TRANSPORT OF CHOLESTEROL INTO MITOCHONDRIA IS RATE LIMITING FOR BILE ACID SYNTHESIS VIA THE ALTERNATIVE PATHWAY IN PRIMARY RAT HEPATOCYTES

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ABSTRACT

Bile acid synthesis occurs mainly via two pathways: the “classic”, initiated by microsomal cholesterol 7α-hydroxylase (CYP7A1), and an “alternative” (acidic) pathway, initiated by sterol 27-hydroxylase (CYP27). CYP27 is located in the inner mitochondrial membrane where cholesterol content is very low. We hypothesized that cholesterol transport into mitochondria may be rate-controlling for bile acid synthesis via the “alternative” pathway. Overexpression of the gene encoding steroidogenic acute regulatory (StAR) protein, a known mitochondrial cholesterol transport protein, led to a 5-fold increase in bile acid synthesis. An increase in StAR protein coincided with the increase in bile acid synthesis. CYP27 overexpression increased bile acid synthesis by less than 2-fold. The rates of bile acid synthesis following the combination of StAR plus CYP27 overexpression were similar to StAR alone. TLC analysis of [14C]-bile acids synthesized in cells overexpressing StAR showed a 5-fold increase in muricholic acid; in chloroform extractable products, a dramatic increase was seen in bile acid biosynthesis intermediates (27- and 7,27-hydroxycholesterol). HPLC analysis showed that 27-hydroxycholesterol accumulated in the mitochondria of StAR overexpressing cells only. These findings suggest that cholesterol delivery to the inner mitochondrial membrane is the predominant rate-determining step for bile acid synthesis via the “alternative” pathway.
INTRODUCTION

The liver plays a pivotal role in the maintenance of cholesterol homeostasis. Under normal physiologic conditions, cholesterol input into the body equals cholesterol output (1,2). Bile acid synthesis in liver is the major pathway for cholesterol output. The biotransformation of cholesterol to primary bile acids occurs via two main pathways. In the “neutral” pathway, metabolism of the sterol nucleus occurs before side-chain modifications, and begins with hydroxylation of cholesterol at the 7α position (3). This reaction is catalyzed by cholesterol 7α-hydroxylase (CYP7A1); the initial and rate-determining step in this pathway of bile acid synthesis. In the alternative pathway of bile acid synthesis, commonly called the “acidic” pathway, side-chain modifications precede modifications in the sterol nucleus. The initial and presumed rate-determining step in the “acidic” pathway is catalyzed by mitochondrial sterol 27-hydroxylase (CYP27).

In contrast to CYP7A1, which is found only in the liver, CYP27 has a wide tissue distribution. The ability of peripheral cells to 27-hydroxylate cholesterol has been proposed to be important in “reverse cholesterol transport” (4-6). According to this hypothesis, CYP27 located in peripheral tissues, generates oxysterols that are more water soluble than cholesterol. These metabolites can then be transported to the liver and converted to bile acids. It is possible that CYP27 in peripheral tissues may both down-regulate cholesterol synthesis, and enhance the ef llux of cholesterol to the liver.
for elimination. Thus, up-regulation of CYP27 could represent a treatment of hyperlipidemia. However, overexpression of the gene encoding CYP27 in primary rat and human hepatocytes, or HepG2 cells only led to a ~50% increase in bile acid synthesis (7). This led us to hypothesize that increasing cholesterol delivery to and/or into the mitochondria where CYP27 is located could potentially increase the rate of bile acid synthesis via the “acidic” pathway. Precedence for this has previously been demonstrated in other steroidogenic tissues. In the adrenal, increased expression of the mitochondrial cholesterol transport protein, steroidogenic acute regulatory protein (StAR), was shown to increase mitochondrial cholesterol delivery and steroidogenesis (8).

The present study shows that overexpression of the gene encoding StAR protein in rat primary hepatocytes dramatically increases bile acid synthesis, which suggests that cholesterol delivery to the inner mitochondrial membrane is the rate-determining step for bile acid biosynthesis via the “alternative” pathway rather than CYP27. Furthermore, it was shown that increasing cholesterol transport to inner mitochondrial CYP27 bypasses the highly regulated CYP7A1 of the “classic / neutral” pathway of bile acid biosynthesis. These findings provide an entirely new insight into thinking about how bile acid biosynthesis is regulated.
EXPERIMENTAL PROCEDURES

Cell culture reagents and supplies were purchased from GIBCO BRL (Grand Island, NY). RPA II Kit was purchased from Ambion (Austin, TX). [14C]-Cholesterol and [3H]-25-Hydroxycholesterol were purchased from New England Nuclear (Boston, MA). 25-Hydroxycholesterol, 7α- and 7β-hydroxycholesterol were purchased from Steraloids, Inc (Newport, RI). Cyclodextrin was purchased from Cyclodextrin Technologies Development Inc. (Gainsville, FL). Silica gel thin-layer chromatography plates (LK6 D) were from Whatman (Clifton, NJ). Silica gel 1B TLC sheets were purchased from VWR (Bridgeport, NJ). HPLC grade solvents were purchased from Fisher Scientific (New Lawn, NJ). All other reagents were from Sigma Chemical Co (St. Louis, MO), unless otherwise indicated.

Isolation and Culture of Primary Rat Hepatocytes:

Hepatocytes were isolated from male Sprague-Dawley rats (250-300 g) as previously described by us using the collagenase-perfusion technique of Bissell and Guzelian (9). Cells were routinely harvested after 72 hours of culture as previously described (10). Unless specified, cells were maintained under conditions where CYP7A1 activity is undetectable (i.e. cultured as previously described in the absence of thyroid hormone) (10).
Generation of Recombinant Adenoviruses and Their Use

The adenoviruses constructs used in this study were obtained through the Massey Cancer Center Shared Resource Facility of Virginia Commonwealth University. The CMV-CYP27 recombinant adenovirus clone (Ad-CMV-CYP27) was constructed as previously described (7,11).

Briefly, the CMV-StAR adenovirus construct (Ad-CMV-StAR) was obtained using a pTG-CMV system as previously described (7,11). A 1.6 kb human adrenal cortex StAR cDNA (a generous gift from Dr. Jerome Strauss, Department of Obstetrics and Gynecology, University of Pennsylvania, Philadelphia, PA) was cloned into the Sal/NotI sites of pZero TG-CMV, a plasmid containing the CMV promoter, multiple cloning site and partial DNA sequence from Ad5dl324 (12). The resulting pZero TG-CMV/HsStar plasmid was co-transformed with ClaI-linearized pTG-CMV (containing the entire Ad5dl 324 genome) into E. coli. Recombinant plasmids were transfected into 293 cells (human embryonic kidney cell line). Adenovirus DNA from resulting plaques was further screened by Southern blot for the presence of the insert.

Propagation of Ad-CMV-StAR and Ad-CMV-CYP27: Large scale production of recombinant virus was accomplished by infecting confluent monolayers of human embryonic kidney “293” cells (American Type Culture Collection, Rockville, MD), grown in 15 cm tissue culture dishes, with stock adenoviruses at a multiplicity of infection
(MOI) of 1 pfu/cell. After 2 hours of infection, unbound virus was removed and Dulbecco’s Modified Eagle Medium (DMEM) with 2% fetal bovine serum (FBS) was added. Infected monolayers were harvested by scraping when >90% of cells showed cytopathic changes. They were then harvested by scraping and centrifuged at 2700 g at 4°C for 10 minutes. The pellet was suspended in DMEM with 2% FBS and subjected to 5 cycles of freeze/thaw lysis to release the recombinant virus. Cell debris were removed by centrifugation at 7700 g for 10 minutes at 4°C. To purify the recombinant virus, the crude supernatant was carefully layered over a two-step gradient containing 3 ml of CsCl (d=1.4 g/ml) in TD buffer (0.14 M NaCl, 5 mM KCl, 19 mM Tris pH 7.4 and 0.7 mM Na₂HPO₄) layered over 3 ml of CsCl (d=1.25 g/ml) in TD buffer, and centrifuged at 155,000 g at 20°C for 1 hour. The viral band was removed, layered over 8 ml of CsCl (d=1.33 g/ml) in TD buffer and centrifuged at 155,000 xg, 20°C for 18 hrs. The pure viral, opalescent band was removed and dialyzed overnight at 4°C, against 10mM Tris-HCl (pH7.4), 1mM MgCl₂ and 10% glycerol. The virus was aliquoted and stored at minus 70°C until used. The virus titer (pfu = plaque forming units) was determined by plaque assay, and viral particles were determined by optical density using spectrophotometry (260nm).

**Infection of Cells with Adenovirus encoding the StAR protein and CMV-CYP27:**

Primary rat hepatocyte cultures, prepared as previously described (10) were plated on
P150 mm tissue culture dishes (~2.5 x 10^7 cells) in Williams’ E media containing dexamethasone (0.1 µM). Unless otherwise specified, cells were maintained in the absence of thyroid hormone, and only the acidic pathway of bile acid synthesis is functional (10). In selected studies, thyroid hormone (L-thyroxine) was added as previously described in a concentration of 1.0 µM, culture conditions in which both bile acid biosynthesis pathways are fully functional. Twenty-four hours after plating, culture medium was removed, and 2.5 ml of fresh medium was added. Cells were then infected with unpurified adenovirus encoding either Ad-CMV-StAR or Ad-CMV-CYP27 at a multiplicity of 10 pfu/cell. All experiments were compared to Ad-CMV control virus and no virus cultures. The virus was allowed to dwell for at least 2 hours in minimal culture medium with shaking the plates gently every 15 minutes. After 2 hours of infection, unbound virus was removed and replaced with 20 ml of fresh medium. The cells were incubated at 37°C, 5% CO₂ for 48 hours. Cells were then harvested as previously described (10).

RNA Preparation and Quantification:

RNA was isolated as previously described (13). CYP7A1 and CYP27 mRNA was quantified using Northern blot assays (20 µg of total RNA).

Protein levels

After infection, cells were either harvested by adding sample buffer as indicated
in the text, or subcellular fractions were separated and isolated by centrifugation as previously described (7). Proteins were then solubilized by adding 2X SDS-PAGE sample buffer (5 mM Tris buffer, pH 8.3, 29% (w/v) SDS, 10% mercaptoethanol, 10% (v/v) glycerol, 38 mM glycine and 0.2% (w/v) bromophenol blue, followed by heating in a boiling water bath for 5 minutes. Three µg of solubilized proteins were analyzed by 10% SDS-PAGE. Electrophoresis was performed at 20 mA for 2 hours in a BioRad (Richmond, CA) mini gel system. StAR protein was identified by Western blot analysis: after electrophoresis, samples were transferred to nitrocellulose membranes. Membranes were blocked with 3% non-fat dry milk in 10 mM HEPES buffer, pH 7.4 containing 25 mM EDTA, 0.5 M NaCl, 0.05% NaN₃ and immunostained with a rabbit polyclonal antibody (1:2000 dilution) against human StAR protein (a generous gift from Dr. Jerome Strauss) in HEPES buffer and washed with the same buffer plus 0.05% Tween 20, incubated with a goat anti-rabbit secondary antibody (1:10,000) purchased from Sigma. Bands were visualized by using a Chemiluminescence reagent (NEN Life Science Products, Boston, MA) and BioMax Kodak film.

CYP27 immunoblotting was performed as previously described (7). Rabbit polyclonal antibody against rat CYP27 protein was a generous gift from Dr. N. Avadhani (University of Pennsylvania, Philadelphia, PA).

**Determination of Enzymatic Specific Activities**
Mitochondria and microsomes were prepared as previously described (7,10). The specific activities of CYP7A1 and CYP27 were determined by HPLC assays as previously described (7,10).

**Quantification of bile acid synthesis rates:**

**In vitro studies:**

Bile acid synthetic rates were determined by the addition of 2.5 µCi of $[^{14}\text{C}]$-cholesterol to each P150 mm plate of confluent primary rat hepatocyte cultures (~2.45 x $10^7$ cells) 24 hours after plating. Media and cells were harvested 48 hours after viral infection. Conversion of $[^{14}\text{C}]$-cholesterol into $[^{14}\text{C}]$-methanol-water soluble products was determined by scintillation counting after Folch extraction (14) with chloroform-methanol (2:1, vol/vol) of cells and of culture media. Rates of bile acid biosynthesis following recombinant adenovirus infection were calculated as the ratio of $[^{14}\text{C}]$-methanol-water soluble counts to the sum of chloroform plus methanol-water counts.

Individual bile acids were identified as previously described (11). Briefly, to identify the individual bile acids, the $[^{14}\text{C}]$-methanol/water phase was first base hydrolyzed, then separated by thin layer chromatography (TLC) in a solvent system of ethyl acetate: cyclohexane: acetic acid (7.7:2.3:1, vol/vol/vol). $[^{14}\text{C}]$-bile acids were visualized with a phosphoimager.
Time points for conversion of $^{14}$C-cholesterol to $^{14}$C-bile acids were carried out using P150 mm tissue culture dishes. Aliquots (100 µl) of media were collected in duplicate in a microfuge tube and kept frozen until analysis. A mini Folch extraction was carried out by adding to the culture medium sample, 50 µl of water, 250 µl of methanol, 537 µl of chloroform ($H_2O$:MeOH:CHCl$_3$, 2:3:7) and 3 µl of 1M Na$_2$CO$_3$ to help separate the phases. The tubes were vigorously vortexed and centrifuged at 16,000 g for 6 minutes. The phases were collected separately and counted. Time points for the $^{14}$C-7α-hydroxycholesterol conversion into bile acids were taken from 60 mm tissue culture dishes plated for that purpose.

In selected studies, the rate of 7α-hydroxycholesterol uptake and subsequent metabolism to bile acids was determined. Twenty-four hours after plating, isolated primary rat hepatocytes were infected with recombinant adenovirus encoding CMV driven StAR gene, null virus (control) or no virus addition. Following infection, $^{14}$C-7α-hydroxycholesterol ((1x10$^5$ dpm/ 60 mm plate) and unlabeled 7±-hydroxycholesterol in [5 µM] was added. Samples were collected in duplicate, extracted, and methanol/water soluble counts determined. Bile acid synthesis was measured as conversion of $^{14}$C-labeled 7α-hydroxycholesterol into $^{14}$C-labeled methanol/water extractable counts.

In vivo studies:
Conjugated bile acids in the bile collected from biliary diverted rats were analyzed by reverse phase high-performance liquid chromatography as previously described (13). In chronic biliary diverted rats, bile acid synthesis is equivalent to biliary bile acid secretion.

**Biliary diverted rat:**

Adult male Sprague-Dawley rats weighing between 250 and 300g were housed under controlled lighting conditions on a natural light-dark cycle. Groups of age-and weight-matched animals were used in all experiments. Under brief methoxyflurane anesthesia, intravenous and biliary fistula cannulas were placed as previously described (13,15,16). After cannula placement, each rat was intravenously infused with 1-1.5 x 1011 virus particles of recombinant adenovirus containing CMV-StAR or control virus. Following surgery, the rats were housed in individual metabolic cages with free access to water and chow. Diverted bile was collected in timed increments throughout the course of the experiment. All animals received a continuous infusion of glucose-electrolyte replacement solution at 1.07 ml/h. Throughout the experiment, dietary intake, activity, and bile flow were monitored as previously described (13). At the end of the experiments, animals were briefly anaesthetized, decapitated, and blood was collected in order to measure serum ALT and alkaline phosphatase levels as previously described (16). Animals were sacrificed at 9-10am. The animal protocol utilized was reviewed and approved annually by the institutional animal care and use committee of the Medical College of Virginia, Richmond, VA; and complies with the “Guide for Care
and Use of Laboratory Animals” published by the USA National Institute of Health.

**Statistics:**

Data are reported as mean ± standard error. Where indicated, data were subjected to t-test analysis and determined to be significantly different if p<0.05.
RESULTS

StAR Overexpression in primary rat hepatocytes

Infection of primary rat hepatocytes with Ad-CMV-StAR produced high StAR mRNA and protein levels with no evidence of cell toxicity. Figure 1 shows the increase in StAR mRNA and StAR protein 48 hrs following infection (see “Experimental Procedures”). Rat adrenal poly A RNA was used as a control. Two mRNA species (1.6 and 4.0 kb) were seen (Fig.1A) representing parental and mature forms of StAR mRNA as previously shown (18,19). Western blot analysis of mitochondria proteins showed one major immunoreactive band with a molecular weight of 30 kDa, consistence with StAR mature protein (Fig. 1B) as previously reported (8,17). Primary rat hepatocyte subcellular fractions were then isolated and the distribution of StAR protein was examined using Western blot analysis. In hepatocytes overexpressing StAR, StAR protein was found widely distributed in the cytosol, microsomes, and mitochondria (data not shown). A comparison of StAR protein levels in StAR-overexpressed primary hepatocytes with those in rat testis and adrenal glands is shown in Fig. 1C. The recombinant StAR protein had a molecular weight similar to that of rat testis and adrenal gland, however, the level of StAR protein in hepatocytes following StAR overexpression was significantly higher than that expressed under normal physiological conditions in rat testis or adrenal.

Sterol 27-hydroxylase (CYP27) overexpression in primary rat hepatocytes
CYP27 is responsible for the 27 hydroxylation of cholesterol as the initial step in the “acidic” pathway of bile acid synthesis. To compare the effects of StAR overexpression with that of CYP27, primary rat hepatocytes were infected with recombinant adenovirus containing CMV-driven CYP27 gene (Ad-CMV-CYP27). The infected cells produced very high CYP27 mRNA and protein levels without inducing any evidence of cell toxicity. Northern blot analysis showed a 2.1 Kb mRNA band representing mature CYP27 mRNA (Fig. 2A). Western blot analysis of mitochondria proteins showed one major immunoreactive band with a molecular weight of 55 kDa (Fig. 2B). Overexpression of StAR, either alone or in combination with CYP27, did not alter CYP27 protein levels or catalytic activity (data not shown).

Effect of StAR on the rate of bile acid synthesis

Overexpression of StAR protein dramatically increased rates of bile acid synthesis in primary rat hepatocytes. Time courses showing the increase in StAR protein levels, bile acid synthesis, and [14C]-cholesterol uptake in primary rat hepatocytes after StAR overexpression are shown in Fig 3. StAR protein was easily detected at 12 hours following infection with recombinant adenovirus, and steadily increased up to 48 hours (Fig 3A). The effects of StAR protein on the rates of bile acid synthesis in the cells were determined via the conversion of [14C]-cholesterol into [14C]-methanol/water extractable products (Fig 3B). CYP27 overexpression only slightly increased bile acid synthesis rates over that observed in controls (cells infected with
control recombinant adenovirus). In contrast, rates of bile acid synthesis increased dramatically with expression of StAR protein (Fig. 3B). Furthermore, overexpression of CYP27 and StAR together did not increase bile acid synthesis rates any more than StAR overexpression alone (Fig. 4). These results show that an increase in StAR protein is capable of increasing bile acid synthesis more efficiently than an increase in CYP27 expression. Effects of StAR protein on cellular cholesterol uptake are shown in Fig. 3C. To determine that this increase in bile acid synthesis was not the result of an increase in cholesterol uptake, the rates of cholesterol uptake were determined as $[1^{4C}]$-cholesterol “disappearance” (chloroform phase) from the cell culture media. As shown, neither StAR and/or CYP27 overexpression affected cellular cholesterol uptake rates.

The rates of bile acid synthesis were further determined by quantifying the bile acid levels in culture media and within cells at 48 hours after infections is shown in Fig. 4. Within the cells, StAR overexpression, and co-overexpression of StAR plus CYP27, produced a >10-fold increase (p<0.001) in the amount of bile acids over that observed in control cells (i.e. infected with control recombinant adenovirus); while CYP27 overexpression alone only led to a 1.4-fold increase (Fig. 4A) over . In the culture media, an ~ 6-fold increase (p<0.001) in bile acids (i.e. less than within the cell) was seen following StAR overexpression, and StAR plus CYP27; with a 76±58% increase following infection with recombinant adenovirus containing CMV-driven CYP27 gene
alone as compared with virus control (Fig. 4B). The differences in rates of bile acid synthesis following overexpression of the gene encoding StAR or StAR plus CYP27 were not significant. The increase in rates of bile acid synthesis, following the addition of unlabeled cholesterol [5 µM] in order to saturate and competitively slow $^{[14}C$-cholesterol uptake into cells, was > 2-fold (data not shown).

The steroid products in chloroform and water/methanol extractable phases were further analyzed by thin layer chromatography (TLC) (Fig. 5). $^{[14}C$-Steroid extractable products in the chloroform phase were mainly composed of cholesterol esters, cholesterol, 25-hydroxycholesterol, 27-hydroxycholesterol, 3-oxo-7-hydroxycholesterol, and 7,27-dihydroxycholesterol (Fig. 5.A). Cholesterol and cholesterol esters were decreased in the culture media of cells overexpressing StAR or StAR plus CYP27. Conversely, 27-hydroxycholesterol and 7,27-dihydroxycholesterol levels increased significantly in the cells overexpressing the StAR or StAR plus CYP27. Because the CYP27 is located in inner mitochondria membrane, it is assumed that the 27-hydroxycholesterol and 7, 27-dihydroxycholesterol are products of the alternative pathway of bile acid synthesis. TLC analysis of products in methanol phase of the culture media is shown in Fig. 5B. A 5-fold increase was seen in soluble steroids, β-muricholic acid and chenodeoxycholic acid (Fig. 5B) in the culture media following StAR or StAR plus CYP27 overexpression.
In selected studies, thyroid hormone was added to the culture medium as described in the “Experiment Procedures”. Under these conditions CYP7A1 and “neutral” (classic) pathway of bile acid synthesis are fully active. Of note is that CYP7A1 mRNA levels under these culture conditions are greater than that found in the up-regulated cholestyramine fed rat model (10). These studies were performed to address the question: Is StAR overexpression capable of increasing the rate of bile acid synthesis over the basal rates found in the presence of a fully functional “neutral pathway.” Using these culture conditions, overexpression of StAR still lead to a > 2-fold increase (p<0.001) in rates of bile acid synthesis (data not shown).

Effect of StAR and/or CYP27 gene overexpression on mitochondrial levels of 27-hydroxycholesterol:

To demonstrate that StAR overexpression leads to an increase in the product of CYP27, mitochondrial 27-hydroxycholesterol levels were determined following StAR and CYP27 overexpression (7). Mitochondrial sterol analysis revealed an easily detectable retention peak for endogenous 27-hydroxycholesterol in mitochondria of StAR overexpressing hepatocytes (Fig. 6). Of note is that neither control cells nor cells overexpressing CYP27 had detectable 27-hydroxycholesterol levels in their mitochondria (previous determined detection sensitivity of ~20 pmol). 27-hydroxycholesterol accumulated only following StAR overexpression. Following the determination of endogenous mitochondrial 27-hydroxycholesterol levels, mitochondrial
were assayed for CYP27 specific activity. Interestingly, no detectable increase in CYP27 activity over controls was found following StAR overexpression. These findings suggest that StAR’s ability to increase mitochondrial cholesterol transport, and its subsequent conversion to 27-hydroxycholesterol, occurred prior to mitochondrial isolation for CYP27 activity analysis. Furthermore, these results show, given the existing basal cellular CYP27 protein levels, that cholesterol delivery to the inner mitochondrial membrane is the key rate-determining step in bile acid synthesis via the “alternative” pathway. The above findings, coupled with the inability of overexpression of the genes encoding CYP27 plus StAR to further increase bile acid synthesis above overexpression of the StAR gene alone, suggests there exists an abundance of CYP27 under normal physiologic conditions.

**Effect of StAR on 7α-hydroxycholesterol metabolism:**

Overexpression of StAR did not alter the neutral pathway of bile acid synthesis. 7α-Hydroxycholesterol is the product of the initial and rate-determining step in the “neutral” (classic) pathway of bile acid synthesis. However, to be metabolized to bile acids, 7α-hydroxycholesterol must first be 27-hydroxylated by CYP27 in the mitochondria. In order to assess whether StAR protein might also induce uptake and metabolism of 7α-hydroxycholesterol to mitochondrial CYP27, [14C]-7α-hydroxycholesterol, was added to cells (Experiment Procedures). Shown in Fig. 7 is the time course for [14C]-7α-hydroxycholesterol in cells overexpressing the gene encoding
StAR. The time course for $[^{14}\text{C}]-7\alpha$-hydroxycholesterol utilization in primary rat hepatocytes showed no effect on the metabolism of $7\alpha$-hydroxycholesterol. These results further suggest that StAR protein up-regulates bile acid synthesis via the “alternative” (acidic) pathway via the delivery of cholesterol and not bile acid intermediates to the inner mitochondrial membrane.

**Effect of StAR overexpression on bile acid synthesis in the biliary diverted rat**

Infection of biliary diverted rats with recombinant adenovirus encoding CMV-StAR markedly increased StAR mRNA and protein levels (data not shown). Biliary diverted rats, infected with StAR three days earlier at the time of their biliary diversion, increased their rates of bile acid synthesis 2.5-fold (n=6; p<0.001) over their basal synthesis rates at 20-24 hours (Fig. 8). This represented a 1.8-fold (n=3; p<0.03) increase over three day biliary diverted controls (i.e. infected with control recombinant adenovirus). Thus overexpression of StAR was able to dramatically increase bile acid synthesis rates over and above the usual ~1.5 to 2-fold increase in basal rates observed in control biliary diverted rats three days following the loss of negative bile acid feedback. The bile acid concentration following both StAR and CYP7A1 overexpression was also similarly increased (data not shown).
DISCUSSION

StAR protein has previously been shown to mobilize cholesterol from the outer to the inner mitochondrial membrane in steroidogenic cells (i.e. adrenal cortex and gonads) (8,17). Cholesterol transport is a principal control point for regulation of steroidogenesis by adrenocorticotropic hormone (ACTH) and other hormones acting through the adenylyl cyclase and Ca\textsuperscript{2+} pathways (18). Observations by Sugawara et al. (19) have subsequently provided evidence for mitochondrial cholesterol transport as being not only rate-determining for steroidogenesis, but that StAR induced mitochondrial cholesterol transport is capable of enhancing mitochondrial cholesterol metabolism by enzymes other than the steroidogenic cytochrome P450scc. There is, however, an abundance of StAR protein within the adrenal mitochondria. In contrast, StAR mRNA or protein has not been detected in liver tissue (18,20). Still, the existence of StAR or a StAR-like protein in liver hepatocytes seems necessary for bile acid synthesis to occur via the alternative pathway as cholesterol must first be transported to the inner mitochondrial membrane before it can undergo 27-hydroxylation. Furthermore, the inability to dramatically increase bile acid synthesis in hepatocytes overexpressing CYP27, suggests that 27-hydroxylation of cholesterol is not rate-limiting for the “alternative” pathway of bile acid synthesis.

The contribution of the “alternative” pathway to total bile acid synthesis is unclear, as under most physiologic conditions the “classic” pathway appears to be the
dominant pathway (3,21). It is currently believed that the “alternative” pathway of bile acid synthesis may play at least three roles in cholesterol homeostasis (3): 1) The 27-hydroxylation of cholesterol, both in the periphery and the liver forming a regulatory oxysterol (i.e. 27-hydroxycholesterol) (3,7). The liver is capable of hydroxylating these regulatory oxysterols; leading to their subsequent metabolism to bile acids. 2) The “alternative” pathway may act as a “backup pathway” when the classic pathway is down-regulated. In CYP7A1 “knockout” animals, alternative pathways appear capable of producing adequate amounts of bile acids for survival and growth (22). 3) The “alternative” pathway may serve to regulate the ratios of bile acid species in the bile, as this pathway is thought to generate mostly chenodeoxycholic acid in humans (3). It has been shown that up to 50% of bile acid biosynthesis may occur via an alternative pathway in the rat (23,24). Studies in humans have found a lower contribution under normal physiologic circumstances (25). However, in human liver cholestatic conditions, this contribution has been found to be much higher; suggesting the alternative pathway can be a major pathway under certain pathophysiologic conditions (26).

Evidence supportive of mitochondrial cholesterol transport as the rate-controlling step of bile acid synthesis via the alternative pathway would give rise to a new hypothesis regarding the regulation of the “alternative” pathway. It would also give strong evidence as to why CYP27 is localized in the mitochondrial under highly regulated cholesterol access. In the “alternative” pathway, the initial and presumed rate-determining step is catalyzed by mitochondria CYP27. The present study shows
that cholesterol transport into the inner mitochondrial membrane is the rate-limiting step in the “alternative” pathway of bile acid synthesis rather than CYP27. Furthermore, in an unregulated state, (i.e. increased expression of StAR protein with increased mitochondrial cholesterol transport) the highly regulated “neutral” (classic) pathway of bile acid synthesis can be bypassed; demonstrating the absolute necessity of tight regulation of mitochondrial cholesterol transport in the liver. Supportive of this statement is the observation made in primary rat hepatocytes cultured in the presence of thyroid hormone. We have previously shown that with addition of thyroid hormone to our standard culture medium CYP7A1 is markedly upregulated to levels greater than that found in the upregulated cholestyramine fed rat (13). StAR overexpression under these culture conditions still led to a greater than 2-fold increase in the rates of bile acid synthesis. In in vivo studies, overexpression of the StAR gene in the biliary diverted rat, also led to a 1.8-fold increase in bile acid synthesis over controls, a model previously believed to have maximal rates of bile acid synthesis (Fig. 8).

Our results show that overexpression of the StAR gene, or co-overexpression of StAR with CYP27, leads to accumulation of 27-hydroxycholesterol in mitochondria, while overexpression of CYP27 alone did not. Meanwhile, StAR gene overexpression increased bile acid synthesis 6-fold, while in direct comparison, CYP27 gene overexpression increased synthesis less than 2-fold. These findings are consistent with the increase in bile acid synthesis seen in HepG2 cells, following CYP27 gene overexpression (7).
StAR protein overexpression increases transport of cholesterol from the outer to inner membrane of the mitochondria; possibly leading to saturating cholesterol concentrations within the inner membrane of mitochondria and allowing maximal rates of bile acid synthesis. However, the accumulation of 27-hydroxycholesterol and other bile acid intermediates in the mitochondria, suggests the possibility of another rate-limiting step in bile acid synthesis; i.e. transport of 27-hydroxycholesterol and/or other bile acid intermediates across mitochondria membranes.

StAR (StARD1) is a member of a family of proteins, each containing an ~200-210 amino acid StAR-related lipid transfer (START) domain (27). Recently, Soccio, Breslow et al. have discovered several more members of a subfamily of START (i.e. “StAR”) domain-containing proteins, StARD4, StARD5, and StARD6 (28). Both StARD4 and StARD5 are ubiquitously expressed, with greatest abundance in the liver and kidney; whereas, StARD6 is exclusively expressed in the testis. Whether one of these liver START-domain proteins could function as a liver mitochondrial cholesterol transporter is currently not clear. Of interest is that most other previously identified START-domain proteins contain a N-terminal domain, which appears important in directing its function (i.e. StARD1) (8,17). StAR 4, 5, and 6, however, are only 205-233 amino acid proteins consisting almost entirely of a START domain (28). Furthermore, StAR 4, 5, and 6 only share an ~ 20% identity with the cholesterol binding StARD1, and ~ 30% identity with each other, allowing one to hypothesize that each may have a distinct function in the maintenance of intracellular lipid homeostasis (28).
In summary, the results reported in this study show that the alternative pathway of bile acid synthesis is primarily regulated by cholesterol transport into the mitochondria, and suggest that the hepatocyte must have StAR or homologues for transporting cholesterol into inner mitochondria membrane. These studies also suggest alternative mechanisms for increasing rates of bile acid synthesis and cholesterol output from the body. Previously unsuspected, this study also demonstrates that a sufficient increase in mitochondrial cholesterol transport within the hepatocyte is capable of bypassing the usually dominant “classic” pathway of bile acid synthesis; obviating the rate-controlling function of the highly regulated CYP7A1.
FIGURE LEGENDS

Figure 1. StAR mRNA and protein levels in primary rat hepatocytes following StAR and CYP27 overexpression. Primary rat hepatocytes were infected with the indicated recombinant adenoviruses as described under “Experiment Procedures”. Cells were harvested 48 hours following infection, and RNA or mitochondria were isolated. A. mRNA levels for StAR and cyclophilin (as control) were determined by Northern analysis. B. and C. StAR protein levels as determined by Western analysis. Rat adrenal and testis RNA and protein were used as positive controls; compared to overexpressed StAR levels.

Figure 2. CYP27 mRNA and protein levels in primary rat hepatocytes following StAR overexpression and CYP27. Primary rat hepatocytes were infected with the indicated recombinant adenoviruses as described in “Experimental Procedures”. Cells were harvested 48 hours following infection, and RNA or mitochondria were isolated. A. mRNA levels for CYP27 and cyclophilin (as control) were determined by Northern analysis. B. CYP27 protein levels as determined by Western analysis.

Figure 3. StAR overexpression increases cholesterol uptake and rates of bile acid synthesis. Primary rat hepatocytes were infected with the StAR recombinant adenovirus as described under “Experimental Procedures.” Cells and tissue culture media were collected at the indicated time points following infection. A. StAR protein in
the mitochondria of infected cells at the indicated time was analyzed by Western analysis. B. Bile acid synthesis rates quantified as conversion of $[^{14}\text{C}]-\text{cholesterol}$ into methanol/water extractable products as described under “Experimental Procedures.”

C. $[^{14}\text{C}]-\text{cholesterol}$ was quantified as a function of change in $[^{14}\text{C}]-\text{cholesterol}$ counts in the media.

**Figure 4. Effect of StAR and/or CYP27 overexpression on the rates of bile acid synthesis in primary rat hepatocytes.** Primary rat hepatocytes were infected with the indicated recombinant adenoviruses and incubated with $[^{14}\text{C}]-\text{cholesterol}$ as described under “Experimental Procedures.” Forty-eight hours after infection, both cells (A) and culture medium (B) were harvested, and bile acid synthesis levels were quantified as described under “Experimental Procedures.” Bile acid synthesis levels are expressed as a % of control (null) virus and represent the mean ± SE of 3 to 9 experiments.

**Figure 5. StAR overexpression increases bile acids and its intermediates.** Primary rat hepatocytes were infected with the indicated recombinant adenoviruses and incubated with $[^{14}\text{C}]-\text{cholesterol}$ as described under “Experimental Procedures.” Forty-eight hours after infection, both cells (A) and culture medium (B) were harvested, extracted with methanol:water, and analyzed by TLC as described under “Experimental Procedures.” The migration of authentic standards is indicated on the right.
Figure 6. 27-hydroxycholesterol accumulates in mitochondria of StAR overexpressing cells, but not with CYP27 overexpression. Primary rat hepatocytes were infected with the indicated adenoviruses. Forty-eight hours after infection, mitochondria were isolated and endogenous 27-hydroxycholesterol levels were determined as described under “Experimental Procedures.” The graphs represent the HPLC tracings showing 27-hydroxycholesterol peaks as determined by an authentic standard.

Figure 7. StAR overexpression does not alter the rate of uptake or conversion of $[^{14}\text{C}]-7\alpha$-hydroxycholesterol to bile acids. Primary rat hepatocytes were infected with the indicated recombinant adenoviruses (NA = no virus) as described under “Experimental Procedures.” Cell culture media was collected at the indicated time points following infection. A. Bile acid synthesis rates quantified as conversion of $[^{14}\text{C}]-7\alpha$-hydroxycholesterol into methanol/water extractable products as described under “Experimental Procedures.” B. $[^{14}\text{C}]-7\alpha$-hydroxycholesterol uptake was quantified as a function of change in $[^{14}\text{C}]-7\alpha$-hydroxycholesterol extractable counts in the media.

Figure 8. Effects of StAR overexpression on the rate of bile acid synthesis in chronic biliary diverted rats. Chronic biliary diverted rats were infected with the indicated recombinant adenoviruses (1.5 x 10^{11} virus particles) as described under “Experimental Procedures.” The time period 20-24 hours represents the time post biliary diversion in which the pre-diversion bile acid pool has drained and bile synthesis
(i.e. secretion) is at a basal level. The 70 hour time period represents the time of maximal up-regulation of bile acid synthesis which occurs following pool drainage with loss of negative bile acid feedback. Data are expressed as the mean ± SE (n=4 for StAR; n=3 for controls).
REFERENCES


FIGURE 2

A

CYP27

Cyclophilin

2.1 Kb

1.0 Kb

Control  StAR  CYP27  StAR+CYP27

B

55 kDa

Control  StAR  CYP27  StAR+CYP27
FIGURE 3

A

Time (h) 0 3 6 12 24 48

StAR

B

[^14C]-Cholesterol DPM/mg X 10^{-3}

Methanol Phase (Bile Acid Synthesis)

Control
StAR
Cyp27

Culture Time (hrs) 0 10 20 30 40 50

[^14C]-Cholesterol DPM/mg X 10^{-4}

Chloroform Phase (Cholesterol uptake)

Control
StAR
Cyp27

Culture Time (hrs) 0 10 20 30 40 50
FIGURE 6

Relative Absorbance at 240 nm

<table>
<thead>
<tr>
<th></th>
<th>Standard</th>
<th>Control</th>
<th>StAR</th>
<th>CYP27</th>
<th>StAR+CYP27</th>
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<tr>
<td><strong>A</strong></td>
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Retention Time (min)

- 15
- 16
- 17
- 18
- 19
- 20
- 21

27-Hydroxycholesterol
FIGURE 7

A

B

[1^4C]-Cholesterol DPM/ml X 10^-3

Methanol Phase (Bile Acid Synthesis)

[1^4C]-Cholesterol DPM/ml X 10^-3

Chloroform Phase (Cholesterol Uptake)

Culture Time (Hrs)

Culture Time (Hrs)

NA
Control Virus
S1AR

NA
Control virus
S1AR
FIGURE 8

Bile Acid Synthesis
umoles/h/100g rat

Control
- StAR

Bile acid pool drainage

Basal rate of bile acid synthesis

Time (hours)
Post Biliary Diversion

0 20 40 60 80

0 1 2 3 4
Transport of cholesterol into mitochondria is rate limiting for bile acid synthesis via the alternative pathway in primary rat hepatocytes
William M. Pandak, Shunlin Ren, Dalila Marques, Elizabeth Hall, Kaye Redford, Darrell Mallonee, Patricia Bohdan, Douglas Heuman, Gregorio Gil and Phillip Hylemon

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