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Indinavir alters sterol and fatty acid homeostatic mechanisms in primary rat hepatocytes by increasing levels of activated sterol regulatory element-binding proteins and decreasing cholesterol 7α-hydroxylase mRNA levels

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Abstract

Human immunodeficiency virus protease inhibitors induce hyperlipidemia in many patients treated with these drugs. We examined the effects of indinavir on cholesterol and bile acid homeostatic mechanisms in a primary rat hepatocyte (PRH) culture model. In PRH, indinavir up-regulated (2.5-fold) 3-hydroxy-3-methylglutaryl-Coenzyme A reductase mRNA levels 24 hr after drug addition. In these same experiments, cholesterol 7α-hydroxylase (CYP7A1) mRNA levels, the rate-limiting enzyme in bile acid biosynthesis, was decreased up to 10-fold. Fatty acid synthase mRNA levels were up-regulated more than 3-fold under these conditions. Indinavir did not alter CYP7A1 transcriptional activity, but decreased CYP7A1 mRNA half-life in PRH from 1.5 hr to less than 0.5 hr. Sterol regulatory element-binding protein-1 (SREBP-1) mature form was increased approximately 6-fold by this drug. Indinavir-induced mRNA changes and SREBP-1 mature protein levels were significantly abated by the addition of cholesterol, solubilized in β-cyclodextrin, to culture medium. Indinavir markedly decreased endogenous cholesterol esterification and increased cholesterol in intracellular membranes in primary hepatocytes. Indinavir gavaged into intact mice also markedly increased SREBP-1 and SREBP-2 (mature forms) in hepatic nuclei. CYP7A1 mRNA was also decreased ~52% in indinavir-treated animals. We propose that indinavir disrupts cellular cholesterol homeostasis by increasing SREBP's and decreasing CYP7A1 mRNA.
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Keywords: Indinavir; Bile acid synthesis; SREBP; CYP7A1; Cholesterol; Fatty acid

1. Introduction

The development and use of human immunodeficiency virus (HIV) protease inhibitors (PIs) in combination with nucleotide analogue reverse transcriptase inhibitors have significantly reduced the morbidity and mortality of patients with HIV infection [1]. Patients on highly active antiretroviral therapy (HAART) have essentially transformed HIV infection into a chronic disease. Unfortunately, the use of HIV PIs has resulted in significant side effects from these drugs [2,3]. Most patients treated with HAART develop significant hyperlipidemias that lead to an increased risk of atherosclerosis [4]. A significant percentage of HIV-infected patients treated with HAART develop high serum levels of cholesterol and triglycerides [5–9].

The cellular/molecular mechanism(s) responsible for HAART-associated hyperlipidemias has not fully elucidated and may be multifactorial [10–15]. Recent studies with ritonavir report induced accumulation of hepatic

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Abbreviations: CYP7A1, cholesterol 7α-hydroxylase; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl-Coenzyme A reductase; LDL-R, low density lipoprotein receptor; CYP27, sterol 27-hydroxylase.

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responsive element-binding proteins (SREBP) 1 and 2 in mice treated with this drug [10]. There was no alteration of SREBP-1 and SREBP-2 mRNA levels in drug-treated animals suggesting a post-translational regulation of these transcription factors by ritonavir. Indinavir decreased the levels of SREBP-1, peroxisome proliferation activated receptor-γ (PPAR-γ), and the insulin receptor in preadiopotes in culture [14]. Nelfinavir also caused a decrease in the mature form of SREBP-1 and other transcription factors in a preadipocytes in culture [13]. Finally, indinavir decreased SREBP1-c in human embryonic biliary and hepatoma cells in culture. This was correlated with a decrease in the fatty acid synthase (FAS) and lipoprotein lipase transcriptional activity [15].

In the present study, we examined the effects of indinavir on cholesterol input and output pathways using PRHs as a model system. The results showed that indinavir markedly increased SREBP-1 and SREBP-2 protein in the nucleus, and significantly decreased the half-life of CYP7A1 mRNA. There was a corresponding increase in 3-hydroxy-3-methyl-glutaryl-Coenzyme A reductase (HMG-CoA reductase), low density lipoprotein receptor (LDL-R) (at 6 hr), and FAS mRNA levels following drug treatment. These results may provide a possible explanation for how indinavir alters cellular cholesterol homeostatic mechanisms.

2. Materials and methods

2.1. Materials

MAXIscript T7 and ribonuclease protection assay (RPA II) kits were purchased from Ambion. β-Cyclodextrin was obtained from Cyclodextrin Technologies Developments, Inc. Indinavir was a generous gift from Merck. SREBP-1 antibodies (SREBP-1 (2A4):sc-13551) were obtained from Pharmingen. Actinomycin D, cholesterol, dexamethasone, L-thyroxine, progesterone, and *N*-α-tosyl-L-lysine chloromethyl ketone (TLCK) were obtained from Sigma Chemical Co. Human LDL was purchased from Sigma Chemical Co and 25-hydroxycholesterol was obtained from Steraloids, Inc.

2.2. PRH isolation and culture

Primary hepatocytes were isolated from adult male Sprague–Dawley rats (250–300 g) using the collagenase-perfusion technique of Bissell and Guzelian [16]. Trypan blue exclusion was used to determine cell viability (>90%) before plating monolayers on collagen-coated plates (60 mm). Unless otherwise indicated, cells were cultured in serum-free Williams' E medium containing dexamethasone (0.1 μM), insulin (100 nM), penicillin (100 units/mL), and thyroxine (1 μM). Cells were incubated from 12 to 24 hr in 5% CO_2 environment at 37° before additions were made to culture medium [17].

2.3. Animal treatment

Five- to 6-week-old male C57BL/6 mice were purchased from the National Cancer Institute and the mice were fed with a standard mouse chow diet containing 5% (w/w) fat and 0.04% (w/w) cholesterol (Harlan Taklad Laboratories) and were maintained according to our university guidelines. Half of the mice were gavaged with 50 μL indinavir (3 mg), while the other half of the mice received equal volume of water as vehicle control. Livers were removed from control and indinavir-treated animal. Tissue was immediately rinsed with ice-cold PBS and then used for preparation of membrane and nuclear protein using the modified procedures as described by Buckley *et al*. [18] and Sheng *et al*. [19].

2.4. RNA isolation and mRNA quantitation

RNA was isolated and quantitated as previously described [17]. All ribonuclease protection (RPA) probes (CYP7A1, HMG-CoA reductase, LDL-R, sterol 27-hydroxylase (CYP27), and rat cyclophilin) were synthesized using a MAXIscript T7 kit from Ambion using a probe-specific DNA fragment. The RPA probes were labeled with [α-^{32}P]UTP and isolated using Qiaquick columns. Overnight hybridization was carried out with 8×10^4 cpm for CYP7A1, CYP27, HMG-CoA reductase, LDL-R, and 4×10^4 cpm for cyclophilin which was used as an internal control. Ten micrograms of total RNA was used in all RPA assays. Following RNase digestion, samples were fractionated on 5% acrylamide/8 M urea gels and bands visualized by autoradiography using Kodak Biomax MS film. Bands were quantitated by laser densitometry and normalized to rat cyclophilin. FAS was quantitated by Northern blot analysis [17].

3. Transient transfection of hepatocytes

The chimeric rat CYP7A1 promoter-luciferase reporter gene construct (pGL3R7α-342) was obtained from Dr. John Chiang's (Northeastern Ohio Universities, Rootstown, OH) laboratory. Cells were plated in Primaria 6-well culture plates (Falcon) in Williams' E medium containing 10% fetal calf serum (FCS) plus penicillin, thyroxine (1 μM), and dexamethasone (0.1 μM). Transient transfections were performed 6- to 8-hr post-plating using Effectene transfection reagent (Qiagen). Post-transfection media did not contain FCS. Transfections were carried out using the following plasmid concentrations: 100 ng/well pGL3R7α-342, 5 ng/well of pCMV-β-Gal (control for transfection efficiency), and p-blue script carrier DNA (Stratagene) to obtain an experimentally determined optimal final DNA concentration of 800 ng/well. Twenty-four hours post-transfection, culture media were changed, indinavir (100 μM) was added, and cultures incubated for an

additional 24 hr. Cells were harvested and assayed for luciferase and β-galactosidase activities using a Dual-Light Kit (Tropix). Luciferase activities were normalized to β-galactosidase activities to obtain final results. Transfections were performed in duplicate and background activity subtracted (obtained from transfection with pGL3-Basic vector (Promega)).

3.1. Cholesterol addition to hepatocyte cultures

PRHs were plated in Williams' E Medium (above), incubated for 24 hr, culture medium changed, and indinavir (100 μM) added. Cultures were incubated for another 12 hr, culture medium changed and the following individual additions made: indinavir alone (100 μM); indinavir (100 μM) + cholesterol (200 μM) solubilized in β-cyclodextrin; indinavir (100 μM) + β-cyclodextrin; β-cyclodextrin alone; and cholesterol (200 μM) solubilized in β-cyclodextrin. Cultures were allowed to incubate for 24 hr, RNA isolated, and mRNA (CYP7A1, HMG-CoA reductase, and LDL-R) quantitated as described above.

3.2. CYP7A1 mRNA half-life determination

PRHs were plated in Williams' E medium (above), incubated for 48 hr, and media changed. Indinavir (100 μM) was added to all plates, except controls, and cultures incubated for 12 hr. Actinomycin D (1 μg/mL final concentration in DMSO) was then added to culture medium, cells harvested (every 30 min) over a 2-hr time course, RNA isolated, and CYP7A1 mRNA determined (above). A control experiment included DMSO alone.

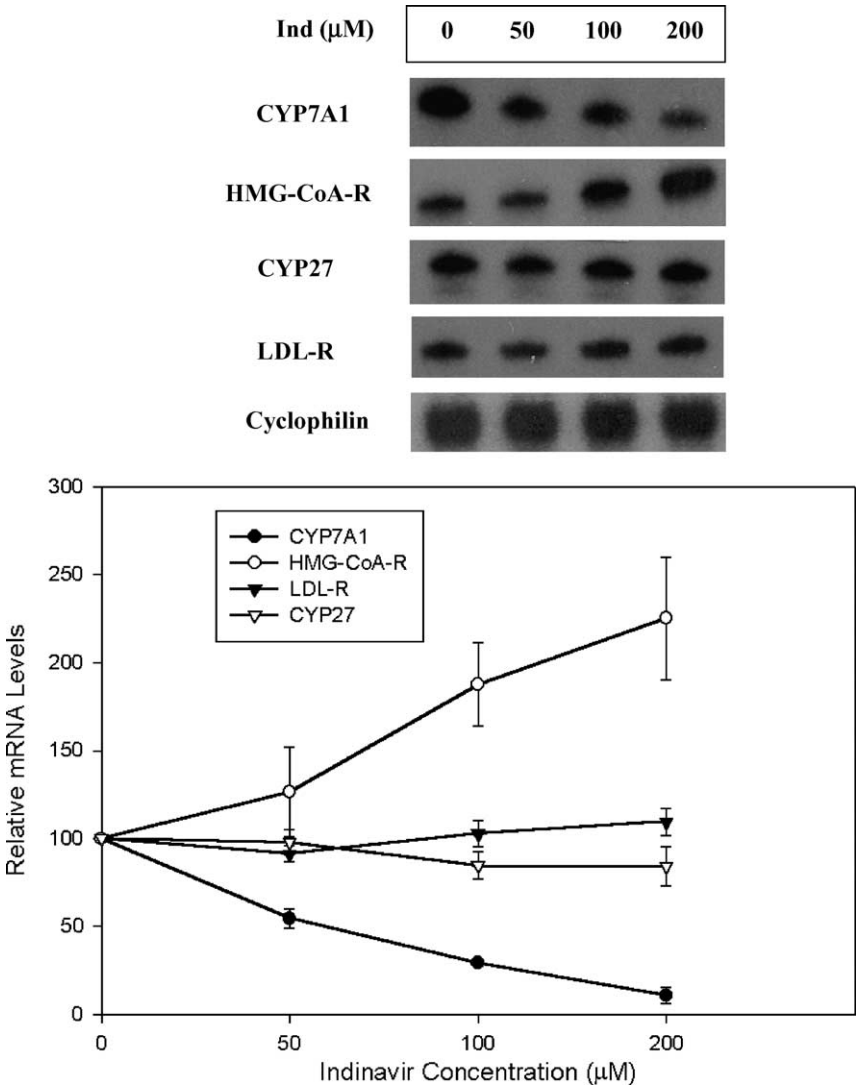

Fig. 1. Effects of indinavir concentration on steady-state mRNA levels of genes involved in cholesterol and bile acid metabolism. Primary rat hepatocytes were isolated as described in Section 2, plated, incubated for 24 hr, culture medium changed, and indinavir added to final concentrations of 50, 100, and 200 μM. After 24 hr of incubation with indinavir, total RNA was isolated and levels of specific mRNA species determined by either Northern blot or RPA analysis. Relative amounts of mRNA was quantitated by laser scanning densitometry and normalized to rat cyclophilin mRNA as loading control. Each experiment was independently repeated at least three times and data presented as ±SEM. CYP7A1, cholesterol 7α-hydroxylase; HMG-CoA-R, 3-hydroxy-3-methylglutaryl-Coenzyme A reductase; LDL-R, low density lipoprotein receptor; and CYP27, sterol 27-hydroxylase.

3.3. Effect of Indinavir on SREBP

Cells were plated (60 mm dishes) in Williams' E medium, incubated for 24 hr, and media changed. Indinavir (100 μM) or buffer was added to culture medium and cells harvested for nuclear extract isolation at 6, 12, and 24 hr after drug or buffer addition. Cells were placed on ice and washed once in PBS. Buffer A (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 2 mM NaF, 2 mM Na_3VO_4, 25 μg/mL leupeptin, 25 μg/mL aprotopin, 10 μg/mL pepstatin, and 0.1 mM PMSF) (0.8 mL) was added to each culture dish and cells harvested using a cell scrapper. Cells were allowed to incubate on ice for 15 min, 50 μL Nonidet NP-40 (10%) was added and vortexed for 10 s. Cell extracts were then placed on ice for 3 min and centrifuged in a microfuge (16,000 *g*) for 10 min at 4°. The supernatant fluid was discarded and the pellets suspended in 50 μL of buffer B (20 mM HEPES, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 2 mM NaF, 2 mM Na_3VO_4, 25 μg/mL leupeptin, 25 μg/mL aprotopin, 10 μg/mL pepstatin, 0.1 mM PMSF). The suspensions were ''rocked'' vigorously at 4° for 20 min and the centrifuged in a microfuge (16,000 *g*) for 5 min at 4°. Supernatants (which contain nuclear extract) were then removed, protein concentrations estimated (5 μL), and the remaining extract stored at −70°.

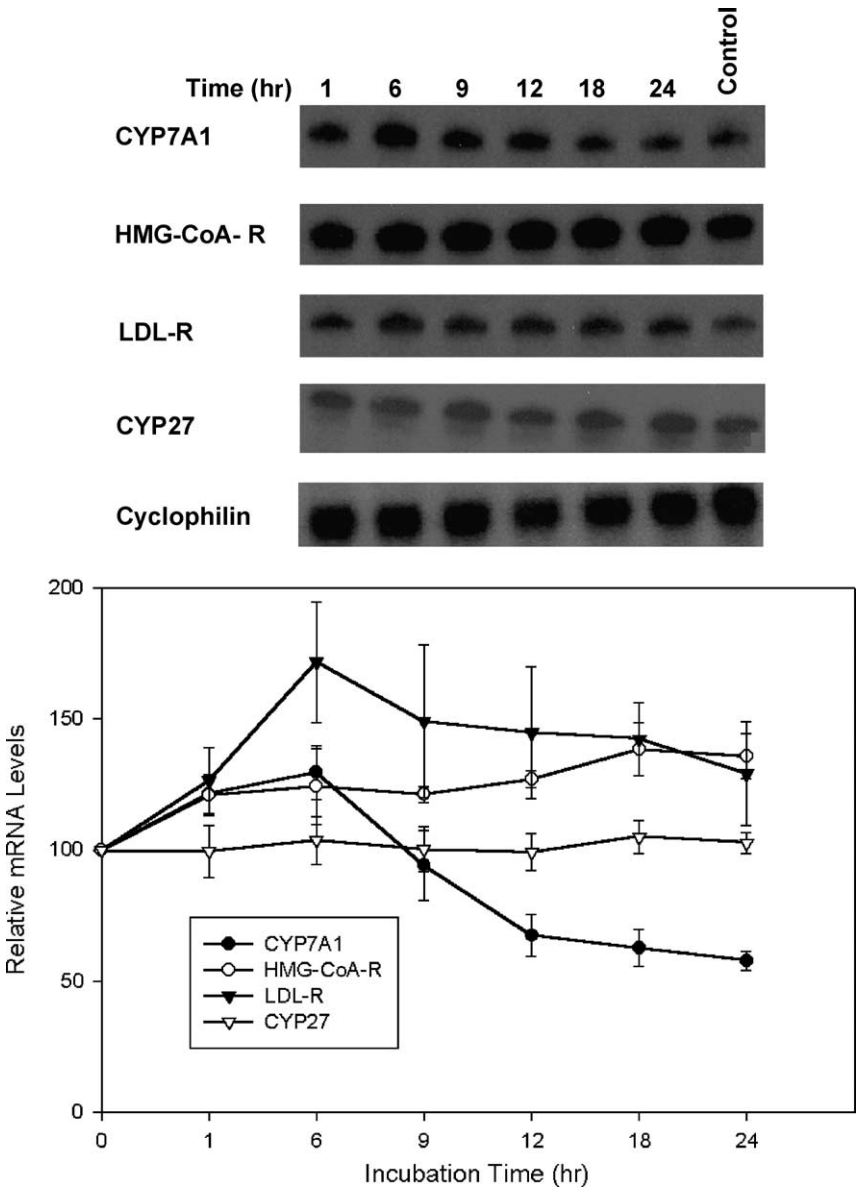

Fig. 2. Effects of time on steady-state mRNA levels of genes involved in cholesterol and bile acid metabolism following the addition of indinavir. Primary rat hepatocytes were isolated as described in Section 2, plated, incubated for 24 hr, culture medium changed, and indinavir (100 μM) added to culture medium. Total RNA was isolated at 1, 6, 9, 12, 18, and 24 hr from drug-treated and control cells. Specific species of mRNA was determined by either Northern blot or RPA analysis as described in Section 2. Values were normalized to rat cyclophilin mRNA. The experiment was independently repeated four times and the data presented as ±SEM. Abbreviations as in Fig. 1.

3.4. Western blot analysis for SREBP-1 and SREBP-2

Fifteen micrograms of nuclear extract protein was mixed with sample buffer and boiled for 2 min. The samples were then separated on a 7.5% SDS–PAGE gels (Bio-Rad Mini-PROTEIN II gels from Bio-RAD) at 150–175 V. Separated proteins were transferred onto nitrocellulose membranes using a Bio-Rad Mini-Blot transfer apparatus. Protein transfer was performed at 100 V for 1 hr after which, the membrane was soaked in 5% non-fat milk blocking solution in TNT buffer (0.02 M Tris base, 0.15 M NaCl, 0.5 mL Tween-20) at room temperature for 1 hr. The membrane was then incubated in a 2% non-fat milk/TNT solution containing 0.5–1 μg/mL primary antibody (purified mouse IgG-2A$_1$κ anti-SREBP-1) overnight at 4° with shaking. The membrane was then washed three times in TNT buffer (10 min/wash at room temperature). Next, the membrane was incubated in a 1:10,000 dilution of secondary antibody (goat anti-mouse IgG-HRP conjugate from Bio-Rad) for 1 hr at room temperature in a 2% non-fat milk/TNT buffer solution. The membrane was again washed three times in TNT buffer solution. Protein bands were visualized using Western Blot Chemiluminescence Reagent Kit (NEN Life Sciences Products) and were developed on X-OMAT AR film (Kodak) following a 30-s to 1-min exposure.

3.5. Cholesterol esterification assay

PRHs were plated in Williams' E medium, incubated for 24 hr and media changed. Indinavir (100 μM), buffer or test lipid was individually added to the culture medium for 24 hr. Cells were pulsed with [^{3}H]oleate (3 μCi/mL) for 2 hr, washed three times with Tris-buffered saline at 4° and lipids extracted with hexane:isopropyl alcohol (3:2, v/v) essentially as described [20]. A recovery standard (30 μg cholesteryl oleate, 30 μg triolein, 0.0005 μCi [^{14}C]cholesteryl oleate) was added and extracted samples dried under a nitrogen gas atmosphere. The lipids were separated by TLC (SIL 1 B gel plates (J.T. Baker)) using heptane:ethylether:acetic acid (90:30:1, v/v/v) and visualized by iodine. The [^{3}H]cholesteryl oleate was quantified by liquid scintillation spectrometry.

3.6. Filipin staining of cellular cholesterol

PRHs were plated in Williams' E medium on 12 mm glass cover-slips in 4-well dishes with 20% confluence for

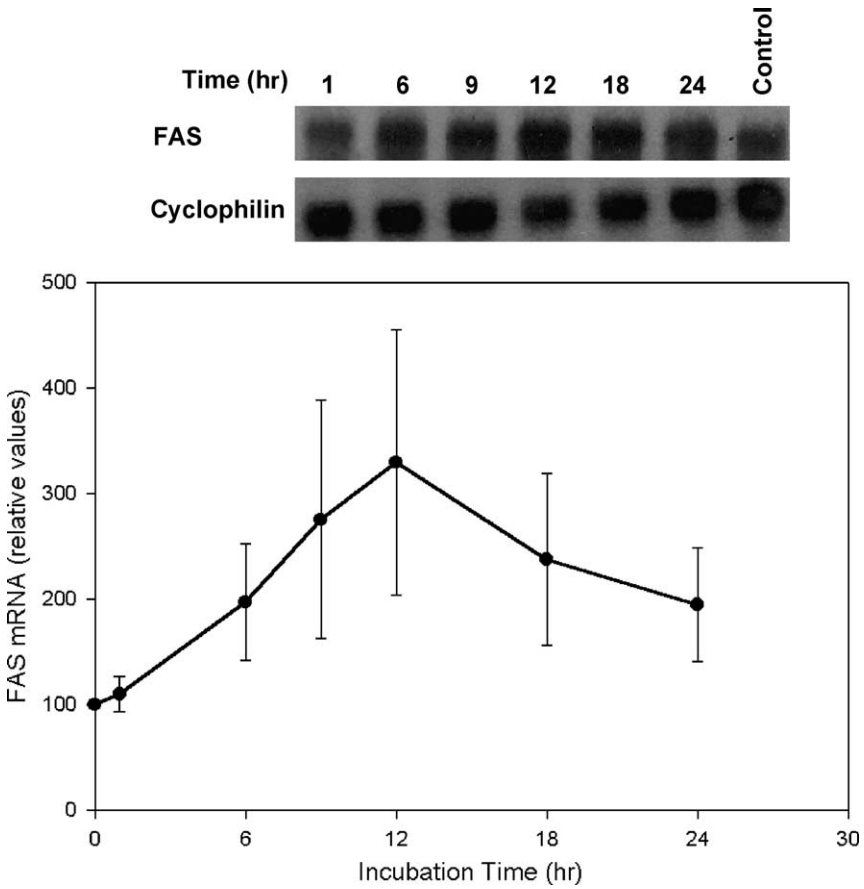

Fig. 3. Effects of time on steady-state mRNA levels of fatty acid synthase (FAS) following the addition of indinavir to culture medium. Primary hepatocytes were isolated as described in Section 2, plated for 24 hr, culture medium changed, and indinavir (100 μM) added to culture medium. Total RNA was isolated at 1,6, 9, 12, 18, and 24 hr from drug-treated and control cells. FAS mRNA was determined by Northern blot analysis and normalized to rat cyclophilin as loading control. The experiment was independently repeated three times and the data presented as ±SEM.

24 hr, media changed, and indinavir (100 μM) or buffer added to the culture medium for 24 hr. Cells were washed with PBS and fixed with 3.7% paraformaldehyde in PBS for 30 min. Cells were washed twice with PBS, permeabilized, and stained with filipin (50 μg/mL) dissolved in 0.5% BSA–PBS solution for 30 min. Cells were examined by fluorescence microscopy using an Olympus epifluorescence microscope. The filter settings used for filipin detection were: excitation filter 380/40 nm, emission filter 485/35 nm.

3.7. Quantitation of X-ray film

All blot analysis (RPA, Northern, and Western) was carried out using the Molecular Dynamics Personal Densitometer SI (mode #PDSI) personal densitometer and Image QuaNT image analysis computer software.

3.8. Statistical analysis

Results from different experiments were expressed as means ± SEM (N = number of independent experiments). Results were compared using Student's t test: $P < 0.05$ was considered statistically significant.

4. Results

4.1. Effects of indinavir on mRNA levels of key genes involved in cholesterol biosynthesis and metabolism

Indinavir was added, in varying concentrations (50, 100, and 200 μM), to the culture medium of PRHs. After 24-hr incubation, RNA was isolated, and specific mRNA species quantitated either by RNase protection assays or Northern

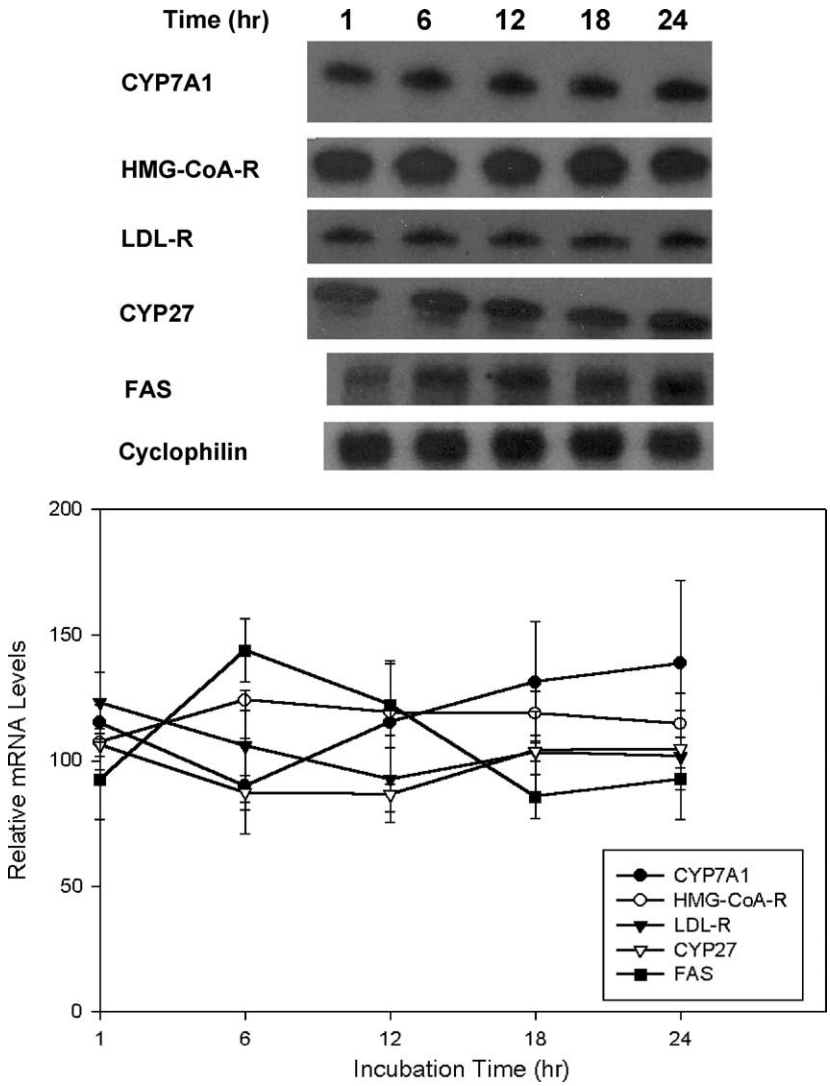

Fig. 4. Effects of TLCK on steady-state mRNA levels of genes involved in cholesterol and bile acid metabolism. Primary hepatocytes were isolated as described in Section 2, plated for 24 hr, culture medium changed, and TLCK (a trypsin-like serine protease inhibitor) was added to the culture medium (100 μM final concentration). Total RNA was isolated at 1, 6, 12, 18, and 24 hr following drug-treated and control cells. Specific mRNA species were quantitated by either Northern or RPA analysis and normalized to rat cyclophilin mRNA. The experiment was independently repeated three times and the data presented as ±SEM.

blot analysis (Fig. 1). Under these experimental conditions, indinavir caused a concentration-dependent increase (126–225% of control) in HMG-CoA reductase mRNA levels ($P < 0.025$ at 100 and 200 μM). In contrast, CYP7A1 mRNA levels decreased (45–89% of control) in these same experiments ($P < 0.005$, $P < 0.001$, $P < 0.001$ at 50, 100, and 200 μM, respectively). CYP27 and LDL-R mRNA levels did not significantly change under these conditions. There was no evidence of cellular toxicity as assessed by Trypan blue exclusion, release of lactate dehydrogenase into the culture medium, or light microscopy (data not shown).

In a time course experiment, the addition of indinavir (100 μM) to the culture medium caused the expected increase in HMG-CoA reductase mRNA levels ($P < 0.001$ at 9 and 12 hr and $P < 0.01$ at 18 and 24 hr) and a decrease in CYP7A1 steady-state mRNA levels ($P < 0.01$, $P < 0.005$, $P < 0.001$ at 12, 18, 24 hr, respectively). However, LDL-R mRNA levels increase within the first 6 hr ($P < 0.025$) of incubation and then declined to near control over the next 18 hr ($P =$ ns) (Fig. 2). CYP27 and cyclophilin (loading control) mRNA levels did not change significantly in this experiment. In these same experiments, the levels of FAS mRNA levels increase (up to ~300% of control) during the first 12 hr after indinavir addition and then declined over the next 12 hr (Fig. 3).

4.2. Effects of TLCK on mRNA levels of key genes in cholesterol biosynthesis and metabolism

TLCK (100 μM), an inhibitor of trypsin-like serine proteases, was added to the culture medium of PRHs and cells harvested over a 24-hr time course. Messenger RNA levels of HMG-CoA reductase, CYP7A1, CYP27, LDL-R, and FAS were determined either by RNase protection assays or Northern blot analysis. The results of this control experiment, using a different proteinase inhibitor,

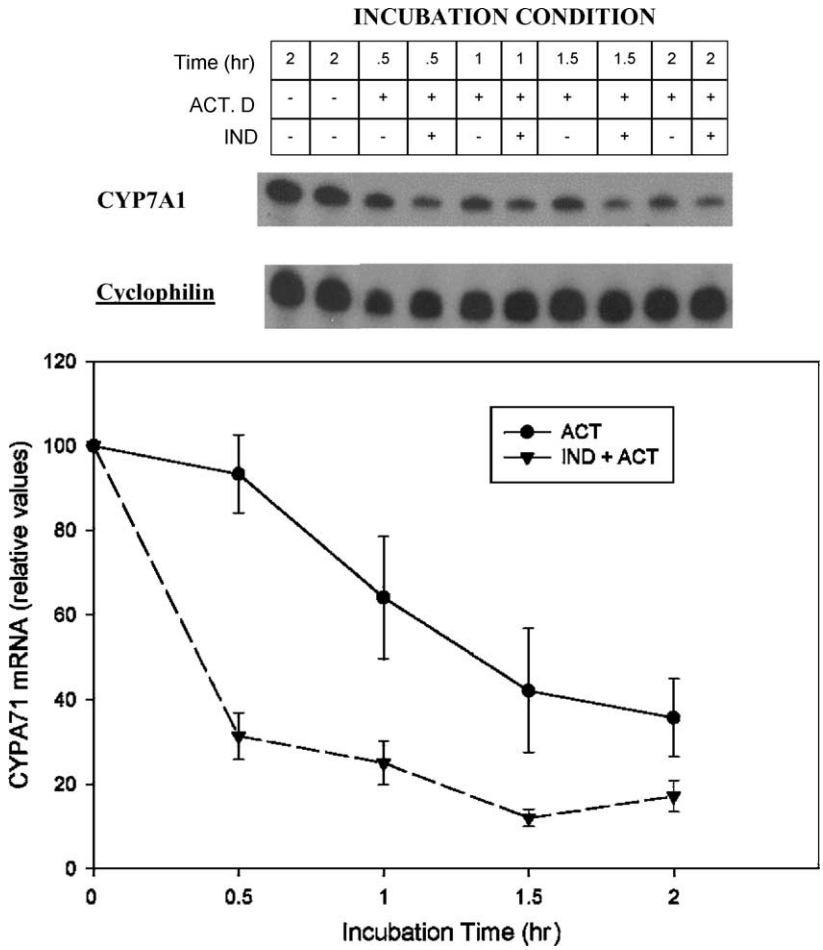

Fig. 5. Indinavir decreases the half-life of cholesterol 7α-hydroxylase (CYP7A1) mRNA in primary rat hepatocytes in culture. Primary hepatocytes were isolated as described in Section 2, plated for 24 hr and the culture medium changed. Indinavir (100 μM) or buffer was added to the culture medium and cells incubated from an additional 12 hr. Then, CYP7A1 half-life was determined under the following conditions: (1) Actinomycin D alone (1 μg/mL final concentration dissolved in DMSO) was added to untreated cells and samples taken at 0.5, 1, 1.5, and 2 hr; (2) Actinomycin D (1 μg/mL) was added to indinavir-treated cells and samples taken at 0.5, 1, 1.5, and 2 hr; DMSO alone and no addition was run as controls. In each experiment, cells were harvested at 0.5, 1, 1.5, and 2 hr and total RNA isolated. CYP7A1 mRNA levels were determined by RPA and normalized to rat cyclophilin mRNA. The experiment was independently repeated three times, data normalized to percent DMSO control, and the data presented as ±SEM.

showed no significant changes in the mRNA levels of these genes (Fig. 4).

4.3. Indinavir decreases the half-life of CYP7A1 mRNA

In order to determine the possible mechanism for the marked decrease in CYP7A1 mRNA levels following the addition of indinavir, primary hepatocytes were transfected with a plasmid containing the rat CYP7A1 promoter (−342 bp) linked to a luciferase reporter gene as described in Section 2. Following transfection of primary hepatocytes with the CYP7A1 promoter construct, indinavir was added (100 μM) and luciferase activity measured over a 24-hr time course. The results showed no decrease in luciferase activity following the addition indinavir (data not shown). These results suggested that indinavir was not decreasing the transcriptional activity of the CYP7A1 gene. Next, the half-life of CYP7A1 mRNA was determined in the presence and absence of indinavir. The half-life of CYP7A1 mRNA was approximately 1.5 hr in untreated primary hepatocytes, which is consistent with literature values [21]. In primary hepatocytes treated with indinavir, the CYP7A1 mRNA half-life was significantly decreased to <0.5 hr (Fig. 5).

4.4. Indinavir markedly increases the mature form of SREBPs

Next, we investigated the effects of indinavir on the protein levels of mature and immature forms of SREBP-1. The mature forms of SREBPs are know to increase the expression of genes involved in cholesterol and fatty acid biosynthesis and metabolism [22]. Following the addition of indinavir (100 μM) to PRHs, the nuclear fraction of hepatocytes was isolated at 6, 12, and 24 hr, and Western blot analysis performed to detect and quantitate SREPB-1 protein. The results showed a dramatic increase (6-fold, $P < 0.025$) in the mature form of SREBP-1 at 6 and 12 hr ($P < 0.001$) after indinavir addition followed by a decline over the next 12 hr (Fig. 6). There was a small increase in the immature form of SREBP-1 at 6 hr (P = ns). The levels of SREBP-2 were not determined in these experiments as the specific antibody for the rat protein is not commercially available. A Northern blot was performed to determine if there was any change in the SREBP-1 mRNA levels following the addition of indinavir (100 μM). The results showed no significant change in steady-state mRNA levels of SREBP-1 (data not shown).

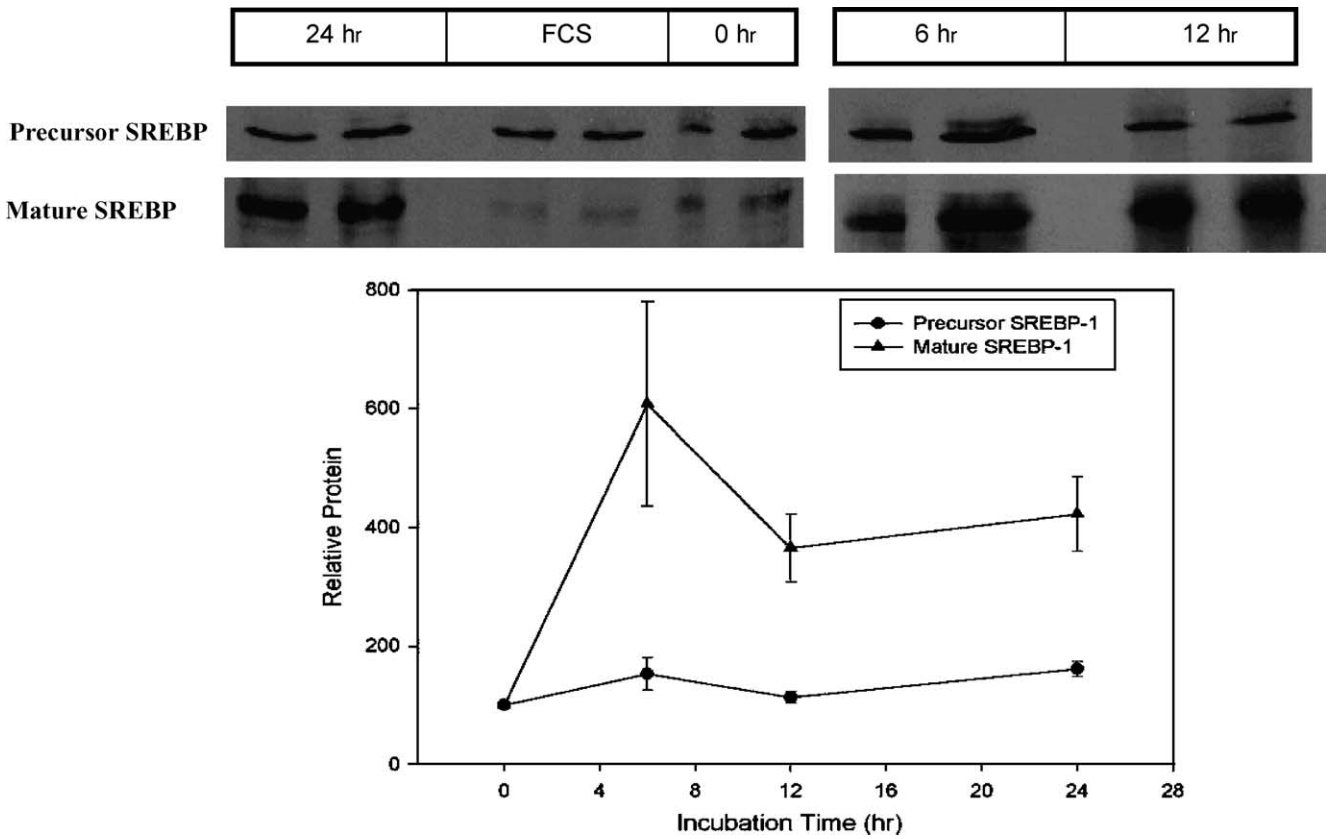

Fig. 6. Effects of indinavir on levels of mature and immature sterol responsive element-binding protein-1 (SREBP-1) in primary rat hepatocytes in culture. Primary hepatocytes were isolated as described in Section 2, plated for 24 hr, and the culture medium changed. Indinavir (100 μM) was added to the culture medium, cells harvested at 0, 6, 12, and 24 hr and nuclear extracts prepared as described in Section 2. The levels of mature and immature SREBP-1 were determined by Western blotting described in Section 2. The experiment was independently repeated four times and the quantitative results (lower panel) presented as ±SEM. The upper panel shows a typical Western blot of SREBP-1 under different experimental conditions.

4.5. Effects of exogenous cholesterol addition on alteration of mRNA and SREBP-1 protein levels by indinavir

The regulation of expression of key genes involved in cholesterol, fatty acid, and bile acid metabolism are sensitive to alteration in cellular cholesterol pools [22,23]. Therefore, we hypothesized that indinavir might effect the intracellular transport of cholesterol to regulatory sites within the hepatocyte. To test this possibility, cholesterol (200 μM), solubilized in β-cyclodextrin, was added to the culture medium along with indinavir. Messenger RNA levels were determined 24 hr after indinavir addition. The results showed that cholesterol (XOL) addition to the culture medium was able to markedly abate the effects of indinavir on HMG-CoA reductase and CYP7A1 mRNA levels (Fig. 7). HMG-CoA reductase mRNA levels were not increased when cholesterol (dissolved in β-cyclodextrin) plus indinavir was added to the culture medium. Moreover, the down-regulation of CYP7A1 was considerably less when cholesterol and indinavir were added in combination. The addition of cholesterol alone resulted in the expected increase in CYP7A1 mRNA and the down-regulation of HMG-CoA reductase and LDL-R mRNA levels (Fig. 7).

In other experiments, we also showed that the mature form of SREBP-1 was markedly increased in hepatocytes treated with 100 μM indinavir in medium containing exogenous cholesterol in the form of lipoproteins (10% FCS). Interestingly, the addition of cyclodextrin alone (1%) to cultures markedly decreased the amount of mature form of SREBP-1 in these cultures (Fig. 8).

4.6. Effects of indinavir on cholesterol esterification and transport

The addition of indinavir to PRHs markedly decreased endogenous cholesterol esterification (Fig. 9). However, there was no effect of indinavir on the esterification of exogenous LDL cholesterol. Filipin staining of cells

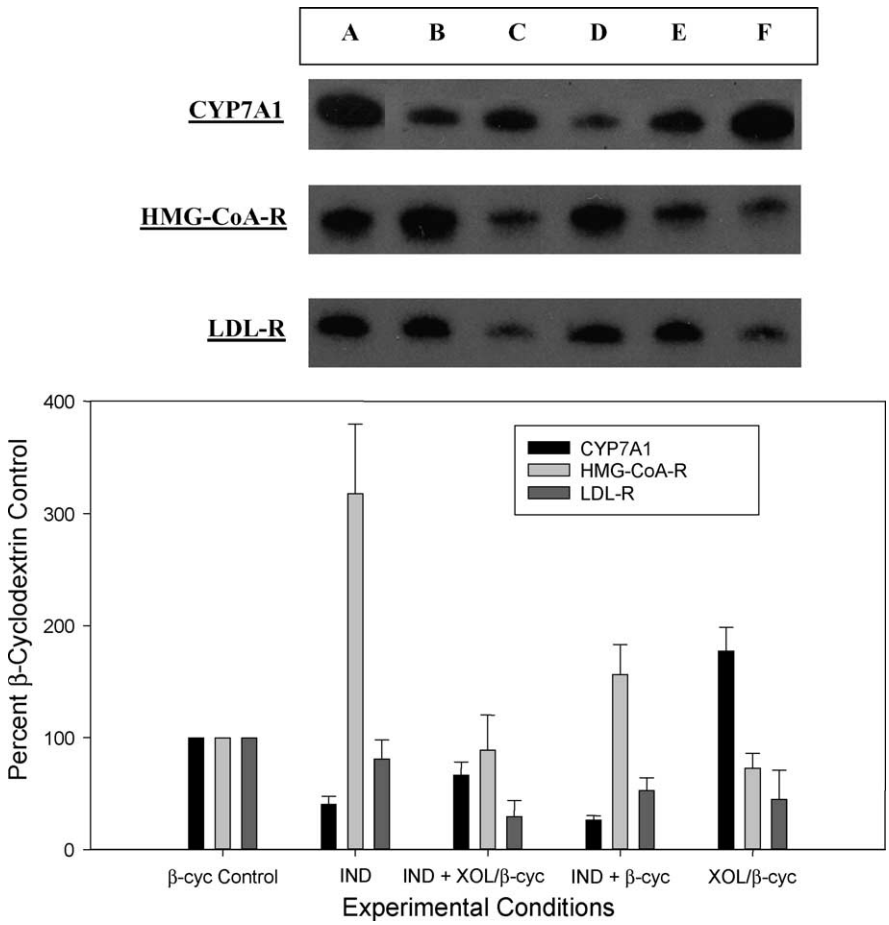

Fig. 7. Exogenous cholesterol (XOL) abrogates the effects of indinavir (IND) on mRNA levels of genes involved in cholesterol and bile acid metabolism. Primary rat hepatocytes were isolated as described in Section 2, plated for 24 hr, and the culture medium changed. The following additions were made to cells: top panel: A, no addition control; B, IND (100 μM) alone; C, IND + XOL dissolved in β-cyclodextrin (210 μL); D, IND + β-cyclodextrin (210 μL); E, β-cyclodextrin (210 μL) alone, F, XOL dissolved in β-cyclodextrin (210 μL). Cultures were then incubated for 24 hr, cells harvested, total RNA isolated, and levels of cholesterol 7α-hydroxylase (CYP7A1), 3-hydroxy-3-methylglutaryl-Coenzyme A reductase (HMG-CoA-R), and low density lipoprotein receptor (LDL-R) mRNA determined (upper panel) as described in Section 2. The experiment was independently repeated three times and the results reported as ±SEM (lower panel). The β-cyclodextrin alone served as the 100% control value.

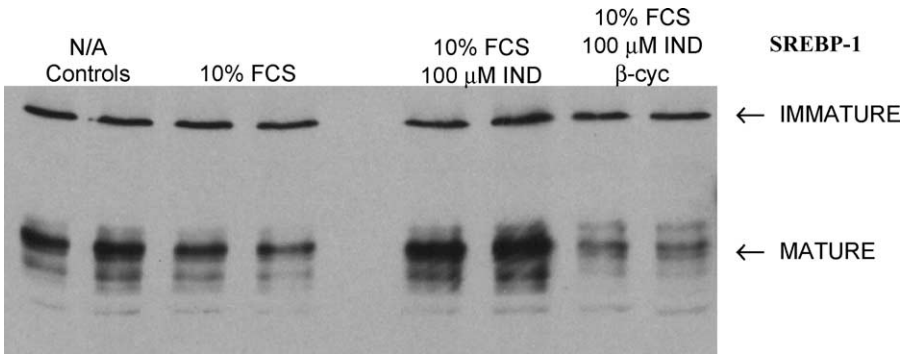

Fig. 8. β-Cyclodextrin blocks the increase in sterol responsive element-binding protein-1 (SREBP-1) by indinavir in serum containing medium. Primary hepatocytes were isolated as described in Section 2, plated in Williams' E medium containing 10% FCS, and the medium changed at 24 and 48 hr. At 48-hr post-plating indinavir (100 μM) or indinavir (100 μM) + β-cyclodextrin (1%) was added to the culture medium, incubated for 6 hr, nuclear extracts prepared, and Western blotted for SREBP-1 as described in Section 2.

treated with indinavir showed an apparent accumulation of cholesterol in the intracellular membrane, but not lysosomal vesicles (Fig. 10).

4.7. Effects of indinavir on SREBP-1 and SREBP-2 and CYP7A1 in mice

Western blot analysis was performed to determine the level of premature and mature (activated forms) of SREBPs in the membrane and nucleus of mouse liver after indinavir treatment. Results shown that indinavir had no influence on the level of the premature form of SREBPs membranes (Fig. 11). However, as compared to controls, increased levels of both mature activated SREBP-1 ($168 \pm 22\%$) and SREBP-2 ($207 \pm 11\%$) were observed in hepatic nuclear extracts from the mice treated with indinavir for 12 hr (Fig. 11). Moreover, CYP7A1 mRNA also decreased (56%) in these same animals (Fig. 12).

5. Discussion

The liver is the main organ responsible for maintaining cholesterol homeostasis within the body. Serum levels of cholesterol are controlled by tightly regulated cholesterol input and output pathways. Cholesterol can be derived from either newly synthesized or dietary cholesterol and is secreted from the body as either biliary cholesterol or following degradation to bile acids (Fig. 13). HMG-CoA reductase is the rate limiting and most highly regulated enzyme in the cholesterol biosynthetic pathway [24]. Regulation of the HMG-CoA reductase gene is linked to

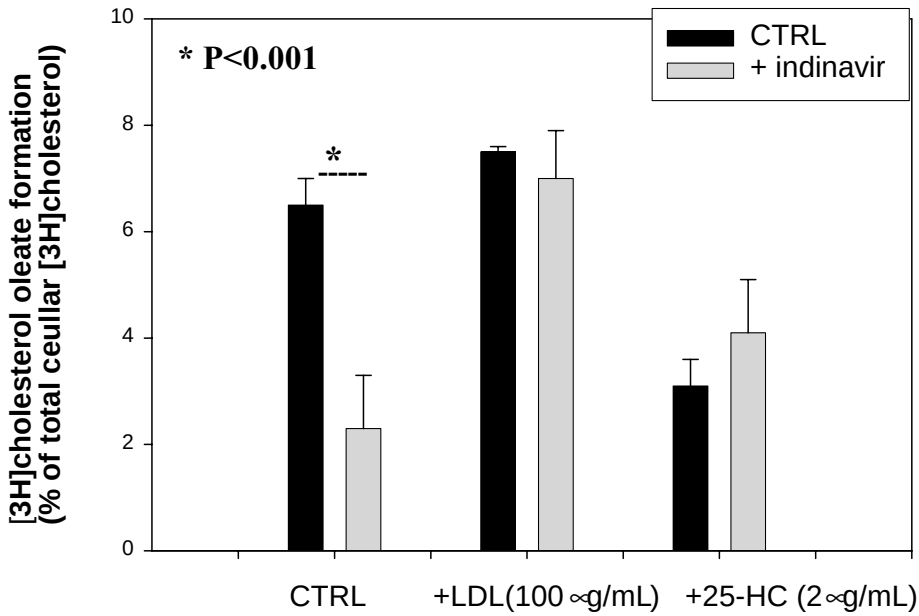

Fig. 9. Effects of indinavir on low density lipoprotein (LDL) and 25-hydroxycholesterol (25-HC) esterification. Primary rat hepatocytes were isolated and grown as described under "Section 2". LDL (100 μg/mL) or 25-HC (2 μg/mL) was added to culture media and incubated for 24 hr [^{3}H]cholesteryl oleate and [^{3}H]cholesterol were analyzed by thin layer chromatography. Data points represent the [^{3}H]cholesteryl oleate formed as a percentage of [^{3}H]cholesterol plus [^{3}H]cholesteryl oleate and are the average ± SEM of duplicate samples from three separated tests. CTRL: control. $^*P < 0.001$ compared with control.

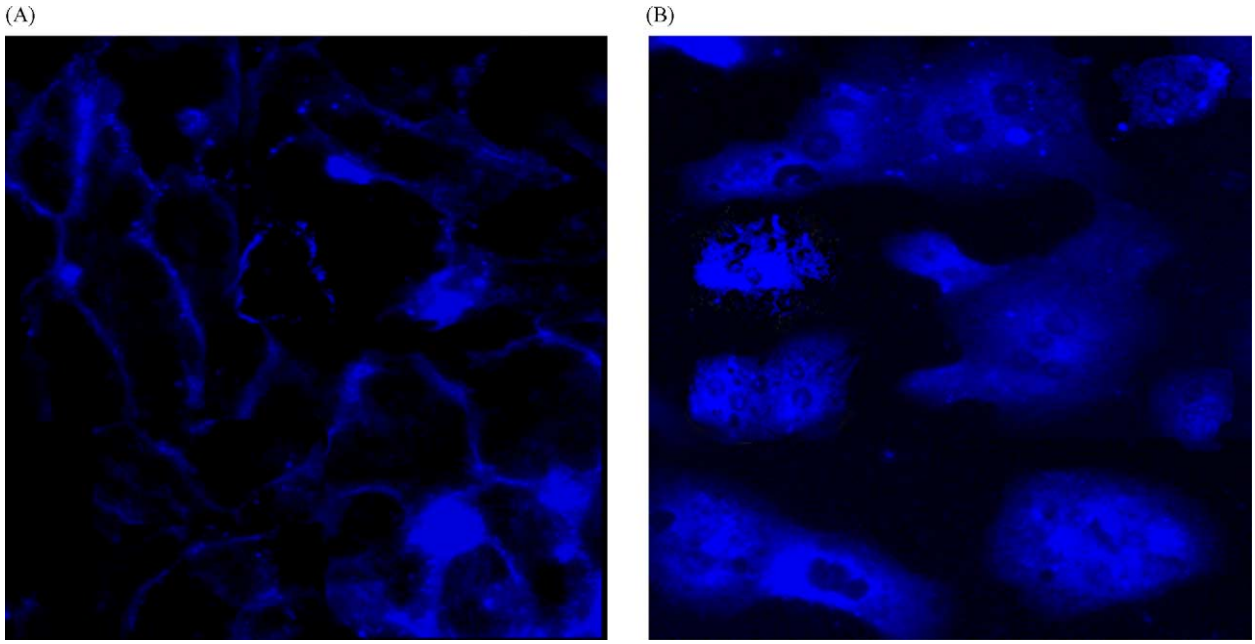

Fig. 10. Localization of cholesterol in primary rat hepatocytes with and without indinavir treatment. Primary rat hepatocytes were isolated and grown as described under ''Section 2''. Control cells (panel A) or indinavir treated (panel B) are shown. Cells were stained with filipin for cholesterol as described under ''Section 2''.

cellular cholesterol (oxysterols) levels. When cellular cholesterol levels are low, mammalian cells respond by increasing the rate of proteolytic processing of SREBPs to mature active transcription factors (reviewed in Ref. [22]). When cellular cholesterol levels are high the processing of SREBPs is decreased resulting in a repression of the gene encoding HMG-CoA reductase and other genes encoding enzymes in the cholesterol biosynthetic pathway. There are three isoforms of SREBPs and they have been reported to regulate a number of genes involved in cholesterol and fatty acid biosynthesis and transport [22]. SREBP-1 primarily regulates genes involved in fatty acid biosynthesis; whereas, SREBP-2 regulates genes involved in cholesterol biosynthesis and metabolism [22]. Transgenic animals overexpressing SREBP-1 or SREBP-2 show significant increases in the mRNA levels of genes involved in fatty acid and cholesterol biosynthesis, respectively [25].

In the current study, indinavir was shown to significantly increase the nuclear protein levels (mature form) of SREBP-1 (Fig. 6). Similar observations have been reported for ritonavir in mice receiving this drug [10]. Moreover, there was no significant change in the levels of SREBP-1 mRNA in hepatocytes treated with indinavir (data not shown). Therefore, indinavir most likely increases the

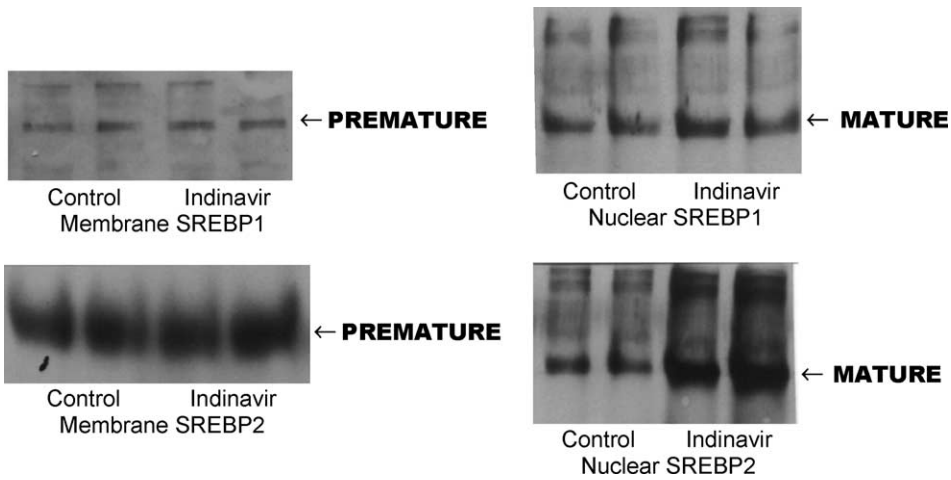

Fig. 11. Hepatic levels of SREBP-1 and SREBP-2 in mice treated with indinavir. Two mice in each group were treated with indinavir (3 mg) by gavages for 12 hr. Nuclear and membrane proteins were isolated and the level of mature and immature SREBP-1 and SREBP-2 were determined by Western blotting in membranes and isolated nuclei as described in Section 2. The experiment was independently repeated twice in two separate groups of mice with similar results.

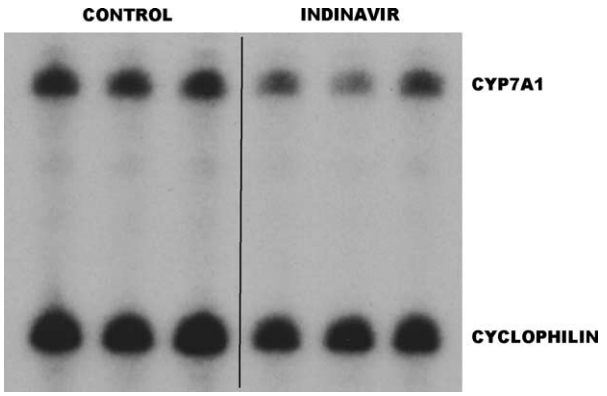

Fig. 12. Effects of indinavir on mouse hepatic cholesterol 7α-hydroxylase (CYP7A1) mRNA Levels. Mice were treated 12 hr with either indinavir (3 mg) or vehicle, hepatic RNA isolated, and levels of CYP7A1 mRNA determined by RPA. There was approximately a $56 \pm 13\%$ ($P < 0.01$) decrease in CYP7A1 mRNA levels in indinavir-treated animals (N = 5).

accumulation of SREBP-1 by decreasing the rate of turnover of the mature form of SREBP-1. In this regard, SREBP turnover has been reported to be mediated by an ALLN-sensitive proteasome-dependent mechanism and some HIV PIs have been shown to inhibit proteasome activities [26]. However, it was surprising that the addition of exogenous free cholesterol, dissolved in β-cyclodextrin, significantly abated the effects of indinavir on mRNA levels of genes involved in cholesterol and bile acid biosynthesis (Fig. 7). Moreover, the addition of cyclodextrin alone to hepatocytes cultured in 10% FCS plus indinavir also markedly decreased the amount of the mature form of SREBP-1 (Fig. 8). These results suggest that indinavir might inhibit the intracellular movement of cholesterol to specific regulatory sites within the cell. The inhibition of endogenous, but not exogenous, cholesterol esterification by indinavir suggests it may be blocking the transport of cholesterol from intracellular membranes to the plasma membrane (Figs. 8–10). Additional studies will be necessary to test this hypothesis. It should be noted that the concentrations of indinavir used in these studies are well above (5- to 10-fold) those that occur in plasma of patients taking indinavir [27]. However, even small changes in cholesterol input and output pathways, with time, may alter serum cholesterol and triglyceride levels.

CYP7A1 and CYP27 are the initial enzymes in the "neutral" and "acidic" pathways of bile acid biosynthesis, respectively [27]. The gene encoding CYP7A1 is down-regulated by bile acid and is up-regulated by cholesterol (oxysterols) at the transcriptional level in rodents [23,27]. CYP7A1 is important in controlling the rate of cholesterol output from the body under normal physiological conditions [28]. In the current investigation, the level of CYP7A1 mRNA was significantly reduced by treatment of primary hepatocytes with indinavir (Fig. 5). Surprisingly, the mechanism responsible for the decrease in CYP7A1 mRNA levels appears to be a marked decrease in the mRNA half-life (Fig. 5). However, it is unclear how indinavir might alter the half-life of CYP7A1 mRNA.

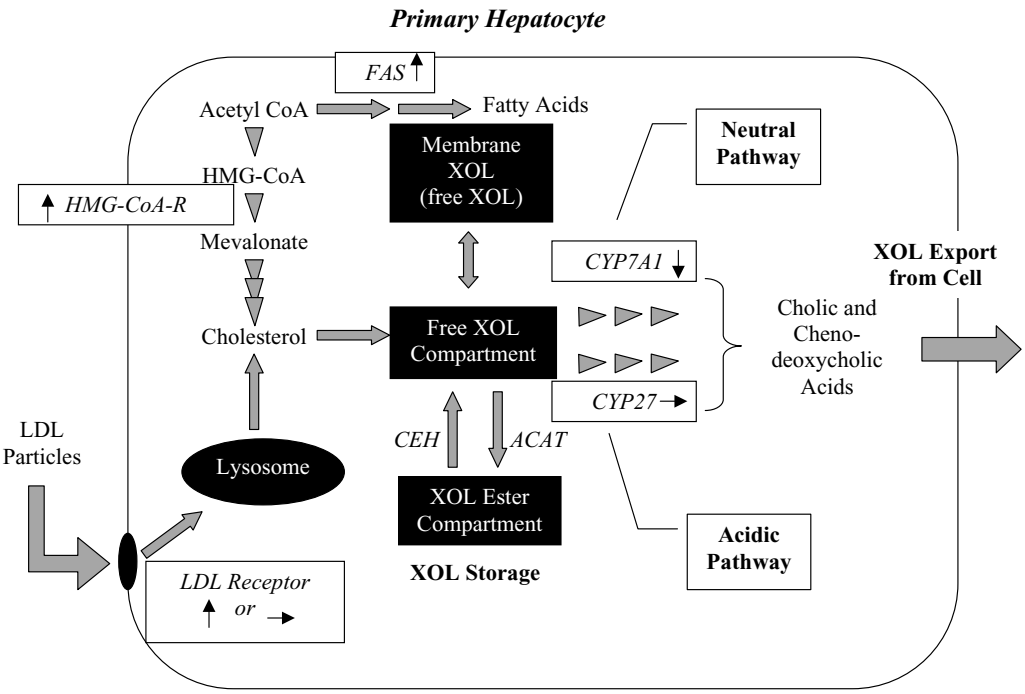

Fig. 13. Alterations of cholesterol, bile acid, and fatty acid metabolism in hepatocytes treated with indinavir. HMG-CoA-R, 3-hydroxy-3-methylglutaryl-Coenzyme A reductase; LDL, low density lipoproteins; FAS, fatty acid synthase; XOL, free cholesterol; CYP7A1, cholesterol 7α-hydroxylase; CYP27, sterol 27-hydroxylase; CEH, neutral cholesterol-ester hydrolase; ACAT, acyl-CoA-cholesterol acyltransferase.

In summary, we hypothesize that the dysregulation of lipid metabolism, at the cellular level, may be the result of indinavir-induced alteration in the movement of intracellular cholesterol to key regulatory pools, and may effect the turnover rates of mature SREBPs.

Acknowledgments

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