Overexpression of CYP27 in hepatic and extrahepatic cells: role in the regulation of cholesterol homeostasis

E. Hall, P. Hylemon, Z. Vlahcevic, D. Mallonee, K. Valerie, N. Avadhani, and W. Pandak. Overexpression of CYP27 in hepatic and extrahepatic cells: role in the regulation of cholesterol homeostasis. Am J Physiol Gastrointest Liver Physiol 281: G293–G301, 2001.—In the liver, sterol 27-hydroxylase (CYP27) participates in the classic and alternative pathways of bile acid biosynthesis from cholesterol (Chol). In extrahepatic tissues, CYP27 converts intracellular Chol to 27-hydroxycholesterol (27OH-Chol), which may regulate the activity of 3-hydroxy-3-methylglutaryl CoA reductase (HMG-CoA-R). This study attempts to better define the role of CYP27 in the maintenance of Chol homeostasis in hepatic and extrahepatic cells by overexpressing CYP27 in Hep G2 cells and Chinese hamster ovary (CHO) cells through infection with a replication-defective recombinant adenovirus encoding for CMV-CYP27. After infection, CYP27 mRNA and protein levels increased dramatically. CYP27 specific activity also increased two- to fourfold in infected cells (P ≤ 0.02), with a marked increase in conversion of [14C]Chol to [14C]27OH-Chol (≈150%; P ≤ 0.01). Accumulation of 27OH-Chol in CHO cells was associated with a 50% decrease in HMG-CoA-R specific activity (P ≤ 0.02). In infected Hep G2 cells, the significant increase in bile acid synthesis (46%; P ≤ 0.006), which prevented the accumulation of intracellular 27OH-Chol, resulted in increased HMG-CoA-R activity (183%; P ≤ 0.02). Overexpression of CYP27 in Hep G2 cells also increased acyl CoA-cholesterol acyltransferase (71%, P ≤ 0.02) and decreased cholesteryl ester hydrolase (55%, P ≤ 0.02). In conclusion, CYP27 generates different physiological responses depending on cell type and presence or absence of bile acid biosynthetic pathways.

Hepatic cholesterol homeostasis is maintained largely through the coordinate regulation of cholesterol uptake, esterification, biosynthesis, secretion, and degradation to bile acids. In humans, conversion of cholesterol to bile acids accounts for approximately half of daily net cholesterol elimination from the body, and most of the remaining 50% is eliminated in the form of biliary cholesterol secretion.

In the liver, the 27-hydroxylation of cholesterol by mitochondrial sterol 27-hydroxylase (CYP27) is the initial step in an alternative or acidic pathway of bile acid biosynthesis (Fig. 1). Once formed, 27-hydroxycholesterol (27OH-Chol) can compete with cholesterol for binding and further oxidation. Under nonsaturating substrate concentrations, 27OH-Chol can be further oxidized by mitochondrial CYP27 to form 3β-hydroxy-5-cholestenol and finally 3β-hydroxy-5-cholestanolic acid (22). Both 27OH-Chol and 3β-hydroxy-5-cholestanolic acid are substrates for the newly discovered microsomal oxysterol 7α-hydroxylase (CYP7B1). Recent in vivo studies in the rat (37) and in primary rat hepatocytes (23, 28) have suggested that this pathway may be responsible for generating up to 50% of bile acid biosynthesis (Fig. 1).

CYP27 is also responsible for the 27-hydroxylation of bile acid intermediates (5β-cholestane-3α, 7α-diol, and 5β-cholestane-3α, 7α, 12α-triol) in the neutral pathway of bile acid biosynthesis, a pathway initiated by cholesterol 7α-hydroxylase (CYP7A1) (Fig. 1) (7). CYP27 has been found in peripheral tissues (i.e., extrahepatic tissues), where it has been shown to convert cholesterol to 27OH-Chol and 3β-hydroxy-5-cholestanolic acid (3, 4, 5, 17). These metabolites efflux from the peripheral cells and are incorporated into the lipoprotein fractions, which are then transported to the liver where they are presumably metabolized via the alternative pathway into bile acids. This mechanism of cholesterol elimination from peripheral cells may represent a second reverse cholesterol transport pathway to the liver (3, 4, 5, 17).

The addition of 27OH-Chol, the product of this reaction, to various cell lines has resulted in repression of 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA-R), the rate-determining enzyme in cholesterol biosynthesis.
biosynthesis (2, 12). On the basis of these observations, it has been hypothesized that circulating 27OH-Chol may be a potent negative feedback regulator of HMG-CoA-R in peripheral and possibly liver cells (2, 5): 27OH-Chol has also been shown to stimulate acyl-CoA-cholesterol acyltransferase (ACAT) activity, thereby increasing the esterification of itself, cholesterol, and other potential regulatory oxysterols.

The mechanism of regulation of CYP27 is just beginning to be elucidated. Studies in the rat (both in vitro and in vivo) have shown that CYP27 is downregulated at the transcriptional level by hydrophobic bile acids (28, 34, 35). In contrast, in the rabbit, CYP27 mRNA is neither repressed by bile acids nor stimulated by cholestyramine. Cholesterol stimulates rabbit CYP27, whereas bile drainage has no effect on mRNA levels (1). Furthermore, CYP27 in the rat has been shown to undergo diurnal variation, is upregulated by glucocorticoids, and is downregulated by insulin (28, 33, 35).

A genetic defect in CYP27 in humans leads to the development of cerebrotendinous xanthomatosis (CTX), a condition characterized by progressive neurological dysfunction, xanthomatosis, and accelerated atherosclerosis (25). As a result of point mutations in the CYP27 gene, patients with CTX exhibit a marked decrease in bile acid synthesis and reduced bile acid pool size (in particular chenodeoxycholic acid) (16). In mice with targeted disruption of CYP27 (CYP27−/−), there is reduced metabolism of cholesterol to bile acids, with increased cholesterol synthesis (24). However, CYP27−/− mice demonstrated no accumulation of cholesterol or bile alcohols. CYP27−/− mice showed no CTX-related pathological abnormalities, suggesting that some of the consequences of CYP27 deficiency are specific for the species being studied. The presence of a 25-hydroxylation pathway in the mouse, as shown by Honda et al. (13), offers an explanation for the lack of a specific mouse phenotype. However, an increased death rate was observed when CYP27−/− mice were fed an atherogenic diet, suggesting that CYP27 may be an antiatherogenic agent (24).

In the current study, we used a replication-defective recombinant adenovirus encoding cytomegalovirus (CMV)-CYP27 to overexpress CYP27 in hepatic and Chinese hamster ovary (CHO) cells and attempted to define the role of this enzyme in the maintenance of cellular cholesterol homeostasis in CHO cells (representative of extrahepatic cells), Hep G2 cells, and primary human hepatocytes.

**MATERIALS AND METHODS**

**Materials.** Hep G2 and CHO cells were obtained from the American Type Culture Collection (Rockville, MD). All cell culture materials were obtained from GIBCO BRL (Grand Island, NY) unless otherwise specified. The CsCl, agarose, and RNA ladder used to size CYP27 mRNA were also purchased from GIBCO BRL. FBS was obtained from Bio Whittaker (Walkersville, MD). Tissue culture flasks were purchased from Costar (Cambridge, MA). The chemicals used in this research were obtained from Sigma Chemical (St. Louis, MO) or Bio-Rad (Hercules, CA) unless otherwise specified. The secondary antibody (horseradish peroxidase-conjugated goat anti-mouse IgG) was also purchased from Bio-Rad. All solvents were obtained from Fisher (Fair Lawn, NJ) unless otherwise indicated. All radionucleotides, Aquasol solution, and enhanced chemiluminescence reagents were purchased from Dupont NEN (Boston, MA). Waters Silica Sep-Paks were obtained from Waters (Milford, MA). The β-cyclodextrin was purchased from Cyclodextrin Technologies Development (Gainesville, FL). The testosterone and 27OH-Chol were obtained from Research Plus (Bayonne, NJ). LK6 20 × 20-cm thin layer chromatography (TLC) plates were purchased from Whatman (Clifton, NJ). Mevalonate was obtained from Aldrich Chemical (Milwaukee, WI). Nylon membranes were purchased from Micron Separation (Westborough, MA). Ammonium persulfate was obtained from Amresco (Solon, OH). Finally, nonfat dry milk was purchased from Carnation (Glendale, CA).
Generation of recombinant adenovirus encoding rat CYP27. The recombinant adenovirus AdCMV-CYP27 was generated by homologous recombination in HEK-293 cells. A rat cDNA encoding CYP27 (1.9 kb) was obtained from N. G. Avadhani (31) and cloned into plasmid (AdCMV, 7.7 kb) constructed from pGEM 4z (Promega) and pcDNA3 (Invitrogen). The CYP27 gene was cloned into the AdCMV vector at the EcoRI I restriction site. Transfection into HEK-293 cells was used to confirm expression by the recombinant adenovirus.

Propagation and purification of AdCMV-CYP27. Large-scale production of recombinant virus was performed by infecting confluent monolayers of HEK-293 cells grown in 15-cm tissue culture dishes with stock adenovirus at a multiplicity of 1 plaque-forming unit (pfu)/cell. After 2 h of infection, the virus was removed and replaced with DMEM with 2% FBS. The infected monolayers were harvested by scraping when >90% of the cells showed cytotoxic changes and were centrifuged at 2,700 g and 4°C for 10 min. The infected cellular pellet was suspended in DMEM-2% FBS and subjected to 5 cycles of freeze/thaw lysis to release the virus. Cell debris was removed by centrifugation at 7,700 g and 4°C for 5 min. To purify, the crude viral supernatant was carefully layered over a two-step gradient containing 3 ml of CsCl (d = 1.33 g/ml) in TD buffer, and 1.4 g/ml in TD buffer, and 0.14 M NaCl, 5 mM KCl, 190 mM Tris, pH 7.4, and 0.7 mM Na2HPO4, pH 7.4, and 0.7 mM Na2HPO4 layer over 3 ml of CsCl (d = 1.25 g/ml in Tris buffer, and centrifuged at 155,000 g and 20°C for 1 h. The viral band was removed, layered over 8 ml of CsCl (d = 1.33 g/ml) in TD buffer, and centrifuged at 155,000 g and 20°C for 18 h. The pure viral opalescent band was removed and dialyzed against 10 mM Tris, pH 7.4, 1 mM MgCl2, and 10% glycerol overnight at 4°C. The virus was aliquoted and stored at –70°C. The virus titer (pfu) was determined by plaque assay, and viral particles were determined by optical density using spectrophotometry (λ = 260 nm).

Cell medium. Hep G2 cells were grown in MEM containing nonessential amino acids, 0.03 M NaHCO3, 10% FBS, 1 mM L-glutamine, 1 mM sodium pyruvate, and 1% Pen/Strep and incubated at 37°C in 5% CO2 atmosphere at 37°C. Unless otherwise indicated, culture medium was maintained containing 0.1 mM dexamethasone and 10 μM 1-thyroxine.

Infection of cells with AdCMV-CYP27. Hep G2 cells and CHO cells were grown in 162 cm2 (25 ml) tissue culture flasks until they were 80–90% confluent. In control flask, (i.e., no adenovirus), the old medium was removed and replaced with 25 ml of fresh medium. In flask to be infected, the old medium was removed and replaced with 12 ml of fresh medium. Hep G2 and CHO cells were infected with AdCMV-CYP27 or the control adenovirus without the CYP27 gene (AdCMV + ) at a multiplicity of infection of 1 to 10. After 3 h of infection, an additional 13 ml of fresh medium was added to the flask and cells were allowed to incubate at 37°C in 5% CO2 for 48 h. Cells (both control and infected) were harvested for the isolation of mitochondria, microsomes, cytosol, and RNA.

Primary human hepatocytes were plated in 152 cm2 plates until they were 80–90% confluent. Twenty-four hours after plating, culture medium was removed and 2.5 ml of fresh medium was added. Cells were then infected with AdCMV-CYP27 at a multiplicity of infection of 10. After 2 h of infection (with gentle shaking every 15 min), the media were discarded and 2 ml of fresh medium was added. The cells were then allowed to incubate at 37°C in 5% CO2 for 48 h before harvesting.

Mitochondrial and microsomal preparation. Mitochondria and microsomes were isolated from cell culture as described previously (28, 35).

Purification of cholesterol. Cholesterol was purified by the method of Winegar et al. (39). Cholesterol, dissolved in hexane (0.6 mg/ml), was loaded dropwise onto a dry Waters Silica Sep-Pak. Cholesterol was eluted with 8 ml 2% isopropanol in hexane, and the eluant was dried under a nitrogen gas atmosphere. For the CYP27 specific activity assay, 375 μl of CYP27 or the control adenovirus without the CYP27 gene was added to the dry extract and vortexed vigorously until the cholesterol was completely dissolved. The determination of bile acid and 27OH-Chol synthesis, 10 μCi of [14C]cholesterol (250 μl) was dried down under nitrogen gas and dissolved in 2 ml hexane. The radiolabeled cholesterol was purified by use of a Waters Silica Sep-Pak as described above. The dry extract was taken up in 250 μl ethanol and added to 50 ml of complete Hep G2 or CHO culture media. Purity was determined by running a small aliquot of the purified [14C]cholesterol (0.05 μCi) on TLC using the buffer system mentioned below for determination of 27OH-Chol synthesis.

CYP27 activity assay. CYP27 specific activity was assayed by the method of Petrak et al. (21), with a few modifications. The assay was performed on 300 μg of freshly prepared mitochondrial protein, to prevent loss of activity due to freeze/thaw. Purified exogenous cholesterol (~80 nmol) in β-cyclodextrin was added to each reaction tube to increase the concentration of substrate available to the enzyme. Testosterone, at a concentration of 0.003 μg/μl, was added to each reaction tube as an internal standard. Control reactions were produced in which 0.003 μg/μl 27OH-Chol was substituted for mitochondrial protein. Samples were suspended in 100 μl of 5% isopropanol in hexane before HPLC analysis. An aliquot of 50 μl was analyzed via normal-phase HPLC on a Beckman ultrasphere silica column (4.6 × 250 mm) by using a mobile phase of hexane-isopropanol-glacial acetic acid (96.5:2.5:1 vol/vol/vol). All assays were carried out in duplicate including the appropriate controls. All 27OH-Chol concentrations were corrected for the amount of testosterone recovered, and CYP27 activity was calculated as nanomoles of product produced per milligram of sample per minute. Finally, CYP27 activities were expressed as percentage of control values.

ACAT specific activity assay. ACAT activity was determined by the method of Burrage et al. (6) with the following modifications. ACAT reactions were carried out using 40 μg microsomal protein in a buffer containing 0.1 M potassium phosphate, 9.8 mM β-mercaptoethanol, and 0.01 μM BSA in a total volume of 50 μl. Purified exogenous cholesterol was added to incubations from cyclohexide stock solutions in 5-μl aliquots (1.2 mg purified cholesterol/0.375 ml β-cyclodextrin). After a 15-min preincubation, [1-14C]oleoyl-CoA (NEN; 55 μCi/μmol) was added to a final concentration of 7.2 pM. Thirty minutes after addition of [1-14C]oleoyl-CoA, reactions were terminated by direct application of a 20-μl aliquot of each sample to silica gel TLC plates (20 × 20 cm; Whatman LK6). Oleoyl-CoA and cholesterol oleate were plated as standards. The chromatograms were developed in hexane-...
diethylether-glacial acetic acid (85:15:1 vol/vol/vol). [14C]Cholesterol olate formation was detected by using a Phosphorimager (Molecular Dynamics), and the substrate and cholesterol olate bands were scraped into scintillation vials containing biodegradable counting scintillant (Amersham, Arlington Heights, IL) and counted in a Beckman liquid scintillation counter. Cholesterol olate concentration was calculated as a percentage of oleyl-CoA substrate to correct for possible loading errors, and values for samples were expressed as a percentage of control values.

Cholesterol ester hydrolase activity assay. Four hundred micrograms of cytosolic protein, obtained when isolating microsomes for the ACAT assay, were used in the cholesterol ester hydrolase (CEH) assay. CEH activity was measured by the method of Ghosh and Grogan (10).

HMG-CoA-R activity assay. Microsomal HMG-CoA-R specific activity was assayed essentially by the method of Shefer et al. (26).

Quantitation of CYP27 mRNA levels. Methods for the isolation of RNA and the determination of mRNA levels by Northern blot have been previously described by Pandak et al. (20). The cDNA probe used in these experiments has also been described previously (31).

Bile acid and 27OH-Chol synthesis. Labeled bile acids and 27OH-Chol were extracted from both media and cells by using the method of Folch et al. (9). Bile acid biosynthesis was measured as conversion of [14C]cholesterol to 14C-labeled bile acids (14). [14C]27OH-Chol was isolated from the cell extract or media CHCl3 phase on TLC (hexane-isopropanol-glacial acetic acid; 95:3:2 vol/vol/vol). [14C]27OH-Chol (Rf 0.19) and [14C]cholesterol (Rf 0.36) bands were scraped from the TLC plates, and radioactive counts were obtained using a Beckman liquid scintillation counter. 27OH-Chol concentration was calculated as a percentage of the cholesterol substrate to correct for possible loading errors. Both bile acid biosynthesis rates and 27OH-Chol concentrations for infected samples were expressed as a percentage of control values.

Immunoblot analysis of CYP27 protein levels. Mitochondrial and cytosolic proteins (30 µg) were separated on a 10% SDS-polyacrylamide denaturing gel according to the method of Laemmli (15). Following electrophoresis, proteins were electrophoretically transferred to Immobilon-P membranes by using a Millipore rapid transfer system. The membranes were blocked overnight (4°C) in blocking buffer (PBS, pH 7.4, 0.1% Tween, and 5% nonfat dry milk). They were then incubated with for 2 h (25°C) with a mouse CYP27 monoclonal IgG (1:1,000) as previously described (31). After membrane washing, a secondary antibody (horseradish peroxidase-conjugated goat anti-mouse IgG; 1:3,000) was added to the blocking solution (25°C, 2 h). Protein bands were detected using the Dupont Renaissance enhanced chemiluminescence kit.

Statistics. Results are reported as means ± SE where possible. Statistical significance was determined where appropriate by Student's t-test.

RESULTS

Figure 2 is a representative Northern blot showing CYP27 mRNA levels in Hep G2 and CHO cells before and after infection with AdCMV-CYP27. As shown, messenger RNA was isolated from Hep G2 and CHO cells harvested at several time points (24, 48, and 72 h) after infection. CYP27 mRNA levels increased with time in both cell lines, from undetectable levels in control cells to easily detectable levels in infected cells. To standardize RNA loading as well as quantitate relative CYP27 mRNA levels, cyclophilin levels were determined as an internal loading standard. The cyclophilin levels shown in this figure were similar in each lane, showing that similar amounts of RNA were loaded into each well.

As a second set of controls, AdCMV-1 was added to cell cultures. No visible changes in CYP27 mRNA levels were observed compared with no-addition controls (Fig. 3). Blots in which control and infected samples were separated by several blank lanes required additional exposure to detect CYP27 mRNA levels in control Hep G2 cells. By using densitometry, it was then estimated that CYP27 mRNA levels increased at least 1,300-fold (P ≤ 0.002; n = 6) following AdCMV-CYP27 infection (data not shown).

CYP27 protein levels in mitochondria were determined by immunoblot analysis by using a mouse CYP27 monoclonal antibody. As seen with CYP27 mRNA levels, mitochondrial CYP27 protein levels increased from undetectable levels in control cells under the conditions employed to easily detectable levels in
infected Hep G2 and CHO cells (Fig. 4). Using Hep G2 cells as an example, we have shown an increase in CYP27 protein levels with time following AdCMV-CYP27 infection (Fig. 4). No CYP27 protein was found in the cytosol, indicating that the protein was localized to the mitochondria, where it is known to be active (data not shown).

The increases in CYP27 mRNA and protein levels observed in cells infected with AdCMV-CYP27 were associated with a significant increase in mitochondrial CYP27 specific activity. Illustrated in Fig. 5 are the effects of adenoviral infection on CYP27 specific activity. Forty-eight hours after infection with AdCMV-CYP27, CYP27 specific activity increased significantly ($P < 0.02$) in both Hep G2 and CHO cells (Hep G2, from 0.018 to 0.050 nmol·mg$^{-1}$·min$^{-1}$; CHO, undetectable to 0.033 nmol·mg$^{-1}$·min$^{-1}$). No increase in CYP27 specific activity was observed in cells infected for 48 h with a control virus (Fig. 5).

Infected CHO cells showed a marked increase in conversion of $^{[14]C}$cholesterol to $^{14}$C-labeled 27OH-Chol, as evidenced by an increase in intracellular and extracellular 27OH-Chol levels ($\sim$150%; $P \leq 0.01$; Fig. 6 and Table 1). In contrast, accumulation of 27OH-Chol in Hep G2 cells was only 16% greater after infection with the CYP27 adenovirus (Fig. 6).

The effect of CYP27 overexpression on the rates of bile acid synthesis in both Hep G2 and primary human hepatocytes is shown in Fig. 7. CYP27 overexpression in Hep G2 cells led to a significant increase in bile acid biosynthesis (46%; $P \leq 0.006$; $n = 4$) (Fig. 7). In one experiment in primary human hepatocytes, the increase in bile acid synthesis was 61% following CYP27 overexpression.

In CHO cells, endogenous accumulation of 27OH-Chol (0.23 ng/1 x $10^6$ cells), due to infection with AdCMV-CYP27, was coupled with a 50% decrease ($P \leq 0.02$) in HMG-CoA-R activity (Fig. 8). HMG-CoA-R specific activity in control CHO cells was 5.2 ± 0.47 nmol·mg$^{-1}$·h$^{-1}$ ($n = 3$). The exogenous addition of 27OH-Chol to CHO cells in concentrations up to 100 times the cellular levels found following infection with the AdCMV-CYP27 had no effect on HMG-CoA-R specific activity. In Hep G2 cells, HMG-CoA-R specific activity increased dramatically (183%; $P \leq 0.02$) following infection with AdCMV-CYP27 (Fig. 8). HMG-CoA-R specific activity in control Hep G2 cells was 2.13 ± 0.96 nmol·mg$^{-1}$·h$^{-1}$ ($n = 3$). No change in HMG-CoA-R specific activity was observed when Hep G2 and CHO cells were infected with control virus (data not shown).

The effects of CYP27 overexpression on other enzymes involved in the regulation of cellular cholesterol homeostasis are shown in Fig. 8. ACAT specific activity increased (71%; $P \leq 0.02$), whereas CEH specific activity decreased (55%; $P \leq 0.02$) when Hep G2 cells were
infected with the AdCMV-CYP27. Overexpression of CYP27 in CHO cells had no significant effect on ACAT or CEH specific activity. Cells infected with a control virus showed no change in either ACAT or CEH specific activity (data not shown). Paired control specific activities in Hep G2 cells for ACAT and CEH were $7.8 \pm 0.85$ and $0.38 \pm 0.03$ nmol/mg/h, respectively ($n = 3$). Paired control specific activities in CHO cells for ACAT and CEH were $9.4 \pm 1.4$ and $1.33 \pm 0.15$ nmol/mg/h, respectively ($n = 3$).

**DISCUSSION**

The overexpression of CYP27 in both CHO and Hep G2 cells led to different alterations in cellular cholesterol metabolism in the two types of cells studied. The observed changes appeared to be a result of increased conversion of cholesterol to 27OH-Chol, which was coupled with an increase in CYP27 specific activities. The cell-specific responses to increased CYP27 specific activities seemed to depend on whether or not the cell in question had a bile acid biosynthesis pathway capable of metabolizing 27OH-Chol. The most important finding of the present study was the observation that overexpression of CYP27 in Hep G2 cells resulted in a significant increase in CYP27 steady-state mRNA levels, protein specific activity, and bile acid synthesis. These results demonstrate that the alternative pathway is fully capable of increasing the rates of bile acid synthesis and hence increasing the rates of cholesterol elimination.

A large discrepancy in the magnitude of increase in CYP27 mRNA and protein levels versus that in CYP27 specific activities was observed in cells infected with AdCMV-CYP27. Even though the increases in CYP27 specific activities were not large, they were consistent following infection and coupled with significant increases in bile acid synthesis. The reason for the disparity in specific activity levels and in protein and mRNA levels is being explored. One possible explanation is the insufficient delivery of the substrate, cholesterol, to the enzyme. Another is lack of sufficient ferridoxin/ferridoxin reductase availability in the mitochondria.

CYP7B1 catalyzes the second step in the alternative pathway by adding a hydroxyl group to the C-7 position. It has been suggested that, as in the neutral pathway of bile acid synthesis, this 7α-hydroxylation step could represent the rate-determining step in this

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**Table 1. 27OH-Chol levels in CHO cell extract and media following infection with AdCMV-CYP27**

<table>
<thead>
<tr>
<th></th>
<th>Control, ng/1×10^6 cells</th>
<th>Infected, ng/1×10^6 cells</th>
<th>% Increase</th>
</tr>
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<tr>
<td>Cell extract</td>
<td>0.15 ± 0.03</td>
<td>0.38 ± 0.04</td>
<td>153</td>
</tr>
<tr>
<td>Media</td>
<td>1.08 ± 0.13</td>
<td>2.71 ± 0.24</td>
<td>151</td>
</tr>
<tr>
<td>Ratio of extract to media</td>
<td>0.14</td>
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Values are means ± SE. Chinese hamster ovary (CHO) cells were harvested 48 h ($n = 3$) after infection. 27-hydroxycholesterol (27OH-Chol) synthesis was determined as conversion of [14C]cholesterol to [14C]-labeled 27OH-Chol. Levels of 27OH-Chol are shown before and after infection with the CYP27 adenovirus (AdCMV-CYP27) in both the cell extract ($P < 0.003$) and the media ($P < 0.007$) phases.
pathway. In preliminary experiments, overexpression of CYP7B1 increased CYP7B1 mRNA and specific activities but failed to increase bile acid synthesis. Furthermore, coinfection of Hep G2 cells with AdCMV-CYP27 and AdCMV-CYP7B1 did not increase bile acid synthesis over CYP27 overexpression alone. These findings suggest that CYP27, not CYP7B1, is more likely to be the rate-determining enzyme in the alternative pathway of bile acid synthesis.

Reasons for the diverse tissue localization of CYP27 have been previously discussed (5, 8). It has been suggested that CYP27 is important in the elimination of cholesterol from extrahepatic (i.e., peripheral cell) tissues through its conversion to the more polar 27OH-Chol and 3β-hydroxy-5-cholestenoic acid (5, 8, 38). Effluxed 27OH-Chol may exert different regulatory effects from exogenous addition of 27OH-Chol. Numerous oxysterols, most notably 27OH-Chol, have been shown in vitro experiments to be potent repressors of HMG-CoA-R and cholesterol biosynthesis (2). On the basis of these findings, it has been hypothesized that circulating 27OH-Chol may be a potent negative feedback regulator of HMG-CoA-R activity both in liver and in the peripheral cells (19). Incubation of cells with control virus did not alter any of the cholesterol homeostatic parameters measured. We believe that the adenovirally mediated increase in CYP27 expression represents a more physiological method of studying the role of 27OH-Chol as a regulator of HMG-CoA-R activity than the addition of 27OH-Chol to the culture medium. It is feasible that intracellularly generated 27OH-Chol may exert different regulatory effects from exogenous addition of 27OH-Chol.

Although in our experiments efflux of 27OH-Chol into the medium increased with adenoviral infection, intracellular 27OH-Chol accumulated in CHO cells (0.23 ng/10^6 cells). The intracellular accumulation of 27OH-Chol following overexpression of CYP27 was coupled with decreased cholesterol biosynthesis (~50% HMG-CoA-R activity). Our results suggest a mechanism by which CYP27 may act as an antiatherogenic enzyme by simultaneously mediating the elimination of exogenous cholesterol from peripheral tissues and by inhibition of peripheral cholesterol synthesis.

Recently, it has been shown that 7α-hydroxylation of 27OH-Chol by CYP7B1 eliminates the ability of 27OH-Chol to repress HMG-CoA-R activity (4). Therefore, CYP27 metabolism of cholesterol to 27OH-Chol and 3β-hydroxy-5-cholestenolic acid may represent a second pathway of reverse cholesterol transport.

In the present study, CHO cells (i.e., no bile acid biosynthesis pathway) in which CYP27 had been overexpressed for 48 h were characterized by increased CYP27 activity, as manifested by increased conversion of [14C]cholesterol to 14C-labeled 27OH-Chol and increased efflux of labeled 27OH-Chol into the culture medium (Figs. 5, 6, and 8 and Table 1). Despite the increased formation of 27OH-Chol, the ratio of intracellular 27OH-Chol to medium 27OH-Chol remained the same as in control cells. Therefore, efflux of 27OH-Chol from the cell is not arbitrary but appears to be under tight regulatory control. Westman et al. (38) also found that the cellular efflux of 27OH-Chol was proportional to total sterol cellular content in human monocyte-derived macrophages.
Chol to downregulate cholesterol biosynthesis (19). This finding suggests that 27OH-Chol would have little or no effect on cholesterol biosynthesis in liver cells, where it is rapidly converted to the cholest-5-ene, 3β, 7α, 27-triol (by microsomal CYP7B1) and subsequently to bile acids. This proved to be the case in Hep G2 cells. Microsomal 7α-hydroxylase activity specific for 27OH-Chol has been found in Hep G2 cells by our lab (unpublished data) and others (18). Even though CYP27 specific activity increased almost twofold, 27OH-Chol levels increased only ~16% (P < 0.01) over control levels in Hep G2 cells following infection with AdCMV-CYP27. The small accumulation of 27OH-Chol in infected Hep G2 cells had no downregulatory effect on HMG-CoA-R specific activity. In fact, HMG-CoA-R specific activity in Hep G2 cells increased almost twofold following infection with AdCMV-CYP27. The increase in HMG-CoA-R specific activity occurs most likely because of a compensatory mechanism of depletion of cholesterol from the intracellular pools resulting from an increased CYP27 specific activity/bile acid biosynthesis. On the basis of these data, it would seem highly unlikely that under physiological conditions 27OH-Chol levels in hepatocytes could accumulate to the point at which they would downregulate hepatic cholesterol biosynthesis.

Increased 27OH-Chol levels in CHO cells had no effect on ACAT activity, implying that 27OH-Chol is not a potent regulator of ACAT activity. In Hep G2 cells, ACAT activity increased with the increase in HMG-CoA-R activity. This upregulation in ACAT specific activity is consistent with the observed increase in newly synthesized cholesterol (183% increase in HMG-CoAR specific activities), which supports previous observations suggesting that microsomal cholesterol availability is a potent regulator of ACAT activity (32).

CEH specific activities have been closely tied to cholesterol availability. Cholesterol feeding and mevalonate infusion both lead to decreased CEH specific activity, whereas inhibition of cholesterol synthesis with lovastatin stimulated CEH activity. An increased cholesterol flux observed with cholestyramine or chronic biliary diversion has also been associated with increased CEH specific activity (11). The increase in bile acid synthesis coupled with higher HMG-CoA-R specific activity following CYP27 overexpression in Hep G2 cells seems to most closely follow these last two experimental models.

In summary, we have successfully overexpressed CYP27 in both hepatic and nonhepatic cells in culture. This type of endogenous overexpression should represent a more physiological method of studying the effects of increased CYP27 specific activity and the role of 27OH-Chol in cholesterol and bile acid metabolism. In Hep G2 cells, overexpression of CYP27 resulted in an increase in bile acid biosynthesis with a compensatory stimulation of cholesterol biosynthesis. In CHO cells, where there is no bile acid biosynthesis pathway, CYP27 overexpression resulted in an increase in the cellular levels of 27OH-Chol with subsequent down-regulation of HMG-CoA-R specific activity and cholesterol biosynthesis, as well as increased efflux of 27OH-Chol into the culture medium (Fig. 9).

These results suggest that CYP27 could act as an antiatherogenic enzyme in a number of tissue-specific ways. More specifically, CYP27 mediates the removal of excess cholesterol from peripheral tissues through its conversion to 27OH-Chol and 3β-hydroxy-5-cholestenoic acid. The ability of peripheral cells to increase CYP27 specific activity and thus the intracellular concentrations of 27OH-Chol may also allow for downregulation of cholesterol synthesis. Within hepatocytes, overexpression of CYP27 facilitates the removal of cholesterol from the body by acting as an upregulator of bile acid biosynthesis. These in vitro data suggest that CYP27 generates different physiological responses in the body depending on the cell type and the presence or absence of bile acid biosynthetic pathways.

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