

# Induction of CYP3A by 2,3-Oxidosqualene:Lanosterol Cyclase Inhibitors Is Mediated by an Endogenous Squalene Metabolite in Primary Cultured Rat Hepatocytes

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## ABSTRACT

The effects of inhibitors of 2,3-oxidosqualene:lanosterol cyclase (cyclase) on cytochrome P450 expression were investigated in primary cultures of rat hepatocytes. Treatment of hepatocyte cultures for 24 h with either of the inhibitors [4'-(6-allyl-methyl-amino-hexyloxy)-2'-fluoro-phenyl]-(4-bromophenyl)-methanone fumarate (Ro 48-8071) or *trans*-*N*-(4-chlorobenzoyl)-*N*-methyl-(4-dimethylaminomethylphenyl)-cyclohexylamine (BIBX 79) selectively increased CYP3A mRNA and immunoreactive protein contents, with maximal accumulations occurring at  $3 \times 10^{-5}$  M Ro 48-8071 and  $10^{-4}$  M BIBX 79. The abilities of Ro 48-8071, BIBX 79, and  $3\beta$ -(2-diethylaminoethoxy)androst-5-en-17-one-HCl (U18666A) to induce murine CYP3A were abolished in hepatocyte cultures prepared from pregnane X receptor (PXR)-null mice, and cotransfection of primary cultured rat hepatocytes with a dominant-negative PXR prevented cyclase inhibitor-inducible luciferase expression from a PXR-responsive reporter plasmid. Cyclase inhibitor-mediated CYP3A mRNA induction was eliminated when primary cultured rat hepato-

cytes were cotreated with any of the following agents that inhibit steps upstream of cyclase in the cholesterol biosynthetic pathway: squalstatin 1 (squalene synthase inhibitor), (*E*)-*N*-ethyl-*N*-(6,6-dimethyl-2-hepten-4-ynyl)-3-[(3,3'-bithiophen-5-yl)methoxy]benzenemethanamine (NB-598, squalene monooxygenase inhibitor), or pravastatin (HMG-CoA reductase inhibitor). Ro 48-8071-inducible CYP3A mRNA expression was restored when pravastatin-treated cultures were incubated with medium containing mevalonate. The concentration-dependence of Ro 48-8071-mediated CYP3A mRNA induction corresponded to the cellular contents of metabolically labeled squalene 2,3-oxide and squalene 2,3:22,23-dioxide, but not 24(S),25-epoxycholesterol. These results indicate that cyclase inhibitors are capable of inducing CYP3A expression in primary cultured rat and mouse hepatocytes and that the effect is mediated as a consequence of cyclase blockade through the evoked accumulation of one or more squalene metabolites that activate the PXR.

CYP3A enzymes are well recognized to catalyze the phase I metabolism of numerous xenobiotic substrates, including a large number of clinically used drugs (Quattrochi and Guzelian, 2001). In the adult human liver, CYP3A4 is the most abundantly expressed of the xenobiotic-metabolizing P450s, although there is substantial interindividual variability in

its content (Lamba et al., 2002). Several of the CYP3A enzymes (e.g., CYP3A23 in rat, CYP3A4 in human) are highly inducible by agents such as PCN, rifampicin, and dexamethasone, which are now known to function by binding to and activating the PXR (Kliewer et al., 1998; Lehmann et al., 1998), a "xenobiotic-sensing" nuclear receptor with an unusually large ligand-binding pocket (Watkins et al., 2001, 2003; Xie and Evans, 2001).

CYP3A is also known to catalyze the biotransformation of various endogenous molecules. For example, it has been known for many years that CYP3A enzymes stereoselectively

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**ABBREVIATIONS:** P450, cytochrome P450; BIBX 79, *trans*-*N*-(4-chlorobenzoyl)-*N*-methyl-(4-dimethylaminomethylphenyl)-cyclohexylamine; cyclase, 2,3-oxidosqualene:lanosterol cyclase; GST, glutathione S-transferase; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl CoA reductase; LXR, liver X receptor; NB-598, (*E*)-*N*-ethyl-*N*-(6,6-dimethyl-2-hepten-4-ynyl)-3-[(3,3'-bithiophen-5-yl)methoxy]benzenemethanamine; PCN, pregnenolone 16 $\alpha$ -carbonitrile; PPAR $\alpha$ , peroxisome proliferator-activated receptor  $\alpha$ ; PXR, pregnane X receptor; Ro 48-8071, [4'-(6-allyl-methyl-amino-hexyloxy)-2'-fluoro-phenyl]-(4-bromophenyl)-methanone fumarate; U18666A,  $3\beta$ -(2-diethylaminoethoxy)androst-5-en-17-one-HCl; DMSO, dimethyl sulfoxide; oap, organic anion transporting polypeptide.

catalyze the hydroxylation of testosterone, at the 6 $\beta$ , 2 $\beta$ , and 15 $\beta$  positions (Sonderfan et al., 1987; Waxman et al., 1988). More recently, the cholestatic secondary bile acid lithocholate has been shown to be both capable of activating the PXR and a substrate for CYP3A-catalyzed metabolism, indicating a protective mechanism whereby the cell recognizes and responds to the accumulation of potentially toxic endogenous molecules (Staudinger et al., 2001; Xie et al., 2001). This has been further illustrated by the recent findings that the bile acid precursor sterol 5 $\beta$ -cholestanoic acid-3 $\alpha$ ,7 $\alpha$ ,12 $\alpha$ -triol, which accumulates in the absence of functional CYP27, has been found both to be an activator of the mouse PXR and a substrate for CYP3A-catalyzed metabolism (Furster and Wikvall, 1999; Honda et al., 2001; Dussault et al., 2003; Goodwin et al., 2003). CYP3A metabolism initiates an alternative pathway of sterol side-chain shortening, permitting the formation of cholic acid (Honda et al., 2001; Goodwin et al., 2003). In contrast, bile acid precursor sterols do not activate the human PXR, thereby explaining why humans with the genetic disease cerebrotendinous xanthomatosis, attributable to CYP27 deficiency, produce reduced levels of normal bile acids, accumulate sterols in various tissues, and exhibit a host of severe pathologies, whereas mice that have been genetically engineered to lack CYP27 do not (Dussault et al., 2003; Goodwin et al., 2003).

We have been investigating the effects of drugs that interfere with cholesterol biosynthesis on the expression of xenobiotic-metabolizing P450s (Kocarek and Mercer-Haines, 2002; Kocarek et al., 2002a). For example, we recently reported that treatment of primary cultured rat hepatocytes with squalstatin 1, an inhibitor of squalene synthase, the first committed enzyme in sterol biosynthesis, selectively induces CYP2B expression, and that this effect requires the ongoing synthesis of an endogenous isoprenoid and constitutive androstane receptor activation (Kocarek and Mercer-Haines, 2002). We have also found that treatment of primary cultures of rat or mouse hepatocytes with the oxysterol 24(S),25-epoxycholesterol, an endogenously synthesized oxysterol and potent activator of the LXR, is also capable of inducing CYP3A expression through activation of the PXR (Shenoy et al., 2004).

24(S),25-Epoxycholesterol is biosynthesized through a pathway that begins at the level of squalene 2,3-oxide, the substrate for cyclase (Fig. 1). Squalene 2,3-oxide can be converted to squalene 2,3:22,23-dioxide (Nelson et al., 1981), which then undergoes the same metabolic conversions as does squalene 2,3-oxide, finally yielding the relatively stable epoxide sterol metabolite. As might be expected, when cyclase is inhibited, there is greater shunting of squalene 2,3-oxide to squalene 2,3:22,23-dioxide. However, because the conversion of squalene 2,3-oxide to lanosterol is more sensitive to chemical inhibition than is the conversion of squalene 2,3:22,23-dioxide to 24(S),25-oxidolanosterol, under conditions of partial cyclase blockade, cholesterol biosynthesis can be abolished, but 24(S),25-epoxycholesterol can accumulate (Mark et al., 1996; Morand et al., 1997).

In this study, we first tested whether cyclase inhibitors are capable of inducing CYP3A expression in primary cultured rat hepatocytes. Finding this to be the case, we examined whether this effect is mediated through the PXR and whether it occurs indirectly, through the evoked accumulation of a squalene metabolite.

## Materials and Methods

**Materials.** Ro 48-8071, BIBX 79, and U18666A were gifts from F. Hoffmann-La Roche (Basel, Switzerland), Boehringer Ingelheim USA (Ridgefield, CT), and Pfizer Inc. (Ann Arbor, MI), respectively. NB-598 was a gift from Banyu Pharmaceutical Co., Ltd. (Tokyo, Japan). Squalstatin 1 and pravastatin were gifts from GlaxoSmithKline (Research Triangle Park, NC) and Bristol-Myers Squibb Co. (Stamford, CT), respectively. 24(S),25-Epoxycholesterol, 24(S),25-oxidolanosterol, and racemic squalene 2,3:22,23-dioxide were prepared as described previously (Chang et al., 1979; Taylor et al., 1986; Spencer et al., 2000). Cholesterol, lanosterol, phenobarbital, PCN, and mevalonate were purchased from Sigma Chemical (St. Louis, MO). Squalene 2,3-oxide was purchased from American Radiolabeled Chemicals (St. Louis, MO). Matrigel and Matrisperse were purchased from BD Biosciences Discovery Labware (Bedford, MA). Vitrogen was purchased from Cohesion Technologies (Palo Alto, CA). Recombinant human insulin (Novolin R) was purchased from Novo Nordisk Pharmaceuticals, Inc. (Princeton, NJ). Culture medium and Lipofectin reagent were purchased from Invitrogen (Carlsbad, CA). Nylon hybridization filters (Gene Screen Plus) were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). Nitrocellulose membranes were purchased from Bio-Rad (Hercules, CA). Enhanced chemiluminescence Western blot reagents and [2-<sup>14</sup>C]mevalonate (55 mCi/mmol) were purchased from Amersham Biosciences Inc. (Piscataway, NJ). Solvents for lipid extraction and chromatography (Burdick and Jackson) and thin-layer chromatography plates (Baker silica gel, 250  $\mu$ m) were purchased from VWR (Chicago, IL). High-performance liquid chromatographic supplies were purchased from Waters Corporation (Milford, MA). Polyclonal antibodies to CYP1A1 and CYP2B1 were purchased from Xenotech LLC (Lenexa, KS), and a CYP4A1 antibody was purchased from BD Gentest (Woburn, MA). A polyclonal antibody to CYP3A1 was a gift from Dr. Janis Hulla (United States Army Corps of Engineers, Sacramento, CA).

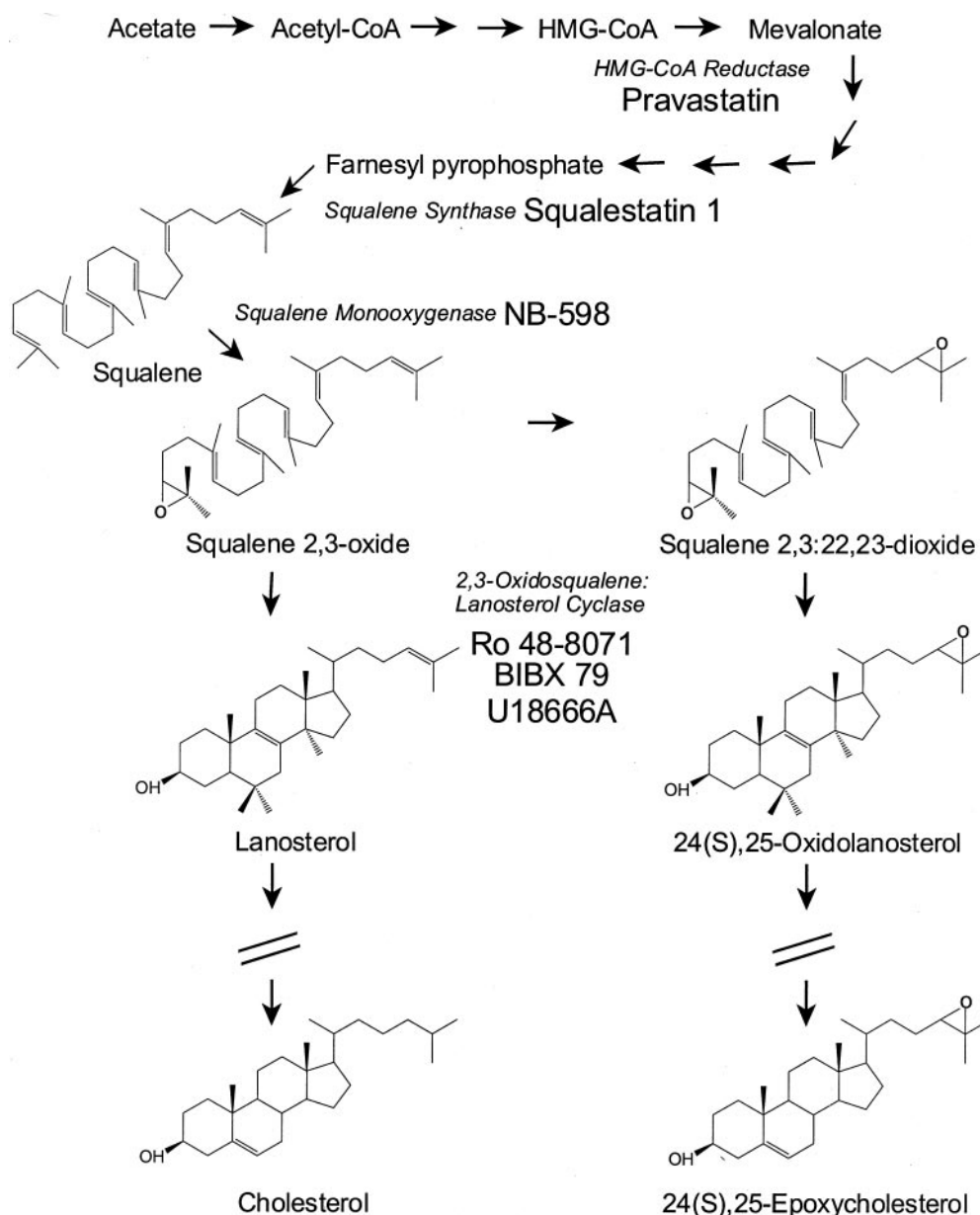
**Primary Culture of Rat and Mouse Hepatocytes.** Hepatocytes were isolated from adult male Sprague-Dawley rats (250–350 g; Harlan, Indianapolis, IN) or from adult male PXR-null mice or their wild-type counterparts (20–30 g; Taconic Farms, Germantown, NY) (Staudinger et al., 2001; Wu et al., 2001). After isolation, three million viable hepatocytes were plated onto 60-mm Matrigel-coated dishes (for Northern blot and metabolic-labeling experiments), or 300,000 viable hepatocytes were plated onto Vitrogen-coated wells in 12-well plates (for transient transfection experiments) and maintained in Williams' E medium supplemented with 0.25 U/ml insulin, 10<sup>−7</sup> M triamcinolone acetonide, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin. Culture medium was renewed every 24 h. Drugs were added to the culture medium as concentrated stock solutions in water (mevalonate, phenobarbital, pravastatin, Ro 48-8071, and squalstatin 1), DMSO (BIBX 79, ciprofibrate, dexamethasone,  $\beta$ -naphthoflavone, NB-598, PCN, and U18666A), or ethanol [24(S),25-epoxycholesterol]. When used, the final concentration of organic solvent in the culture medium was 0.1%.

**Northern Blot Analysis.** Beginning 48 h after plating, hepatocyte cultures were treated with drugs or sterols (three dishes per treatment group for rat hepatocytes, 1–2 dishes per treatment group for mouse hepatocytes), as described in the individual figure legends. After treatment, the dishes representing each treatment group were pooled for preparation of total RNA using the Totally RNA Kit (Ambion, Inc., Austin, TX). Samples (10  $\mu$ g) of the pooled RNAs were resolved on denaturing agarose gels and analyzed by Northern blot hybridization, as described previously (Kocarek and Reddy, 1996). The cDNA probes to CYP1A1, CYP2B1, CYP3A23, and CYP4A1 have been described previously (Kocarek and Reddy, 1996). Plasmid pGTB32, containing a ~500 nucleotide cDNA insert to GST A2 (Pickett et al., 1984), was a gift from Dr. Thomas Rushmore (Merck Research Laboratories, West Point, PA). A cDNA insert corresponding to a region of the rat oap2 3'-untranslated region, exhibiting low

sequence similarity to other rat organic anion transporting polypeptides (i.e., oatp1, GI 8394293; oatp3, GI 13540641; and oatp4, GI 13928899), was prepared by reverse transcriptase-polymerase chain reaction amplification with the use of reverse-transcribed total RNA from PCN-treated primary cultured rat hepatocytes as template, TaqPlus Precision Polymerase (Stratagene, La Jolla, CA), and primers corresponding to nucleotides 2320 to 2337 and 2745 to 2726 of the published oatp2 sequence (GI 18777754). The sequence of the forward primer was 5'-GCCTTCACCCTTAACCTT-3', and the sequence of the reverse primer was 5'-CCCAATCATCTTTGCTACAT-3'. After amplification for 30 cycles, the 426-base pair fragment was A-tailed and ligated into the pGEM-T Easy plasmid, according to the manufacturer's instructions (Promega, Madison, WI). The identity of the amplified sequence with rat oatp2 was verified by sequence analysis, using services provided by the Center for Molecular Medicine and Genetics DNA Sequencing Facility (Wayne State University, Detroit, MI). The insert was excised with EcoRI for Northern blot analysis. After autoradiography, radiolabeled probes were removed from the filters, and blots were re-hybridized with 7S cDNA to control for RNA loading and transfer.

**Western Blot Analysis.** Microsomes were isolated from five pooled dishes of primary cultured rat hepatocytes per treatment group, and Western blot analysis was performed as described previously (Kocarek et al., 1998).

**Transient Transfection of Primary Cultured Rat Hepatocytes.** Primary cultures of rat hepatocytes were transiently transfected with reporter and expression constructs as described previously (Kocarek and Mercer-Haines, 2002). The luciferase reporter plasmid contained three concatamerized copies of the CYP3A23-DR3 motif ligated upstream of a minimal herpes simplex virus thymidine kinase promoter (Kocarek and Mercer-Haines, 2002). Plasmids expressing wild-type or dominant-negative PXR or PPAR $\alpha$  have been recently described (Kocarek et al., 2002b). The dominant-negative PXR and PPAR $\alpha$  cDNAs lack the 3'-terminal nucleotides encoding the activator function-2 subdomains, resulting in the expression of receptors presumed to be capable of undergoing normal heterodimerization and DNA binding but incapable of producing transcriptional activation. Transient transfection data were analyzed by one-way analysis of variance followed by the Newman-Keuls multiple comparison test (GraphPad Software Inc., San Diego, CA).



**Fig. 1.** Cholesterol biosynthetic pathway. The chemical inhibitors that were used in this study are indicated.



**Thin-Layer and High-Performance Liquid Chromatographic Detection of Metabolically Labeled Lipids.** Forty-eight-hour-old cultures of hepatocytes were treated as described in the legend to Fig. 7. One hour later, [ $^{14}\text{C}$ ]mevalonate was added to each dish (2  $\mu\text{Ci}$ ), and incubations were quenched after 4 h at 4°C by the addition of 3 ml Matrisperse containing 1 mM butylated hydroxytoluene. The cells were dispersed and collected by low-speed centrifugation at 4°C, and cell pellets were washed three times with 5 ml of cold phosphate-buffered saline. For thin-layer chromatographic analysis, hepatocyte pellets from individual dishes were incubated with 2 ml of 40% aqueous potassium hydroxide solution and 2 ml of ethanol for 45 min at 80°C and then cooled to room temperature. After exhaustive extraction of the samples with hexanes, the extracts were combined and evaporated to dryness under nitrogen. Residues were resuspended in 100  $\mu\text{l}$  hexanes-chloroform (70:30 v/v) and spotted onto a silica gel thin-layer chromatography plate, which was developed using hexane-diethyl ether (50:50 v/v). Authentic samples (30–100  $\mu\text{g}$ ) of cholesterol, lanosterol, 24(S),25-epoxycholesterol, 24(S),25-oxidolanosterol, squalene 2,3-oxide, and squalene 2,3:22,23-dioxide were spotted onto adjacent lanes, and migration positions of these standards were visualized by exposing the developed and dried plate to iodine vapor. The plate was then exposed to X-OMAT AR film (Eastman Kodak, Rochester, NY) for 4 days at room temperature. Levels of radioactivity corresponding to squalene 2,3-oxide and squalene 2,3:22,23-dioxide were determined by liquid scintillation counting of the scraped bands. For specific analysis of [ $^{14}\text{C}$ ]24(S),25-epoxycholesterol content, hepatocyte pellets prepared from individual radiolabeled dishes were hypotonically lysed with 1 ml of water at room temperature for 5 min, and 100  $\mu\text{g}$  of naphthalene was added to each lysate as an internal standard. A fraction containing 24(S),25-epoxycholesterol and internal standard and was then prepared using a C18 Sep-Pack Light cartridge (Waters). After loading of a cell lysate onto a methanol-equilibrated Sep-Pak, the sorbent was washed with 1 ml of methanol-water (50:50 v/v), and elution was achieved using 7 ml of acetonitrile. The acetonitrile was evaporated under nitrogen at room temperature, and the residue was dissolved in 100  $\mu\text{l}$  of mobile phase for analysis by high-performance liquid chromatography. Separations were achieved under isocratic conditions at room temperature using a 4.6  $\times$  50-mm C18 Symmetry column (Waters) and a mobile phase consisting of acetonitrile-water (52:48 v/v). Mobile phase flow rate was 0.25 ml/min for 1 min and 1.6 ml/min for 11 min, and ultraviolet absorption of the eluate was monitored at 210 nm. Under these conditions, naphthalene and authentic 24(S),25-epoxycholesterol eluted with retention times of 4.0 and 8.2 min, respectively. For quantification of radioactivity, eluant samples were collected at 0.5-min intervals and counted by liquid scintillation spectrometry; cpm versus elution time was plotted, and the cpm contained in the peak corresponding to 24(S),25-epoxycholesterol, as measured by ultraviolet absorption, was estimated as  $0.5 \times \text{base} \times \text{height}$ .

## Results

In initial experiments, primary cultured rat hepatocytes were treated with either of two cyclase inhibitors, BIBX 79 or Ro 48-8071, and amounts of CYP1A1, CYP2B,<sup>1</sup> CYP3A, and CYP4A mRNAs, and immunoreactive proteins were determined (Fig. 2). For comparison, other dishes of cultured hepatocytes were treated with the squalene monooxygenase inhibitor NB-598. Northern blot analyses revealed that treatment with either Ro 48-8071 or BIBX 79 produced a concentration-dependent increase in CYP3A mRNA levels, with

maximal effects observed at  $3 \times 10^{-5}$  M Ro 48-8071 and  $10^{-4}$  M BIBX 79 (Fig. 2A). Higher concentrations of Ro 48-8071 were overtly toxic to the hepatocyte cultures. Maximal increases were approximately 50% of that produced by treatment of the cultured hepatocytes with  $10^{-5}$  M dexamethasone. The only other effect on P450 expression that was observed after cyclase inhibitor treatment was a slight (relative to the increase produced by  $10^{-4}$  M ciprofibrate) concentration-dependent increase in CYP4A mRNA content by Ro 48-8071 treatment. Only the highest concentration of BIBX 79 produced any up-regulation of HMG-CoA reductase mRNA expression. By comparison, NB-598 treatment most markedly increased CYP2B mRNA levels, produced modest elevations in CYP3A mRNA content and strongly up-regulated HMG-CoA reductase expression. Western blot analyses indicated that the drug treatment effects on P450 mRNA expression were recapitulated at the protein level (Fig. 2B). These results therefore indicated that the cyclase inhibitors BIBX 79 and Ro 48-8071 were selective and effective inducers of CYP3A expression in primary cultured rat hepatocytes.

Next, to determine whether the observed effects on CYP3A expression were mediated through the PXR, we wished to use the PXR-null mouse model. However, as a preliminary step, we considered it necessary to verify that the effects of the cyclase inhibitors on CYP3A expression which were observed in primary cultured rat hepatocytes were conserved in mouse hepatocyte cultures. We therefore examined CYP3A mRNA inducibility in primary cultured mouse hepatocytes prepared from the C57BL/6, DBA/2, BALB/c, and C3H strains and included cultures prepared from male and female mice in the analysis (data not shown). Cultures were treated either with  $3 \times 10^{-5}$  M Ro 48-8071 or, for comparison, with  $3 \times 10^{-5}$  M of the oxysterol 24(S),25-epoxycholesterol. Results were comparable for all of the mouse strains and both sexes. In these studies, we noted that CYP3A mRNA was always clearly detectable in untreated control cultures (in comparison with rat hepatocytes, in which CYP3A mRNA expression is extinguished by 3 days of culture) and that treatment with 0.1% ethanol vehicle always produced a slight suppression in the amount. Treatment with either Ro 48-8071 or 24(S),25-epoxycholesterol consistently induced CYP3A mRNA content in the mouse hepatocyte cultures, and the response magnitudes produced by these two treatments were nearly identical (data not shown).

Having established that Ro 48-8071-inducible CYP3A expression is conserved from rat to mouse, we next tested the abilities of Ro 48-8071, BIBX 79, and U18666A, an additional cyclase inhibitor (Sexton et al., 1983), to induce CYP3A mRNA in primary hepatocytes prepared from PXR-null mice relative to their wild-type counterparts. Whereas treatment of wild-type mouse hepatocyte cultures with any of the three cyclase inhibitors markedly induced CYP3A mRNA levels, no such effects occurred in the cultures prepared from the PXR-null mice (Fig. 3A). As a control to demonstrate the functionality of the PXR-null cultures, phenobarbital treatment increased CYP2B mRNA expression as effectively in hepatocyte cultures from the PXR-null as in the corresponding wild-type cultures. To obtain evidence that the cyclase inhibitor treatments induced CYP3A in rat through the PXR, the effects of cotransfecting primary cultured rat hepatocytes with a plasmid expressing a dominant-negative PXR were evaluated on luciferase expression from a PXR-responsive

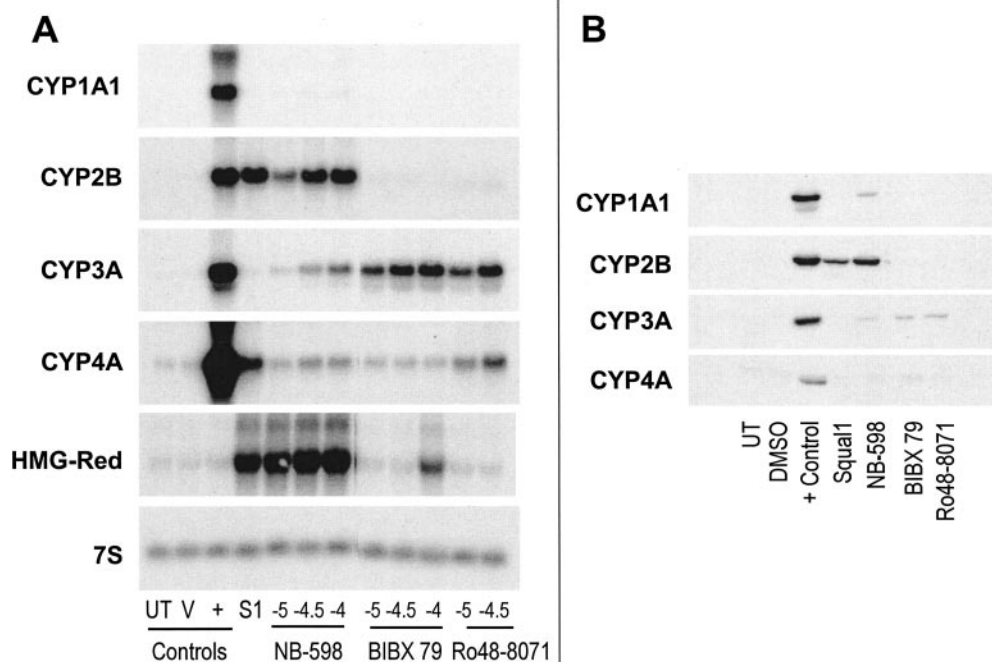
<sup>1</sup> Because the P450 cDNA and antibody probes detect multiple related mRNA or protein species, the specific bands detected on the Northern or Western blot with the CYP2B1, CYP3A1/23, and CYP4A1 probes are referred to as CYP2B, CYP3A, and CYP4A, respectively.

reporter plasmid containing three copies of the CYP3A23 DR3 motif (Fig. 3B). Whereas cotransfection with wild-type PXR had no effect on Ro 48-8071-inducible luciferase reporter-gene expression, transfection with the dominant-negative PXR completely abolished induction. As a control, cotransfection with a plasmid expressing wild-type or dominant-negative PPAR $\alpha$  had no effect on Ro 48-8071-mediated luciferase induction. In addition, treatment of primary cultured rat hepatocytes with Ro 48-8071 increased the levels of mRNAs encoding two other PXR targets, GST A2 (the rat ortholog of mouse GST $\alpha$ 1) and oatp2 (Falkner et al., 2001; Maglich et al., 2002), over the same concentration range that effectively increased CYP3A mRNA levels (Fig. 3C). These results clearly indicate that cyclase inhibitor-inducible expression of CYP3A is mediated through the PXR in rat and mouse hepatocytes.

We demonstrated previously that treatment of primary cultured rat hepatocytes with squalenstatin 1, a squalene synthase inhibitor (Fig. 1), induces CYP2B expression indirectly through a mechanism that requires the ongoing production of an endogenous isoprenoid (Kocarek and Mercer-Haines, 2002). We have reported recently that certain ligands of the LXR, including the endogenously synthesized oxysterol 24(S),25-epoxycholesterol, are capable of activating the PXR and inducing CYP3A expression in primary cultured rat and mouse hepatocytes (Shenoy et al., 2004). In addition, the treatment of hepatic cells with a cyclase inhibitor has been shown to cause the accumulation of 24(S),25-epoxycho-

lesterol (Mark et al., 1996; Morand et al., 1997). We therefore considered the possibility that the cyclase inhibitor treatment effects on CYP3A expression might also be mediated indirectly by causing the accumulation of an endogenous metabolite, perhaps 24(S),25-epoxycholesterol. In our earlier study, one piece of evidence that led us to determine that squalene synthase inhibitors worked indirectly is that treatments with these agents induced CYP2B mRNA expression with a distinctly slower time course than was observed after treatment with a direct-acting agent. Therefore, in an initial test of our hypothesis, we compared the time courses for CYP3A induction after treatment with either PCN, as a prototypical PXR ligand inducer; 24(S),25-epoxycholesterol, as a possible endogenous mediator; or Ro 48-8071 (Fig. 4). PCN treatment produced a clearly detectable increase in the CYP3A mRNA level after only 3 h of treatment, and levels continued to increase through 24 h of treatment. Similarly, increases in CYP3A mRNA levels were observed shortly after beginning treatment with 24(S),25-epoxycholesterol; an increase was possibly evident by 3 h and was clearly detectable by 6 h. In contrast, treatment with Ro 48-8071 did not produce a clear increase in the amount of CYP3A mRNA until 12 h of treatment.

Next, because we determined previously that squalenstatin 1 treatment does not itself induce CYP3A mRNA levels in primary cultured rat hepatocytes (Kocarek et al., 1998), we examined the effect of shutting down flux through the cholesterol biosynthetic pathway, by pre- and cotreating cul-

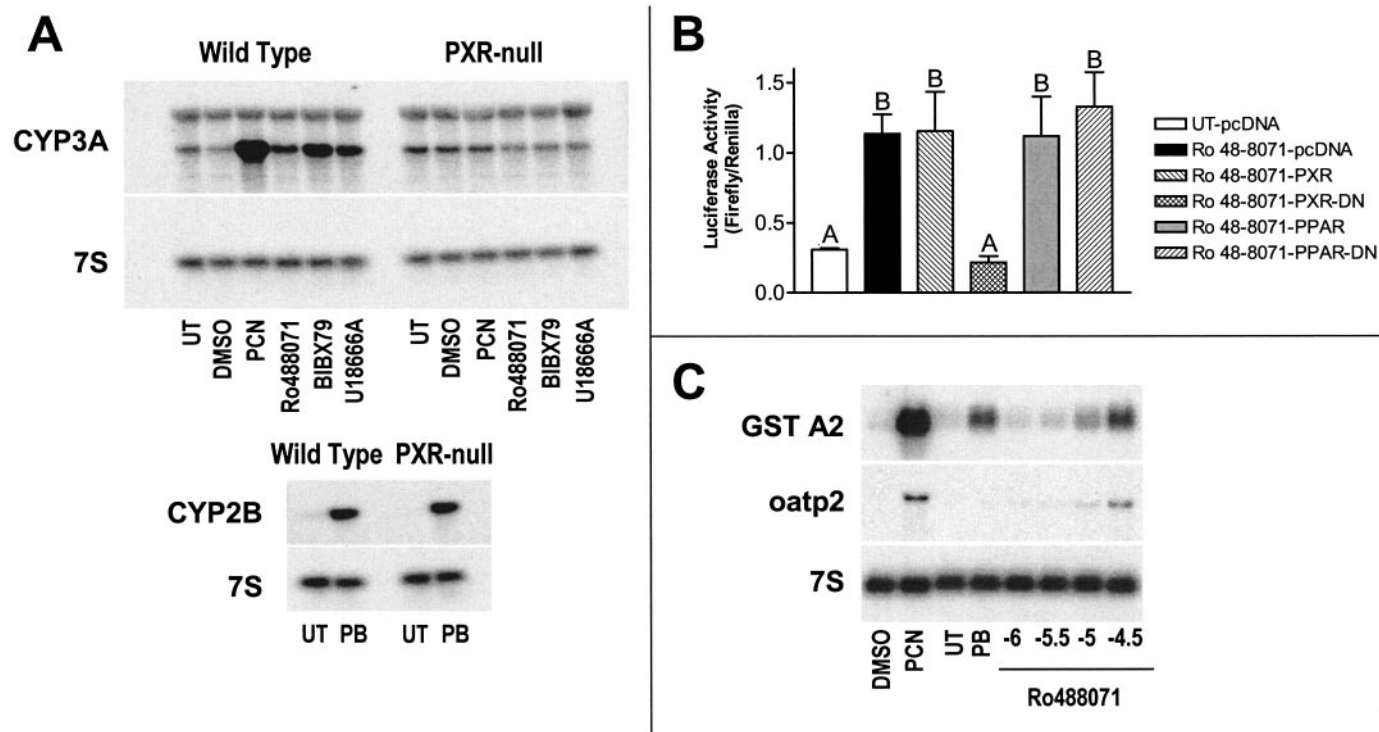


**Fig. 2.** Effects of squalene monooxygenase inhibitor and cyclase inhibitor treatments on P450 and HMG-CoA reductase mRNA and protein levels in primary cultures of rat hepatocytes. **A**, 48-h-old primary cultures of rat hepatocytes were incubated with supplemented Williams' E medium alone (UT); medium containing 0.1% DMSO (V, vehicle control); medium containing one of the following positive control (+) P450 inducers:  $10^{-5}$  M  $\beta$ -naphthoflavone (CYP1A1),  $10^{-4}$  M phenobarbital (CYP2B),  $10^{-5}$  M dexamethasone (CYP3A), or  $10^{-4}$  M ciprofibrate (CYP4A); or medium containing either the squalene synthase inhibitor squalenstatin 1 (S1, at  $10^{-7}$  M), the squalene monooxygenase inhibitor NB-598, or one of the cyclase inhibitors, BIBX 79 or Ro 48-8071 (at the indicated concentrations, shown as log molarities). After 24-h treatment, hepatocytes were harvested for the isolation of total RNA and levels of CYP1A1, CYP2B, CYP3A, CYP4A, and HMG-CoA reductase (HMG-Red) mRNAs were analyzed by Northern blot hybridization. A representative autoradiograph of a blot that was rehybridized with 7S cDNA to control for variations in loading and transfer among samples is also shown. **B**, hepatocyte cultures were treated as described except that a single concentration of NB-598 ( $10^{-4}$  M), Ro 48-8071 ( $3 \times 10^{-5}$  M), or BIBX 79 ( $10^{-4}$  M) was used. After treatment, hepatocytes were harvested for the preparation of microsomes, and amounts of CYP1A1, CYP2B, CYP3A, and CYP4A immunoreactive proteins were measured by Western blot hybridization. Data are representative of results observed in two independent hepatocyte culture experiments.

tures with squalstatin 1, on the subsequent abilities of cyclase inhibitors and other agents to induce CYP3A mRNA (Fig. 5A). Whereas the elevations in CYP3A expression evoked by PCN or phenobarbital treatment were unaffected by squalstatin 1, the induction produced by any of the cyclase inhibitors (i.e., Ro 48-8071, BIBX 79, or U18666A) was completely abolished by squalene synthase inhibition. 24(S),25-Epoxycholesterol-mediated CYP3A induction was also not affected by squalstatin 1 treatment. These results indicated that the abilities of the cyclase inhibitors to induce CYP3A were dependent on the ongoing biosynthesis of an endogenous metabolite of the sterol biosynthetic pathway, the identity of which was either squalene or a downstream metabolite. To obtain further data in support of this conclusion, we performed essentially the same experiment but used NB-598, a potent inhibitor of squalene monooxygenase (Horie et al., 1990), the next step in the cholesterol biosynthetic pathway after squalene synthase (Fig. 1), to block sterol biosynthesis (Fig. 5B). Because our data indicated that treatment with this agent alone could produce some CYP3A induction in rat hepatocyte cultures at concentrations of 10  $\mu$ M and higher (Fig. 2), we performed a concentration-response

analysis of the effects of cotreatment with this agent on Ro 48-8071-inducible CYP3A mRNA expression. Incubation of hepatocyte cultures with NB-598 concentrations of 1 or 3  $\mu$ M alone had no detectable effect on CYP3A mRNA content but concentration-dependently suppressed Ro 48-8071-inducible CYP3A expression. These low concentrations of NB-598 progressively inhibited cholesterol biosynthesis, as reflected by the incremental up-regulation of HMG-CoA reductase mRNA levels. Cotreatment with 10  $\mu$ M NB-598 produced an even greater suppression of Ro 48-8071-mediated CYP3A induction, with the residual CYP3A mRNA level being comparable with that seen in cultures treated with 10  $\mu$ M NB-598 alone. Finally, hepatocytes cotreated with 30  $\mu$ M NB-598 and Ro 48-8071 contained approximately the same amount of CYP3A mRNA as cells treated with 30  $\mu$ M NB-598 alone, possibly reflecting an ability of this agent to activate the PXR directly. These findings further support the concept that Ro 48-8071-mediated CYP3A induction requires the ongoing biosynthesis of an endogenous metabolite and implicate squalene 2,3-oxide or a downstream metabolite as the bioactive mediator.

As final support, we next took advantage of our previous



**Fig. 3.** Role of PXR in cyclase inhibitor-mediated CYP3A induction in primary cultured mouse and rat hepatocytes. **A**, effects of cyclase inhibitor treatments on CYP3A mRNA levels in primary cultures of PXR-null mouse hepatocytes. Forty-eight-hour-old primary cultures of hepatocytes prepared from either a wild-type or PXR-null mouse were incubated with Williams' E medium alone (UT) or containing 0.1% DMSO,  $10^{-6}$  M PCN,  $10^{-4}$  M phenobarbital (PB),  $3 \times 10^{-5}$  M Ro 48-8071,  $3 \times 10^{-5}$  M BIBX 79, or  $10^{-5}$  M U18666A. After 24-h treatment, hepatocytes were harvested, total RNA was isolated and levels of CYP3A or CYP2B mRNA were analyzed by Northern blot hybridization. Blots were rehybridized with 7S cDNA to normalize for variations in loading and transfer among samples. Data are representative of results observed in two independent hepatocyte culture experiments. **B**, effect of dominant-negative PXR on Ro 48-8071-inducible reporter expression in primary cultured rat hepatocytes. Primary cultured rat hepatocytes were transiently transfected with a PXR-responsive reporter together with either pcDNA 3.1 (pcDNA, empty vector), plasmid expressing either wild-type (PXR) or dominant-negative (PXR-DN) PXR, or plasmid expressing either wild-type (PPAR) or dominant-negative (PPAR-DN) PPAR $\alpha$ . Transfected cultures were incubated with medium alone (UT) or containing  $3 \times 10^{-5}$  M Ro 48-8071. After 24-h treatment, the hepatocyte cultures were harvested for measurement of luciferase activities. Bars represent the mean  $\pm$  S.D. of luciferase activity values from three wells of hepatocytes per treatment. Groups not sharing a letter are significantly different from each other ( $p < 0.05$ ). Data are representative of results observed in two independent rat hepatocyte culture experiments. **C**, induction of PXR target gene expression by Ro 48-8071 treatment in primary cultured rat hepatocytes. Forty-eight-hour-old primary cultures of rat hepatocytes were incubated with Williams' E medium alone (UT) or containing 0.1% DMSO,  $10^{-6}$  M PCN,  $10^{-4}$  M phenobarbital (PB), or Ro 48-8071 (at the indicated concentrations, shown as log molarities). After 24-h treatment, hepatocytes were harvested for the isolation of total RNA, and levels of GST A2 and oatp2 mRNAs were analyzed by Northern blot hybridization. A representative autoradiograph of a blot that was rehybridized with 7S cDNA is also shown.

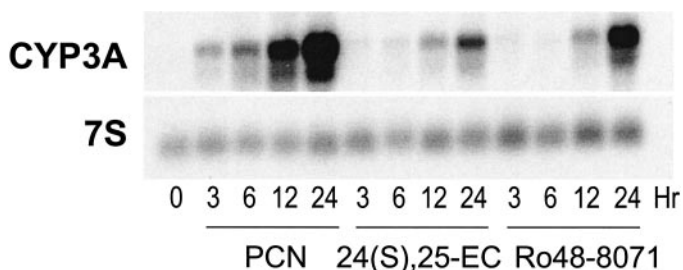


finding that, unlike certain other drugs of its class, the HMG-CoA reductase inhibitor pravastatin does not have any effect on P450 expression in primary cultured rat hepatocytes (Kocarek and Reddy, 1996), as well as the fact that the product of the HMG-CoA reductase-catalyzed reaction, mevalonate, can be readily supplemented into culture medium and used to bypass the effects of HMG-CoA reductase blockade (Fig. 6). As seen for the other sterol synthesis inhibitors, cotreatment of rat hepatocyte cultures with pravastatin completely blocked the ability of Ro 48-8071 to induce CYP3A mRNA. In this case, supplementation of cultures with exogenous meva-

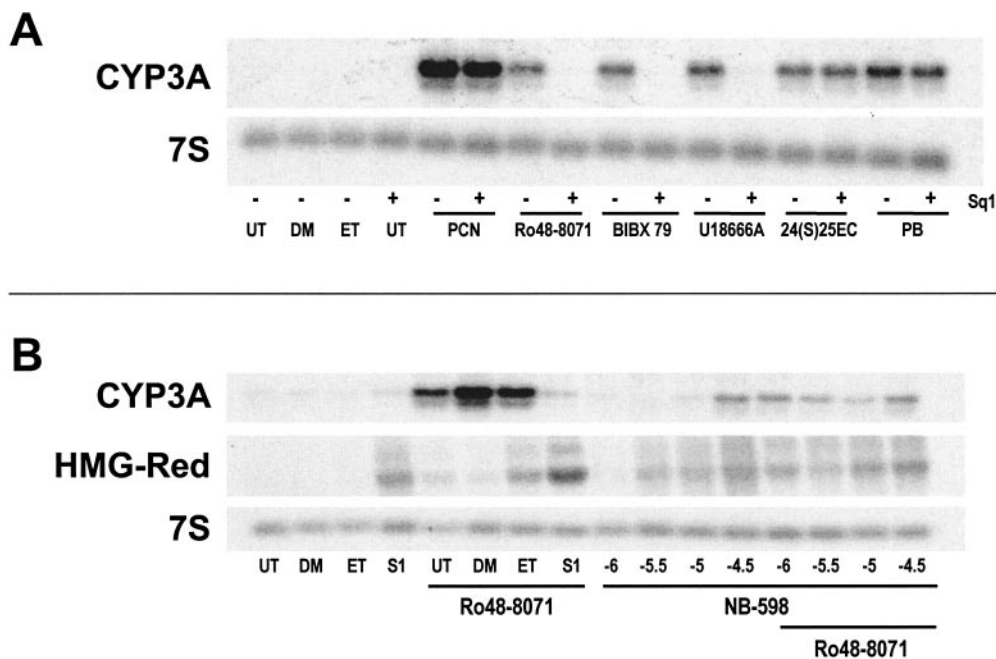
lonate readily reversed the pravastatin-mediated blockade. As controls, although squalstatin 1 treatment once again inhibited Ro 48-8071-inducible CYP3A mRNA expression, mevalonate supplementation did not reverse this effect, reflecting its inability to be metabolized beyond the squalene synthase block, and none of the inhibitor/mevalonate combinations had any effect on PCN-mediated CYP3A mRNA induction.

Because of existing reports that cyclase inhibitor treatments can, while completely blocking conversion of squalene 2,3-oxide to lanosterol, cause the accumulation not only of squalene 2,3-oxide and squalene 2,3:22,23-dioxide but also of downstream metabolites including 24(S),25-epoxycholesterol (Mark et al., 1996; Morand et al., 1997), in an additional experiment, we investigated the effects of cotreatments with agents known to be capable of inhibiting CYP51 (lanosterol 14 $\alpha$ -demethylase), such as ketoconazole and azalanstat (Kraemer and Spilman, 1986; Swinney et al., 1994; Lamb et al., 1999). However, we found that treatment of rat hepatocyte cultures with either of these agents alone caused marked induction of CYP3A mRNA expression, making it impossible to uncover meaningful effects in cotreatment experiments.

Finally, to examine the concurrence of Ro 48-8071-evoked changes in CYP3A mRNA content with the levels of individual lipid metabolites, primary cultured rat hepatocytes were treated with various concentrations of Ro 48-8071. Treated cultures were then either harvested after 24 h for measurement of CYP3A mRNA levels or were metabolically labeled



**Fig. 4.** Time courses for the induction of CYP3A mRNA after treatment with PCN, 24(S),25-epoxycholesterol, or Ro 48-8071 in primary cultured rat hepatocytes. Forty-eight-hour-old primary cultures of rat hepatocytes were either harvested (0 h) or treated with either  $10^{-5}$  M PCN,  $3 \times 10^{-5}$  M 24(S),25 epoxycholesterol [24(S)25-EC], or  $3 \times 10^{-5}$  M Ro 48-8071. The hepatocytes were harvested after treatment for 3, 6, 12, or 24 h for isolation of total RNA. CYP3A mRNA levels were analyzed by Northern blot hybridization. The blot was rehybridized with 7S cDNA to normalize for loading and transfer differences among samples. Data are representative of results observed in two independent hepatocyte culture experiments.



**Fig. 5.** Effect of squalene synthase and squalene monooxygenase inhibition on cyclase inhibitor-mediated CYP3A mRNA induction in primary cultured rat hepatocytes. A, forty-eight-hour-old primary cultures of rat hepatocytes were incubated with either medium alone (–) or containing  $10^{-7}$  M of the squalene synthase inhibitor squalstatin 1 (Sq1) (+). One hour later, the cultures were either left undisturbed (UT) or were treated with either 1) one of the following vehicles: 0.1% DMSO (DM) or 0.1% ethanol (ET); 2) one of the following CYP3A inducers:  $10^{-5}$  M PCN or  $2 \times 10^{-3}$  M phenobarbital (PB); 3) one of the following cyclase inhibitors:  $3 \times 10^{-5}$  M Ro 48-8071,  $3 \times 10^{-5}$  M BIBX 79, or  $10^{-5}$  M U18666A; or 4)  $3 \times 10^{-5}$  M of the oxysterol 24(S),25 epoxycholesterol [24(S)25EC]. B, 48-h-old primary rat hepatocyte cultures were incubated with medium alone (UT) or containing 0.1% DMSO (DM) or 0.1% ethanol (ET) vehicle,  $10^{-7}$  M of squalstatin 1 (S1), or the squalene monooxygenase inhibitor NB-598 at the indicated concentrations (shown as log molarities). One hour later, the cultures were either left undisturbed or were treated with  $3 \times 10^{-5}$  M Ro 48-8071. All of the treated hepatocyte cultures were harvested after 24 h for isolation of total RNA. CYP3A and HMG-CoA reductase (HMG-Red) mRNA levels were analyzed by Northern blot hybridization. The blots were rehybridized with 7S cDNA to normalize for loading and transfer differences among samples. All data are representative of results observed in two independent hepatocyte culture experiments.

with [ $^{14}\text{C}$ ]mevalonate and harvested 4 h later for analysis of labeled nonsaponifiable lipid contents by thin-layer chromatography (Fig. 7A). As controls, squalostatins 1 treatment completely blocked cholesterol biosynthesis, whereas pravastatin treatment had no effect on the conversion of mevalonate to downstream products. Concentrations of Ro 48-8071 ranging from 1 to 30  $\mu\text{M}$  produced a progressive increase in CYP3A mRNA levels, with the response being strongest at 30  $\mu\text{M}$  (Fig. 7B). This same range of Ro 48-8071 concentrations produced a progressive inhibition of lanosterol and cholesterol biosynthesis (Fig. 7A). In comparison, the amount of metabolite comigrating with squalene 2,3-oxide was already markedly increased after treatment with 1  $\mu\text{M}$  Ro 48-8071 and continued to accumulate at higher concentrations (Fig. 7, A and C). Also, the amount of metabolite comigrating with squalene 2,3:22,23-dioxide progressively accumulated over the range of Ro 48-8071 concentrations that was tested (Fig. 7, A and C). Finally, the amount of radiolabeled product comigrating with 24(S),25-epoxycholesterol seemed to be lower in the sample prepared from hepatocytes treated with 30  $\mu\text{M}$  Ro 48-8071 than it was in hepatocytes treated with 10  $\mu\text{M}$  of the cyclase inhibitor. High-performance liquid chromatographic analysis, directed at specifically evaluating the levels of metabolically-labeled 24(S),25-epoxycholesterol, supported this observation (Fig. 7D). Although progressive accumulation of labeled 24(S),25-epoxycholesterol was detected after treatment of hepatocyte cultures with Ro 48-8071 at concentrations up to 10  $\mu\text{M}$ , the amount of this sterol was decreased at 30  $\mu\text{M}$  Ro 48-8071 (Fig. 7D), a concentration at which the CYP3A mRNA level continued to increase (Fig. 7B). These results indicate that the cellular contents of squalene 2,3-oxide and squalene 2,3:22,23-dioxide increased over the same range of Ro 48-8071 concentrations that caused CYP3A mRNA induction, but the profile of 24(S),25-epoxycholesterol accumulation diverted from this trend.

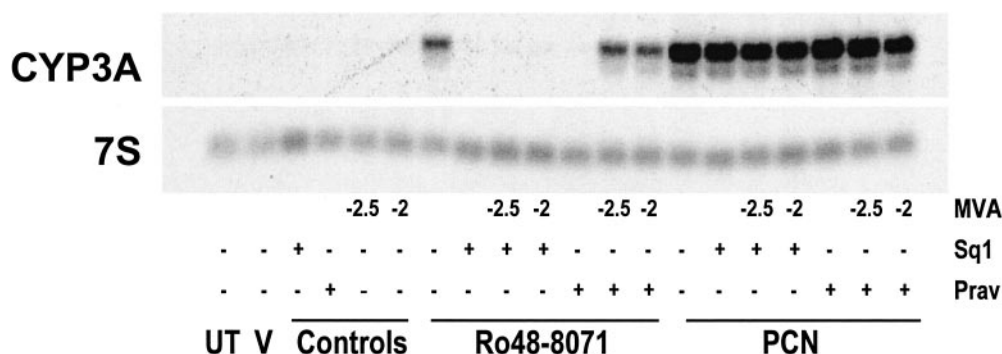
## Discussion

Our results strongly support the growing evidence that there are endogenous molecules within the hepatocyte that, when caused to accumulate, are recognized as hazardous to the cell and trigger metabolic events that facilitate their elimination. Two previously recognized examples of this par-

adigm are provided by certain bile acid precursor sterols (e.g., 5 $\beta$ -cholestanoic acid-3 $\alpha$ ,7 $\alpha$ ,12 $\alpha$ -triol) and cholestatic bile acids (e.g., lithocholate), both of which activate the PXR and induce CYP3A, which catalyzes their metabolism (Furster and Wikvall, 1999; Honda et al., 2001; Staudinger et al., 2001; Xie et al., 2001; Dussault et al., 2003; Goodwin et al., 2003). Because these molecules are produced through the further metabolism of cholesterol, the effects of cyclase inhibitors, which inhibit cholesterol biosynthesis, on CYP3A expression cannot be attributed to the accumulation of any such metabolites.

The original reports describing the pharmacological properties of Ro 48-8071 (Morand et al., 1997) and BIBX 79 (Mark et al., 1996) indicated that these agents were both comparably potent inhibitors of cyclase, completely blocking enzyme activity in cell-free preparations at a concentration of  $\sim 100$  nM, and likewise completely inhibiting the incorporation of [ $^{14}\text{C}$ ]acetate into cholesterol in intact HepG2 cultures. In comparison to the effective concentrations of these agents in the hepatoma cell line, higher concentrations (i.e., maximal effects occurred at approximately 30  $\mu\text{M}$ ) were required to produce CYP3A induction in the primary rat and mouse hepatocyte cultures. At first consideration, such concentration differences might suggest that the effects of the cyclase inhibitors on CYP3A expression were likely to be independent of cyclase inhibition. However, our cotreatment studies, in which cyclase inhibitor-mediated CYP3A induction was abolished by cotreatment of hepatocyte cultures with each of three inhibitors, targeting different enzymes, that shut off flux through the sterol biosynthetic pathway upstream of cyclase, demonstrate unequivocally that the effects of the cyclase inhibitors on CYP3A expression are mediated indirectly through the action of an endogenous squalene metabolite. We therefore suggest that the higher concentration requirements in the primary cultured rodent hepatocytes relative to the HepG2 cells may reflect differences in drug-metabolizing and/or transporting enzyme contents and consequent abilities to inactivate or eliminate the drugs from the cells.

Although the nature of the bioactive metabolite has not been firmly established in this study, clear candidate molecules are apparent. One possibility would be the oxysterol 24(S),25-epoxycholesterol, because we have recently reported



**Fig. 6.** Inhibition of Ro 48-8071-mediated CYP3A mRNA induction by pravastatin cotreatment and rescue by mevalonate supplementation in primary cultured rat hepatocytes. Forty-eight-hour-old primary cultures of rat hepatocytes were incubated with either medium alone or containing  $10^{-7}$  M squalostatins 1 (Sq1, +) or  $3 \times 10^{-5}$  M pravastatin (Prav, +). One hour later, the cultures were either left untreated (UT) or were treated with either DMSO vehicle (V),  $3 \times 10^{-5}$  M Ro 48-8071, or  $10^{-5}$  M PCN. Supplemental mevalonate (MVA) was added to the indicated cultures (concentrations are shown as log molarities). The hepatocytes were harvested after 24-h incubation for isolation of total RNA. CYP3A mRNA levels were analyzed by Northern blot hybridization. The blots were rehybridized with 7S cDNA to normalize for loading and transfer differences among samples. Data are representative of results observed in two independent hepatocyte culture experiments.

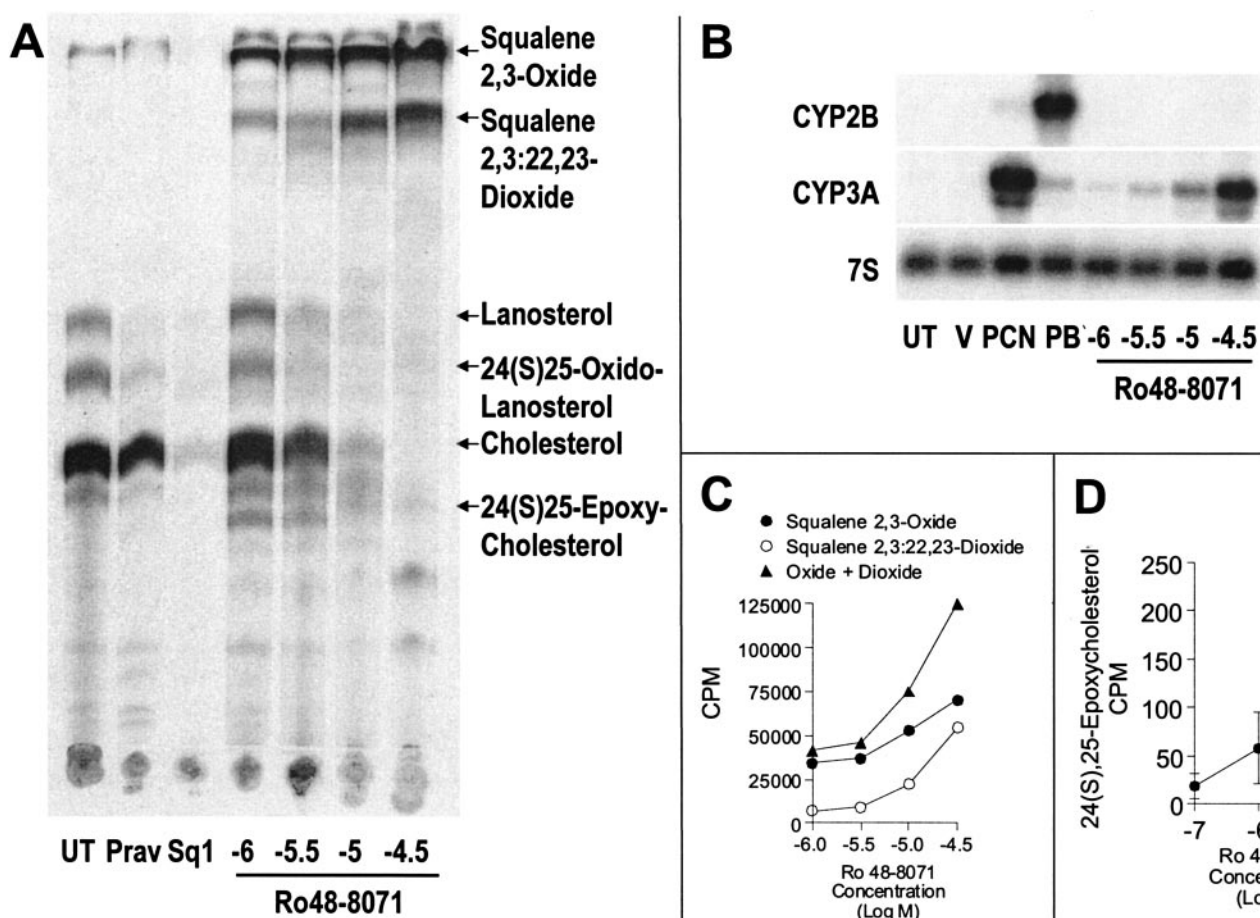


that this sterol is capable of activating the PXR at concentrations only slightly higher than those necessary to activate the LXR (Shenoy et al., 2004). However, our results indicated that the concentration (30  $\mu$ M) of Ro 48-8071 which most effectively induced CYP3A produced complete inhibition of cyclase activity, blocking not only the conversion of squalene 2,3-oxide to lanosterol, but also the conversion of the shunted product squalene 2,3:22,23-dioxide to 24(S),25-oxido-lanosterol. These results, taken together with our finding that cotreatment of primary cultured rat hepatocytes with the squalene monooxygenase inhibitor NB-598 blocked cyclase inhibitor-mediated CYP3A induction, implicate squalene 2,3-oxide and squalene 2,3:22,23-dioxide, rather than 24(S),25-epoxycholesterol, as likely mediators of cyclase inhibitor-inducible CYP3A expression.

As further support, the two squalene metabolites were noted to accumulate to relatively high levels in cultured hepatocytes after cyclase blockade. Indeed, after Ro 48-8071 treatment, the amounts of these two metabolites increased

with concentration-dependencies comparable with the observed changes in CYP3A mRNA levels. Other than the aforementioned differences in drug concentration requirements between the primary hepatocytes and the HepG2 cells, our findings are completely consistent with the earlier data regarding Ro 48-8071 and BIBX 79. Thus, in HepG2 cells, treatment with either of these agents was found to evoke the accumulation of 24(S),25-epoxycholesterol at a drug concentration of  $\sim$ 3 to 10 nM (at which partial inhibition of cyclase occurred), whereas treatment with higher drug concentrations (i.e.,  $\sim$ 100 nM to 1  $\mu$ M, causing complete cyclase inhibition) reduced the production of 24(S),25-epoxycholesterol to below detectable levels (Mark et al., 1996; Morand et al., 1997). In comparison, the cellular contents of squalene 2,3-oxide and squalene 2,3:22,23-dioxide continued to increase at these higher drug concentrations.

Triona et al. (2003) recently reported that effective CYP3A4 induction requires the action of hepatic nuclear factor-4 $\alpha$  in addition to PXR and demonstrated marked ri-



**Fig. 7.** Concentration-dependent effects of Ro 48-8071 treatment on metabolically labeled lipid content and CYP3A mRNA levels in primary cultured rat hepatocytes. Forty-eight-hour-old primary cultures of rat hepatocytes were incubated with supplemented Williams' E medium alone (UT) or containing 0.1% DMSO (V, vehicle control),  $10^{-5}$  M PCN,  $10^{-4}$  M phenobarbital (PB),  $10^{-7}$  M squalestanin 1 (Sq1),  $3 \times 10^{-5}$  M pravastatin (Prav), or Ro 48-8071 at the indicated concentrations (shown as logs). A, after 1 h of drug treatment, cultures were metabolically labeled for 4 h with [ $^{14}$ C]mevalonate, and nonsaponifiable lipids were analyzed by thin-layer chromatography. The migration positions of unlabeled standards are indicated. B, after 24-h drug treatment, hepatocytes were harvested for isolation of total RNA. CYP2B and CYP3A mRNA levels were analyzed by Northern blot hybridization. The blots were rehybridized with 7S cDNA to normalize for loading and transfer differences among samples. C, the amounts of radioactivity corresponding to squalene 2,3-oxide and squalene 2,3:22,23-dioxide in the Ro 48-8071 treatment lanes (shown in A) were determined by liquid scintillation counting of the scraped silica bands. D: The cellular contents of metabolically labeled 24(S),25-epoxycholesterol in hepatocyte cultures treated with various concentrations of Ro 48-8071 were measured by high-performance liquid chromatography. Each point represents the mean cpm  $\pm$  range of [ $^{14}$ C]24(S),25-epoxycholesterol that was measured in one treated dish of cultured hepatocytes from two independent hepatocyte culture experiments.

fampicin-inducible expression of a PXR-responsive CYP3A reporter in HepG2 cells that had been cotransfected with the human PXR and hepatic nuclear factor-4 $\alpha$ . In preliminary studies (H.-L. Fang, S. Shenoy, M. Runge-Morris, and T. Kocarek, manuscript in preparation), we have found that Ro 48-8071 treatment of HepG2 cells transfected in this manner strongly induces CYP3A reporter expression, with the increases occurring at Ro 48-8071 concentrations of  $10^{-7}$  to  $10^{-6}$  M, in the same range of concentrations known to produce the accumulation of squalene 2,3-oxide and squalene 2,3:22,23-dioxide in HepG2 cells (Morand et al., 1997). These findings further support a role for one or both of these squalene metabolites in mediating cyclase-inducible CYP3A expression and also suggest that the effects of cyclase inhibitors on CYP3A expression are likely to be conserved in the human hepatocyte.

It was also noted previously that under conditions of partial cyclase inhibition, not only was cholesterol biosynthesis blocked, but HMG-CoA reductase activity was not up-regulated (Mark et al., 1996; Morand et al., 1997), whereas when cyclase activity was completely inhibited, HMG-CoA reductase activity was increased (Mark et al., 1996). These results suggest that the maximal therapeutic benefits of this class of agent may, in fact, be produced by lower doses that incompletely inhibit cyclase activity and permit the accumulation of regulatory oxysterols, such as 24(S),25-epoxycholesterol (Mark et al., 1996; Morand et al., 1997). From our findings, such doses should result in little, if any, CYP3A induction.

These results have implications not only for understanding the fundamental mechanisms controlling CYP3A expression and for comprehending the reasons underlying the evolution of these superfamily members, but also for the pragmatic task of determining whether a new drug candidate is a CYP3A inducer. Our results suggest that it is not sufficient to use an assay that simply detects the ability of an agent to bind to the PXR. Rather, it is important to consider the possibility that a drug may affect CYP3A expression by somehow interfering with the intricately regulated system by which the hepatocyte controls its content of endogenous bioactive lipid molecules.

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