatocytes were harvested and analyzed for levels of CYP2B1/2 and CYP3A mRNA by slot blot hybridization, as described in the legend to Fig. 3. Normalized data are shown plotted as percentages of the values obtained for the Fluva + DMSO treatment. All values are presented as means ± SD (N = 3). Error bars that are not visible are contained within the boundaries of the bars. *Significantly different from Fluva, $P < 0.05$.

elevation produced by treatment of hepatocytes with either of the lower-efficacy CYP inducers lovastatin or simvastatin [1] was abolished completely when culture medium was supplemented with 10^{-7} M of Dex (data not shown). By contrast, when 10^{-7} M of triamcinolone acetonide, a potent glucocorticoid that produces little (relative to Dex) CYP3A induction, was substituted for Dex, Fluva was able to produce approximately the same level of CYP2B1/2 mRNA induction that was observed in the absence of glucocorticoid. These findings illustrate the importance of the choice of glucocorticoid, and the concentration that is used, in performing experiments with primary cultured hepatocytes.

Many of the HMG-CoA reductase inhibitors in clinical use, including Fluva, undergo extensive first pass metabolism following oral administration. However, little information is available concerning the identities of the specific forms of CYP that are responsible for metabolizing the HMG-CoA reductase inhibitors. Production of a 6′β-hydroxylated metabolite of lovastatin has been demonstrated to be catalyzed by CYP3A [21]. However, there is no evidence demonstrating that Fluva is metabolized by CYP3A. Transon *et al.* [22] used *in vitro* inhibition studies with human liver microsomes to demonstrate that Fluva has a moderate affinity for CYP2D6 and CYP3A4, but has high affinity for CYP2C9, suggesting that this latter CYP form may be of primary importance for drug interactions in humans. The same authors provided evidence that Fluva administration to humans could inhibit the metabolism of CYP2C9 substrates [23], although they did not demonstrate that CYP2C9 actually metabolized Fluva.

If CYP3A-catalyzed metabolic inactivation of Fluva were the only mechanism responsible for the suppressive effects of Dex on Fluva-induced gene expression, then all of the effects produced by Fluva should have been decreased under conditions of CYP3A induction. Furthermore, it should have been possible to overcome the suppressive effects of Dex simply by incubating the hepatocyte cultures with higher concentrations of Fluva. Neither of these predicted effects occurred, suggesting that the actual consequences of Dex supplementation on Fluva-induced gene expression are likely to be more complex than simple metabolic inactivation of the parent drug. Thus, there was not a reduction of HMG-CoA reductase mRNA levels in Dex- or PCN-

treated hepatocyte cultures. There are several reports that Dex stimulates HMG-CoA reductase activity in rat liver [24] and in cultured cells [25, 26]. While this might suggest that CYP3A-inducing agents directly elevate HMG-CoA reductase mRNA levels, thereby counterbalancing any suppressive effects on Fluva-inducible HMG-CoA reductase mRNA expression, we found that incubation of hepatocyte cultures with PCN alone produced little, if any, change in HMG-CoA reductase mRNA levels (unpublished observations), suggesting that CYP3A inducers are not, as a class, effective inducers of HMG-CoA reductase. Alternatively, it is possible that Fluva is metabolized to a species that remains active as an HMG-CoA reductase inhibitor, but is no longer capable of inducing CYP2B. Such a mechanism would imply that the ability of a drug to inhibit HMG-CoA reductase does not predict its ability to induce CYP2B. In this light, we previously reported that pravastatin, another potent HMG-CoA reductase inhibitor, was not an effective inducer of CYP2B in primary cultured rat hepatocytes [1].

Several changes in CYP expression were observed when hepatocytes were treated with Fluva concentrations in excess of the standard 3×10^{-5} M. Thus, in the absence of Dex, Fluva concentrations of $\sim 7 \times 10^{-5}$ M and higher resulted in the almost complete reversal of the CYP2B1/2 mRNA levels that were induced at lower Fluva concentrations. Also, the CYP3A induction produced by treatment with 10^{-6} M of Dex alone was suppressed in the presence of these higher concentrations of Fluva. In view of these substantial changes in gene expression, perhaps it is not surprising that the suppressive effects of Dex on CYP2B mRNA induction were not overcome by the elevated Fluva concentrations. It is also likely that Dex and other glucocorticoids suppress Fluva-mediated CYP2B induction through additional mechanisms that are unrelated to accelerated Fluva metabolism. It has been reported previously [8, 9], and is reproduced in this study, that high concentrations of Dex suppress PB-induced CYP2B expression, although there is no evidence that PB is metabolized by CYP3A.

In summary, we have demonstrated that the ability of Fluva to induce CYP2B1/2 mRNA in primary cultured rat hepatocytes is diminished markedly when Dex is included in the culture medium at concentrations that are often used as standard. This effect appeared to be largely attributable to induction of CYP3A, with a consequent metabolic inactivation of Fluva to species that were no longer capable of inducing CYP2B, although participation of additional mechanisms cannot be dismissed. Metabolism of Fluva by CYP3A may at least partially explain the lower level of CYP2B induction that occurred in the livers of rats treated with Fluva *in vivo* relative to that which occurred in rat hepatocyte cultures that we previously reported [1], since relatively high levels of CYP3A2 are expressed in untreated adult male rat liver [27]. Our results provide further support for the utility of primary cultured rat hepatocytes for studying regulation of CYP gene expression, but emphasize the need for care when selecting culture conditions.

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