

Effect of increasing the expression of cholesterol transporters (StAR, MLN64, and SCP-2) on bile acid synthesis

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Abstract There are two major pathways of bile acid synthesis: the “neutral” pathway, initiated by highly regulated microsomal cholesterol 7 α -hydroxylase (CYP7A1), and an “alternative” pathway, initiated by mitochondrial sterol 27-hydroxylase (CYP27A1). In hepatocyte cultures, overexpression of CYP7A1 increases bile acid synthesis by >8-fold. However, overexpression of CYP27A1 in hepatocytes only increases it by 1.5-fold, suggesting that additional rate-limiting steps must be involved in the regulation of this pathway. The effects of intracellular cholesterol transport proteins on bile acid synthesis have been investigated in the current study. Under culture conditions in which the neutral pathway was inactive, selective overexpression of the gene encoding steroidogenic acute regulatory protein (StAR), MLN64 (StAR homolog protein), and sterol carrier protein-2 (SCP-2) led to 5.7-, 1.2-, and 1.7-fold increases, respectively, in the rates of bile acid synthesis in primary rat hepatocytes. Surprisingly, co-overexpression of MLN64 with StAR, SCP-2, or CYP7A1 blunted the upregulated bile acid synthesis by 48, 47, and 45%, respectively. These results suggest that MLN64, in its full-length form, is not responsible for the transport of cholesterol to the mitochondria or the endoplasmic reticulum, where CYP27A1 or CYP7A1 is located, respectively.—Ren, S., P. Hylemon, D. Marques, E. Hall, K. Redford, G. Gil, and W. M. Pandak. **Effect of increasing the expression of cholesterol transporters (StAR, MLN64, and SCP-2) on bile acid synthesis.** *J. Lipid Res.* 2004. 45: 2123–2131.

Supplementary key words metabolism • sterol carrier protein-2 • MLN64 • sterol 27-hydroxylase • cholesterol 7 α -hydroxylase • steroidogenic acute regulatory protein • mitochondria

The liver plays a pivotal role in the maintenance of cholesterol homeostasis. Under physiologic conditions, cholesterol input into the body equals cholesterol output (1). Bile acid synthesis in the liver is the major pathway for cholesterol output. The biotransformation of cholesterol to primary bile acids occurs via two main pathways. The “neutral” pathway begins with a reaction catalyzed by cho-

lesterol 7 α -hydroxylase (CYP7A1), the initial and rate-determining step in this pathway (2, 3). In an “alternative” pathway, the initial and presumed rate-determining step is catalyzed by mitochondrial sterol 27-hydroxylase (CYP27A1). However, overexpression of the gene encoding CYP27A1 in primary rat and human hepatocytes only led to 147% and 173% increases, respectively, in bile acid synthesis (Table 1) (4). By comparison, a >8-fold increase was seen after the overexpression of CYP7A1 (5). The uneven distribution of cholesterol among intracellular membranes coupled with cholesterol’s limited solubility in water makes it unlikely that cholesterol can be transported to or into mitochondria within the cell unaided. This led us to hypothesize that increasing cholesterol delivery to or into the inner mitochondrial membrane where CYP27A1 is located could potentially increase the rate of bile acid synthesis via the alternative pathway. In steroidogenic tissues, the transport of cholesterol to mitochondria and into the inner membrane is the rate-limiting step in steroidogenesis (6). The protein believed responsible and rate-limiting for this process is the steroidogenic acute regulatory protein (StAR). StAR contains a N-terminal mitochondrial targeting sequence and a C-terminal cholesterol binding domain, allowing it to bind and direct cholesterol to the mitochondria. Recently, overexpression of StAR was found to dramatically increase bile acid synthesis in primary rat hepatocytes, suggesting that cholesterol delivery from the outer and the inner membranes of mitochondria is the rate-determining step for bile acid synthesis via the alternative pathway (7).

Besides StAR, several other proteins are believed to be involved in mitochondrial cholesterol trafficking, but their role(s) in bile acid synthesis remain(s) poorly defined. MLN64, a protein found in many human tissues, including the liver, is highly homologous to StAR and highly conserved (8). Mutations that inactivate StAR have been correlated with amino acid residues that are identical or

Manuscript received 16 June 2004 and in revised form 11 August 2004.

Published, JLR Papers in Press, September 1, 2004.

DOI 10.1194/jlr.M400233JLR200

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TABLE 1. Comparison of the overexpression of CYP27A1 and CYP7A1 on bile acid synthesis in primary rat and human hepatocytes

Protein/Cell	PRH	PHH
CYP7A1	1,096 ± 179% (<i>P</i> < 0.001, <i>n</i> = 12)	1,768 ± 549% (<i>P</i> < 0.001, <i>n</i> = 10)
CYP27A1	147 ± 13% (<i>P</i> = 0.01, <i>n</i> = 4)	173 ± 42% (<i>P</i> < 0.001, <i>n</i> = 7)

CMV, cytomegalovirus promoter; PHH, primary human hepatocytes; CYP7A1, cholesterol 7 α -hydroxylase; CYP27A1, sterol 27-hydroxylase; PRH, primary rat hepatocytes. Twenty-four hours after plating, hepatocytes were infected with recombinant adenovirus encoding the CMV-driven CYP27A1 or CYP7A1 gene. Cultures were then harvested at 48 h after infection. Bile acid synthesis rates were determined as conversion of [¹⁴C]cholesterol into [¹⁴C]bile acid intermediate, methanol/water-extractable counts from cell-cultured media after Folch partitioning. Rates of bile acid synthesis are expressed as percentages of virus (null) controls (means ± SEM).

similar to those of MLN64, indicating that conserved motifs are important for steroidogenic activity (8). Overexpression of N-terminal truncated MLN64 has been found to dramatically increase steroidogenesis in vitro (9). However, these increases have not been observed with the full-length protein. Sterol carrier protein-2 (SCP-2) enhances sterol cycling and facilitates cholesterol translocation between intracellular organelles and the plasma membrane in cultured hepatocytes (10). To replenish cholesterol in the mitochondrial outer membrane, SCP-2 is believed to mediate a slower transfer of cholesterol from other intracellular sites (plasma membranes, lysosomes) to the outer mitochondrial membrane (11), suggesting that SCP-2 might be involved in bile acid synthesis through the alternative pathway.

The present study shows that selective overexpression of the genes encoding StAR, MLN64, and SCP-2 in hepatocytes all increase bile acid synthesis, but to different levels. Interestingly, co-overexpression of MLN64 with StAR, SCP-2, or CYP7A1 blunted the StAR-, SCP-2-, or CYP7A1-mediated upregulation of bile acid synthesis. These results suggest that MLN64 does not direct cholesterol to places where bile acid synthesis occurs.

EXPERIMENTAL PROCEDURES

Cell culture reagents and supplies were purchased from GIBCO BRL (Grand Island, NY). The RPA II Kit was purchased from Ambion (Austin, TX). [¹⁴C]cholesterol and [³H]25-hydroxycholesterol were purchased from New England Nuclear (Boston, MA). Cyclodextrin was purchased from Cyclodextrin Technologies Development, Inc. (Gainesville, 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). 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 using the collagenase-perfusion technique (7). Cells were routinely harvested after 72 h of culture as previously described (12). Unless specified, cells were main-

tained under conditions in which CYP7A1 activity is undetectable (i.e., cultured in the absence of thyroid hormone) (13).

Generation of recombinant adenoviruses and their overexpression

The adenovirus constructs used in this study were obtained through the Massey Cancer Center Shared Resource Facility of Virginia Commonwealth University. The cytomegalo promoter (CMV)-CYP27A1 and CMV-CYP7A1 recombinant adenovirus clones (Ad-CMV-CYP27A1 and Ad-CMV-CYP7A1) were constructed as previously described (4); the recombinant cDNAs MLN64 and StAR were a generous gift from Dr. J. Strauss (14), and SCP-2 was a generous gift from Dr. J. Glick (15, 16).

Briefly, the CMV-StAR, CMV-MLN64, and CMV-SCP-2 recombinant adenovirus clones (Ad-CMV-StAR, Ad-CMV-MLN64, and Ad-DMV-SCP-2) were obtained using a pTG-CMV system as previously described (4). Human cDNA of StAR, MLN64, or SCP-2 was cloned individually into the *SalI/NotI* sites of pZero TG-CMV, a plasmid containing a CMV promoter, multiple cloning sites, and partial DNA sequence from Ad5d1324. The resulting pZero TG-CMV/StAR, pZero TG-CMV/MLN64, and pZero TG-CMV/SCP-2 recombinant plasmids were cotransformed with *ClaI*-linearized pTG-CMV (containing the entire Ad5d1324 genome) into *Escherichia coli*. Resulting plasmids were screened for inserts before being transfected into 293 cells (a human embryonic kidney cell line). Adenovirus DNA from resulting plaques was further screened by Southern blot for the presence of polylinker cassette without any insert DNA.

Propagation of Ad-CMV-StAR, Ad-CMV-CYP27A1, Ad-CMV-MLN64, and Ad-CMV-SCP-2. 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 of 1 plaque-forming unit (pfu)/cell. After 2 h of infection, unbound virus was removed and DMEM with 2% 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 2,700 *g* at 4°C for 10 min. The pellet was suspended in DMEM with 2% FBS and subjected to five cycles of freeze/thaw lyses to release the recombinant virus. Cell debris was removed by centrifugation at 7,700 *g* for 10 min 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 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* at 20°C for 18 h. The pure viral opalescent band was removed and dialyzed overnight at 4°C against 10 mM Tris-HCl, pH 7.4, 1 mM MgCl₂, and 10% glycerol. The virus was divided into aliquots and stored at –70°C until used. The virus titer was determined by plaque assay, and viral particles were determined by optical density using spectrophotometry (260 nm).

Infection of cells with adenovirus encoding StAR, MLN64, SCP-2, CYP7A1, or CYP27A1. Primary rat hepatocyte cultures, prepared as previously described (12), were plated on P150 cm² tissue culture dishes (~2.5 × 10⁷ cells) in Williams' E medium containing dexamethasone (0.1 μM). Unless otherwise specified, cells were maintained in the absence of thyroid hormone. We have previously shown that, in the absence of thyroid hormone, CYP7A1 activity is undetectable and only the alternative pathway of bile acid synthesis is functional (12). As noted, in selected studies, thyroid hormone (L-thyroxine) was added as previously described at a concentration of 1.0 μM. With the addition of thyroid hormone

to culture medium, CYP7A1 and the neutral pathway are fully functional. Previously, we have demonstrated that CYP7A1 mRNA levels under these culture conditions (i.e., with thyroid hormone) are upregulated to the level of cholestyramine-fed rats (12). Twenty-four hours after plating, culture medium was removed, and 2.5 ml of fresh medium was added. Cells were then infected with unpurified recombinant adenovirus encoding Ad-CMV-StAR, Ad-CMV-MLN64, Ad-CMV-SCP-2, Ad-CMV-CYP7A1, or Ad-CMV-CYP27A1 at 10 pfu/cell. All experiments were compared with Ad-CMV control virus infections. Ad-CMV control virus infections were also compared with no-virus-addition cultures. The virus was allowed to dwell for at least 2 h in minimal culture medium with shaking of the plates gently every 15 min. After 2 h of infection, unbound virus was removed and replaced with 20 ml of fresh medium. The cells were incubated at 37°C and 5% CO₂ for 48 h. Cells were then harvested as previously described (12).

Protein levels

After infection, cells were either harvested by adding sample buffer or subcellular fractions were separated and isolated by centrifugation as previously described (4). Mitochondrial subcellular fractions were not further purified to separate lysosomal contaminant. Proteins were then solubilized by adding 2× 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 min. Three micrograms of solubilized proteins was analyzed by 10% SDS-PAGE. Electrophoresis was performed at 20 mA for 2 h in a Bio-Rad (Richmond, CA) mini gel system. StAR, SCP-2, CYP27A1, and MLN64 proteins were identified by Western blot analysis. After electrophoresis, samples were transblotted onto a 0.2 µm nitrocellulose membrane in 25 mM Tris buffer, pH 8.3, containing 192 mM glycine and 20% methanol. Western blotting was performed with a Bio-Rad system at 100 V constant voltage at 4°C for 1 h. Membranes were blocked with 3% nonfat dry milk in 10 mM HEPES buffer, pH 7.4, containing 25 mM EDTA, 0.5 M NaCl, and 0.05% NaN₃ and immunostained with rabbit polyclonal antibodies (1:2,000 dilution) against human StAR and MLN64 proteins (a generous gift from Dr. Jerome Strauss, Department of Obstetrics and Gynecology, University of Pennsylvania, Philadelphia, PA), against rat CYP27A1 protein (a generous gift from Dr. N. Avadhani, University of Pennsylvania, Philadelphia, PA) (4), and against SCP-2 protein (a generous gift from Dr. J. Glick) (14, 15) in HEPES buffer, washed with the same buffer plus 0.05% Tween 20, and 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 Kodak BioMax film (Sigma).

Bile acid biosynthesis and analysis

Bile acid synthetic rates and individual bile acids were determined and identified as previously described (4). Briefly, to identify the individual bile acids, the [¹⁴C]methanol/water phase was first base hydrolyzed, then separated by TLC in a solvent system of ethyl acetate-cyclohexane-acetic acid (7.7:2.3:1, v/v/v). ¹⁴C-labeled bile acids were visualized with a phosphorimager.

Statistics

Data are reported as means ± SEM. Where indicated, data were subjected to *t*-test analysis and determined to be significantly different if *P* < 0.05.

RESULTS

Overexpression of the genes encoding StAR, CYP27A1, MLN64, and SCP-2 proteins

Infection of primary rat hepatocytes with recombinant adenovirus containing a CMV-driven gene encoding StAR, CYP27A1, MLN64, or SCP-2 produced high StAR, CYP27A1, MLN64, and SCP-2 mRNA (data not shown) and protein levels without inducing any evidence of cell toxicity. Cell viability was more than 95% based on trypan blue staining for all conditions studied (data not shown). **Figure 1** shows Western blot analysis of StAR, CYP27A1, MLN64, and SCP-2 protein levels 48 h after infection. Figure 1Aa, showing a Western blot of mitochondrial proteins, shows one major immunoreactive band with a molecular mass of 57 kDa after overexpression of the gene encoding CYP27A1. Western blot analysis of mitochondrial proteins (30 µg) after StAR overexpression showed one major immunoreactive band with a molecular mass of 30 kDa and a second band with the molecular mass of 34 kDa (Fig. 1Ab), consistent with StAR maturing proteins as previously reported (6, 17). Figure 1Ba shows Western blot analysis with antibody against SCP-2 with one major band (13 kDa) and one minor band (15 kDa), consistent with mature SCP-2 and pro-SCP-2 protein after overexpression or co-overexpression of the gene(s) encoding SCP-2 and StAR, suggesting that ~65% of the overexpressed pro-SCP-2 was processed to the mature 13 kDa form. Western blot analysis of mitochondrial protein (5 µg) with antibody against StAR protein again showed the major band with a molecular mass of 30 kDa (Fig. 1Bb), consistent with StAR mature protein (much less total protein was loaded compared with those in Fig. 1A). Figure 1Ca, showing Western blot analysis with antibody against MLN64, shows one band with a molecular mass of 54 kDa in control cells, a band that represents a cross-reacting protein (nonspecific binding protein); two bands of 54 and 30 kDa, the latter molecule of which is consistent with mature StAR protein, in the cells with StAR overexpression; two bands of 54 and 50 kDa, the latter molecule of which is consistent with recombinant MLN64 protein, in cells with MLN64 overexpression; and three bands of 54, 50, and 30 kDa, the latter two of which are consistent with recombinant MLN64 and mature StAR proteins, in StAR and MLN64 co-overexpression cells. Because the 30 kDa protein only appears in the cells infected with virus encoding StAR or StAR plus MLN64 genes, we believe that the 30 kDa protein is StAR and the antibody against MLN64 is cross-reactive with StAR protein. Western blot analysis with StAR antibody shows only one band with a 30 kDa molecular mass in the cells with StAR overexpression or StAR/MLN64 co-overexpression (Fig. 1Cb). Based on the band densities, co-overexpression of StAR with SCP-2 or MLN64, either alone or in combination, did not alter the levels of the other proteins.

Subcellular distribution of overexpressed cholesterol transport proteins in primary rat hepatocytes

Primary rat hepatocyte subcellular fractions were then isolated (see Experimental Procedures) and the distribu-

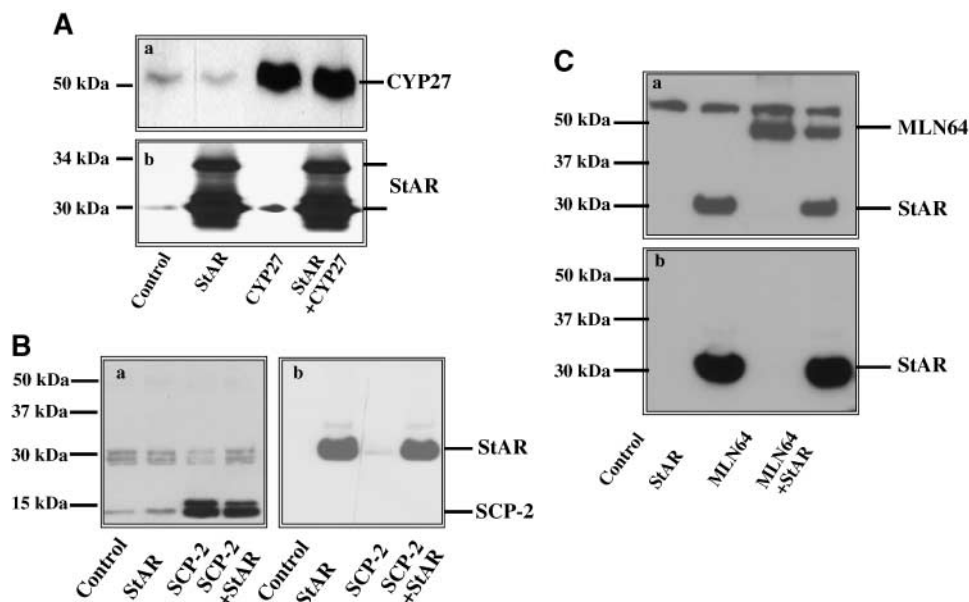


Fig. 1. Transport protein levels in primary rat hepatocytes after steroidogenic acute regulatory protein (StAR), sterol carrier protein-2 (SCP-2), MLN64, and sterol 27-hydroxylase (CYP27A1) overexpression. Primary rat hepatocytes were incubated in culture medium containing 0.1 μ M dexamethasone and infected with either control virus or recombinant adenovirus as described in Experimental Procedures. Cells were harvested at 48 h after infection. A: Western blot analysis of CYP27A1 (a) and StAR (b) protein levels in mitochondria. Mitochondrial protein (30 μ g) was loaded onto each lane for each condition. Cells were infected with the indicated viruses. B: Western blot analysis of SCP-2 (a) and StAR (b) protein levels in mitochondria. Mitochondrial protein (5 μ g) was loaded onto each lane for each condition. Cells were infected with the indicated viruses. C: Western blot analysis of MLN64 (a) and StAR (b) protein levels in mitochondria. Mitochondrial protein (5 μ g) was loaded onto each lane for each condition. Cells were infected with the indicated viruses.

tions of the proteins examined using Western blot analysis after the cells were infected with the recombinant adenovirus. In the hepatocytes infected with the recombinant MLN64 gene, MLN64 protein was present in the mitochondrial and microsomal enriched fractions but not in the cytosol. In the cells infected with the SCP-2 gene, SCP-2 protein was distributed in all cellular fractions (**Fig. 2B**). In cells infected with recombinant CMV-driven StAR gene overexpression, StAR protein was found widely distributed in the cytosol, microsomes, and mitochondrial enriched fractions (**Fig. 2C**). These results suggest that all of these three recombinant cholesterol transporters may have the ability to interact with mitochondrial membranes and that the distribution of MLN64 is different from that of StAR protein.

Effects of cholesterol transport proteins on the rate of bile acid synthesis

The effects of the three cholesterol transport proteins on the rates of bile acid synthesis in cells were determined via the conversion of [14 C]cholesterol into [14 C]methanol/water-extractable products. Overexpression of the gene encoding StAR protein dramatically increased rates of bile acid synthesis by day 3 after infection with the recombinant adenovirus encoding CMV-driven StAR gene in both primary rat hepatocytes and primary human hepatocytes (**Table 2**). Overexpression of SCP-2 and MLN64 protein

also increased rates of bile acid synthesis at day 3 after infection, but to a significantly lower level. Infection with recombinant adenovirus encoding the CMV-driven CYP27A1 gene only slightly increased bile acid synthesis rates in both cells.

To pursue the association between StAR and other cholesterol transport proteins, combination experiments using overexpression of StAR with SCP-2 or MLN64 were performed in primary rat hepatocytes. In addition, StAR was overexpressed with CYP27A1 or CYP7A1 (**Fig. 3**). In the absence of thyroid hormone (T₄) and the presence of dexamethasone, culture conditions in which the neutral pathway is inactive, coinfection with recombinant StAR and CYP27A1 adenovirus did not significantly increase bile acid synthesis more than StAR alone. Either infection or coinfection with recombinant CYP7A1 and/or StAR increased bile acid synthesis by 7- to 10-fold. These findings may suggest that the available cholesterol supply is sufficient for a bile acid synthesis increase of up to 10-fold in the system studied and that StAR and CYP7A1 may compete for cholesterol from the same location, the endoplasmic reticulum membrane.

Surprisingly, coinfection of MLN64 with the StAR gene limited StAR upregulation of bile acid synthesis by 48% ($P < 0.0001$, $n = 6$) (**Fig. 4**), whereas coinfection of SCP-2 with StAR did not significantly affect StAR upregulation of bile acid synthesis ($P = 0.29$) compared with StAR alone. These

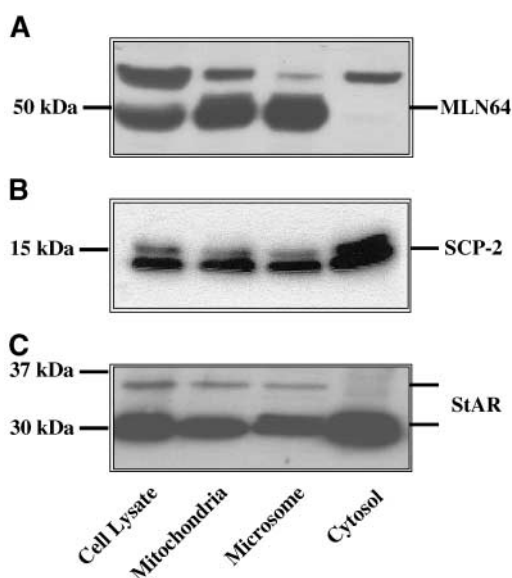


Fig. 2. Western blot analysis of transport protein intracellular distribution after overexpression of StAR, SCP-2, or MLN64 in primary rat hepatocytes. Subcellular fraction protein (3 μ g) was loaded onto each lane for each condition on a 12% SDS-PAGE gel. Lane 1, cell lysate; lane 2, mitochondrial fraction; lane 3, microsome; lane 4, cytosol. A: Antibody against MLN64 protein staining. B: Antibody against SCP-2 protein staining. C: Antibody against StAR protein staining.

three proteins have been reported to be able to transport cholesterol into mitochondria from different subcellular organelles (18–21). However, our results suggest that each of these cholesterol transport proteins may transport cholesterol in a selective direction. To study the effect of MLN64 on the neutral pathway, MLN64 was co-overexpressed with CYP7A1. Interestingly, MLN64 inhibited CYP7A1 upregulation of bile acid synthesis by 47% ($P < 0.0001$, $n = 4$) (Fig. 4). Furthermore, MLN64 also inhibited SCP-2 upregulated bile acid synthesis by 45% ($P < 0.0001$, $n = 4$) (Fig. 4). Those results strongly suggested that MLN64 protein does not transport cholesterol to the

mitochondria or to the endoplasmic reticulum, where CYP27A1 and CYP7A1 are located.

To further study whether co-overexpression of MLN64 with StAR altered the cholesterol-cholesteryl ester ratio, the cells were stained with Filipin for the detection of free cholesterol, and the cholesteryl ester content from the cells was characterized by TLC analysis. StAR overexpression led to a significant increase in Filipin-stained cholesterol and an associated decrease in cholesteryl ester content within cells (data not shown). MLN64 overexpression alone did not change the levels of free cholesterol or cholesteryl ester content (data not shown) compared with control virus infection. Co-overexpression of MLN64 with StAR did not change StAR's effects on cellular cholesterol or cholesteryl ester content (data not shown). These results suggest that MLN64-inhibited, StAR-upregulated bile acid synthesis was not caused by either decreasing free cholesterol or the formation of cholesteryl esters.

To test the effects of cholesterol transport proteins on bile acid synthesis through the neutral pathway, T4 was added to the culture medium as described in Experimental Procedures. Under these conditions, CYP7A1 and the neutral pathway of bile acid synthesis are fully active (5, 12). Of note is that CYP7A1 mRNA levels under these culture conditions are greater than those found in the upregulated cholestyramine-fed rat model (12). Using these culture conditions, the basal rates of bile acid synthesis increased by ~ 2 -fold compared with cells cultured in the absence of T4. Overexpression of the StAR gene still led to an ~ 2 -fold increase ($P < 0.001$) in the rates of bile acid synthesis (Fig. 5). Figure 5 also shows the effects of the other cholesterol transport proteins on the rates of bile acid synthesis in the presence of T4 and dexamethasone in primary rat hepatocytes. Infection with recombinant CYP7A1 and/or StAR genes increased bile acid synthesis by 4-fold. There was no significant difference between CYP7A1 and the combination of CYP7A1 and StAR co-overexpression on the regulation of bile acid synthesis. Infection with CYP27A1 or SCP-2 gene alone only slightly increased bile acid synthesis by ~ 1.2 - or 1.3 -fold, respectively, compared with StAR overexpression, which increased the rate of bile acid synthesis by ~ 2 -fold. Co-overexpression of CYP27A1 or SCP-2 with StAR did not affect the rates of StAR-upregulated bile acid synthesis ($P = 0.17$ or $P = 0.8$, respectively). Interestingly, infection with MLN64 significantly decreased the rates of bile acid synthesis ($79 \pm 9\%$; $P = 0.005$, $n = 4$), and when coinfecting with StAR, MLN64 significantly inhibited StAR-upregulated bile acid synthesis by 20% (paired t -test: $P = 0.029$).

Characterization of bile acid synthesis products

The steroid products in water/methanol-extractable phases were further analyzed by TLC as shown in Fig. 6. [14 C]steroid-extractable products in the chloroform phase were mainly composed of cholesteryl esters, cholesterol, 25-hydroxycholesterol, 27-hydroxycholesterol, 3-oxo-7-hydroxycholesterol, and 7,27-dihydroxycholesterol. Cholesterol and cholesteryl esters were decreased in the culture media of cells overexpressing the gene encoding CMV-driven StAR

TABLE 2. Effects of cholesterol transport proteins on the alternative pathway of bile acid synthesis in hepatocytes

Protein/Cell	PRH	PHH
CYP27	147 \pm 13% ($P = 0.01$, $n = 4$)	173 \pm 48% ($P < 0.01$, $n = 7$)
StAR	568 \pm 48% ($P < 0.001$, $n = 10$)	614 \pm 77% ($P < 0.001$, $n = 7$)
SCP-2	168 \pm 19% ($P < 0.01$, $n = 6$)	268 \pm 29% ($P < 0.001$, $n = 6$)
MLN64	124 \pm 6% ($P = 0.001$, $n = 7$)	150 \pm 34% ($P = 0.1$, $n = 6$)

Twenty-four hours after plating, hepatocytes were infected with recombinant adenovirus encoding CMV-driven steroidogenic acute regulatory protein (StAR), CYP27A1, sterol carrier protein-2 (SCP-2), MLN64 gene, or null virus (control). Cultures were then harvested at 48 h after infection. Bile acid synthesis rates were determined as conversion of [14 C]cholesterol into [14 C]bile acid intermediate, methanol/water-extractable counts from cell-cultured media after Folch partitioning. Rates of bile acid synthesis are expressed as percentages of virus (null) controls (means \pm SEM).

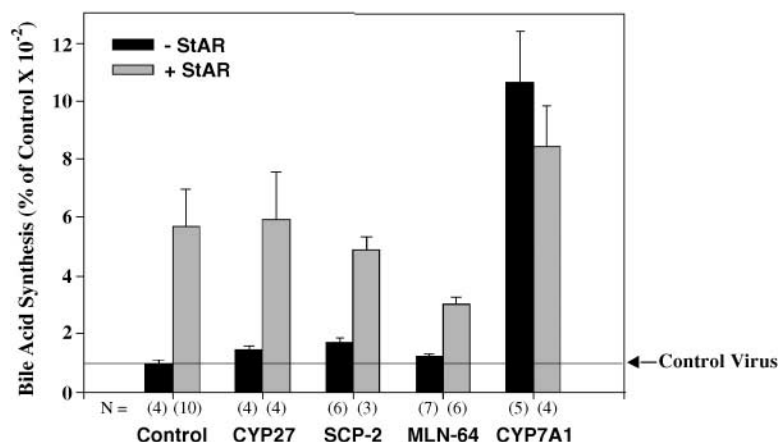


Fig. 3. Effect of cholesterol transporter combination on the alternative pathway of bile acid synthesis in primary rat hepatocytes. Twenty-four hours after plating, hepatocytes were infected with recombinant adenovirus encoding a cytomegalovirus promoter (CMV)-driven StAR gene, CMV-driven CYP27A1 gene, CMV-driven SCP-2, CMV-driven MLN64, CMV-driven cholesterol 7 α -hydroxylase (CYP7A1), StAR plus CYP27A1, StAR plus SCP-2, StAR plus MLN64, StAR plus CYP7A1, or null virus (control). Cultures were then harvested at 48 h after infection. Bile acid synthesis was measured as conversion of [¹⁴C]cholesterol into [¹⁴C]bile acid intermediate, methanol/water-extractable counts from cell-cultured media after Folch partitioning. Rates of bile acid synthesis are expressed as percentages of virus (null) controls (means \pm SEM).

and SCP-2. Conversely, 27-hydroxycholesterol and 7,27-dihydroxycholesterol levels increased significantly in the cells overexpressing StAR, SCP-2, or StAR plus CYP27A1 (data not shown), consistent with previously described findings (7). Because CYP27A1 is located in the inner mitochondrial membrane, it is assumed that 27-hydroxycholesterol and 7,27-dihydroxycholesterol are products of the alternative pathway of bile acid synthesis. TLC analysis of products in the methanol-extracted phase of the cells is shown in Fig. 6. The largest increase in soluble steroids in cultured media after StAR and/or SCP-2 was seen in β -muricholic acid and chenodeoxycholic acid, consistent with previous findings (7). The compositions of sterol products in the cells with overexpression of StAR, SCP-2, MLN64, StAR/SCP-2, StAR/MLN64, or StAR/CYP27A1 demonstrated no significant difference (data not shown), suggesting that all of these transporters increase bile acid synthesis through the same pathway.

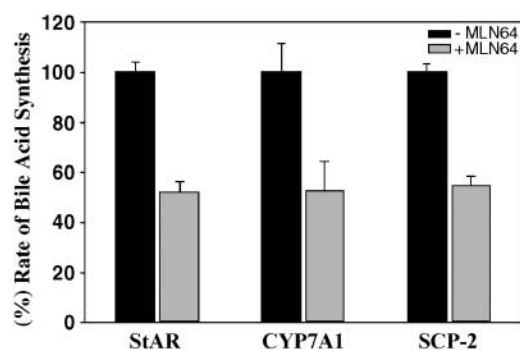


Fig. 4. Effect of overexpression of MLN64 on the rates of bile acid synthesis upregulated by StAR, CYP7A1, and SCP-2 in primary rat hepatocytes. Twenty-four hours after plating, hepatocytes were infected with recombinant adenovirus encoding CMV-driven StAR gene, CMV-driven CYP7A1 gene, and CMV-driven SCP-2 with or without CMV-driven MLN64. Cultures were then harvested at 48 h after infection. Bile acid synthesis was measured as conversion of [¹⁴C]cholesterol into [¹⁴C]bile acid intermediate, methanol/water-extractable counts from cell-cultured media after Folch partitioning. Rates of bile acid synthesis are expressed as percentages of the rates in cells without MLN64 gene expression (means \pm SEM). By paired *t*-test, *P* < 0.001 for all three groups.

DISCUSSION

Evidence for mitochondrial cholesterol transport as a rate-limiting step of bile acid synthesis via the alternative pathway gave rise to a new hypothesis and understanding of the regulation of bile acid synthesis (7). In the current study, the effects of selectively increasing the expression of three cholesterol transport proteins, either alone or in combination, on the rates of bile acid synthesis were investigated and compared in primary rat and primary human hepatocytes. StAR, SCP-2, and MLN64, which have been reported to bind and transport cholesterol from intracellular locations to mitochondria, all increased bile acid biosynthesis, but only StAR dramatically increased synthesis (6-fold). Surprisingly, with co-overexpression, MLN64 inhibited StAR-, SCP-2-, or CYP7A1-upregulated bile acid biosynthesis.

MLN64 is a gene product of unknown function. The N terminus of MLN64 contains four potential transmembrane domains and a C terminus with striking homologies to StAR (8, 22). N-terminal truncated MLN64 led to an \sim 2-fold increase in pregnenolone secretion over COS-1 cells transfected with the cholesterol side chain cleavage enzyme, whereas StAR increased pregnenolone production by nearly 7-fold (9). In the present study, despite overexpression of full-length MLN64 protein in hepatocytes leading to a large increase in the protein levels in the mitochondria fraction, bile acid synthesis was increased by only 1.2-fold (Fig. 3). Although removal of the N-terminal mitochondrial targeting sequence and the putative transmembrane domains for both StAR and MLN64 increased steroidogenesis, the physiological significance of these truncated cholesterol-binding proteins is unclear (9). Thus, the true difference in the function of StAR and StAR homologous MLN64 may be secondary to their proteolytic processing.

StAR protein can process spontaneously to the mature 30 kDa form after overexpression in hepatocytes and has been reported to become a molten protein in the membranes, allowing it to aid in the transport of cholesterol from outer to inner membranes of mitochondria (17, 23, 24). Moreover, under conditions of increased concentration, the N-terminal truncated form of StAR (30 kDa) has

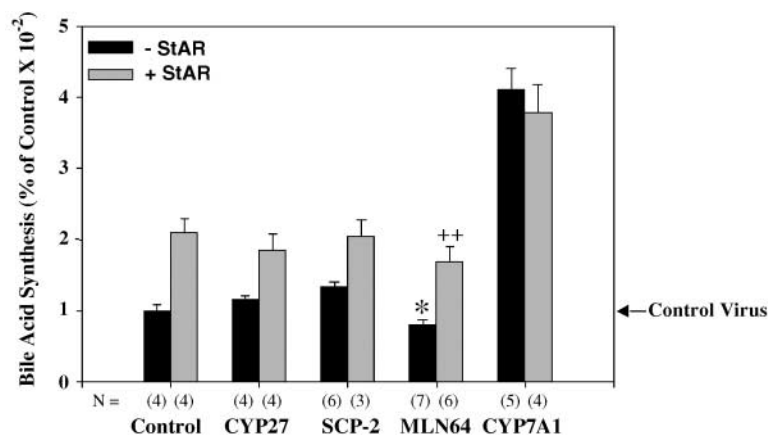


Fig. 5. Effect of cholesterol transporter combination on bile acid synthesis in the presence of thyroid hormone and dexamethasone in primary rat hepatocytes. Twenty-four hours after plating, hepatocytes were infected with recombinant adenovirus encoding CMV-driven StAR gene, CMV-driven CYP27A1 gene, CMV-driven SCP-2, CMV-driven MLN64, CMV-driven CYP7A1, StAR plus CYP27A1, StAR plus SCP-2, StAR plus MLN64, StAR plus CYP7A1, or null virus (control). Cultures were then harvested at 48 h after infection. Bile acid synthesis was measured as conversion of [14 C]cholesterol into [14 C]bile acid intermediate, methanol/water-extractable counts from cell-cultured media after Folch partitioning. Rates of bile acid synthesis are expressed as percentages of virus (null) controls (means \pm SEM). By paired *t*-test, * $P = 0.005$ and ** $P = 0.029$.

been shown to have the same biological activity as the full-length wild-type protein (20, 25). In contrast, Western blot analysis of MLN64 after overexpression showed no proteolytic processing of the protein (50 kDa), suggesting that MLN64 might not be able to generate a molten protein in cells (Fig. 1). This offers an explanation for why overexpression of StAR increases bile acid synthesis by 6-fold, whereas MLN64 overexpression increases it by only 1.2-fold. It is interesting that the combination of overexpression of StAR/MLN64 inhibited StAR upregulation of bile acid synthesis by 48% compared with StAR alone (Fig. 3), that overexpression of CYP7A1/MLN64 inhibited CYP7A1 upregulation of bile acid synthesis by 47% compared with CYP7A1 alone, and that overexpression of SCP-2/MLN64 inhibited SCP-2 upregulation of bile acid synthesis by 47%. The mechanism responsible for these reductions in rates of bile acid synthesis is currently not known. One could hypothesize that MLN64 may decrease the cellular cholesterol pool for mitochondria and the endoplasmic reticulum. A recent report shows that MLN64 constitutively traffics via dynamic NPC-1-containing late endosomal tubules in normal cells and that this dynamic movement was inhibited in cholesterol-loaded cells, where MLN64 appears to be trapped at the periphery of cholesterol-laden lysosomes (19). A truncated form of MLN64, which contains N-terminal transmembrane domains but lacks the start domain, caused free cholesterol accumulation in lysosomes and inhibited late endocytic dynamics (19). These findings suggest that MLN64 is important in the

movement of cholesterol between late endosomes and lysosomes (Fig. 7). The present results show that the overexpression of MLN64 dramatically inhibits StAR-, SCP-2-, and CYP7A1-upregulated bile acid synthesis via both the alternative and neutral pathways after overexpression. The results suggest that MLN64 does not efficiently transport cholesterol to the mitochondria or to the endoplasmic reticulum. Instead, MLN64 more likely is involved in the directional movement of cholesterol from the late endosomes to the lysosomes, and then to the plasma membrane (Fig. 7). Thus, a reasonable interpretation of our results is that overexpressed MLN64 protein takes more cholesterol from late endosomes to the lysosomes and then to the plasma membrane, making less cholesterol available for StAR and CYP7A1. These results are consistent with the mechanism proposed by Strauss and colleagues (19).

Since its discovery three decades ago, SCP-2 has remained a fascinating protein whose effect on cholesterol trafficking is still not fully understood. It participates in mediating cholesterol transfer from lysosomes to plasma membranes (10), from lysosomes to mitochondria, and from mitochondria to lysosomes (11). Therefore, it is unlikely that SCP-2 should have a unique transport direction in cholesterol trafficking. Fuchs et al. (26) reported that C57BL/6 (SCP-2 $^{-/-}$) mice decrease bile salt secretion. Moreover, overexpression of SCP-2 in mice increased bile acid output (15). In the present study, we showed that overexpression of SCP-2 only modestly (1.7-fold) increased the rate of bile acid synthesis, suggesting that SCP-2 may not

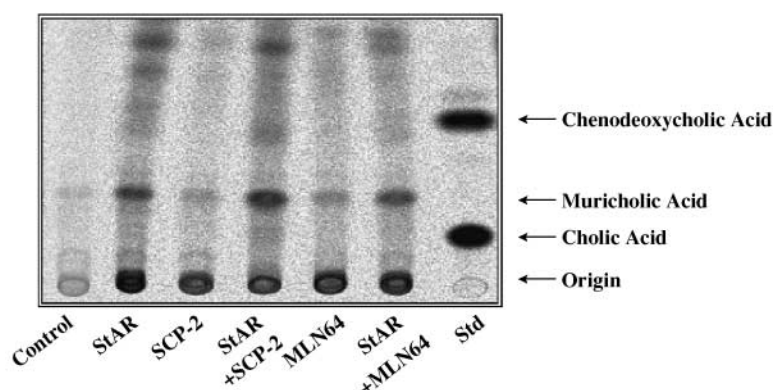


Fig. 6. Thin-layer chromatography analysis of [14 C]methanol/water-extractable sterol products. From cell-cultured media, conjugated bile acids were extracted into the methanol/water phase, base hydrolyzed, and analyzed by TLC in a solvent system of ethyl acetate-cyclohexane-acetic acid (7:2.3:1, v/v/v). [14 C]-labeled bile acids were visualized in a phosphorimager. Cells were infected with control (null) virus (lane 1), Ad-CMV-StAR (lane 2), Ad-CMV-SCP-2 (lane 3), StAR plus SCP-2 (lane 4), AdCMV-MLN64 (lane 5), or AdCMV-StAR plus MLN64 (lane 6). Radiolabeled standards (Std) were chenodeoxycholic acid and cholic acid.

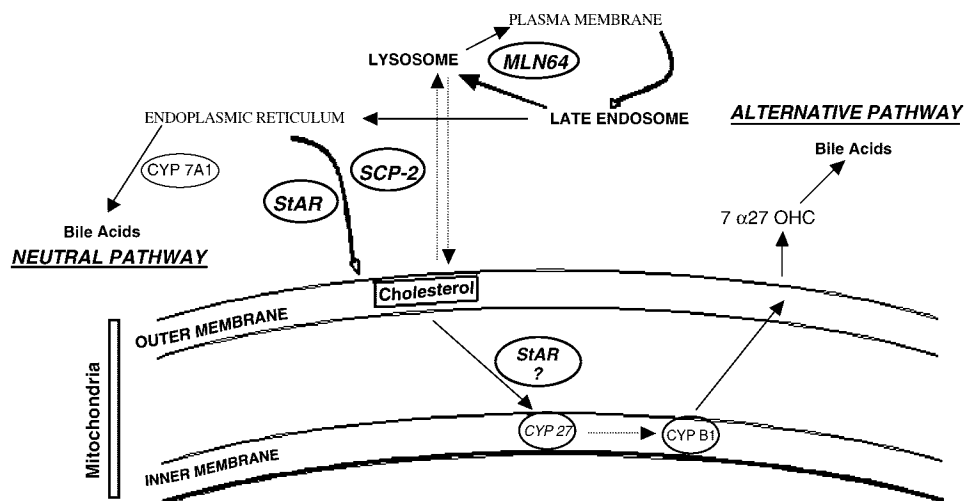


Fig. 7. Proposed role of cholesterol transport proteins in bile acid synthesis. There are two major pathways of bile acid synthesis: the neutral pathway, initiated by highly regulated microsomal CYP7A1, and an alternative pathway, initiated by mitochondrial CYP27A1. Our data suggest that full-length MLN64, a highly homologous protein to StAR, does not deliver cholesterol to the mitochondria or to the endoplasmic reticulum. MLN64, a protein previously localized to the endosomes, may only play a role in the delivery of cholesterol among the late endosomes, lysosomes, and plasma membrane. In contrast, StAR protein facilitates cholesterol delivery from the endoplasmic reticulum to the inner mitochondrial membrane, and changes in its cellular content are capable of significantly altering the rates of bile acid synthesis initiated by the alternative pathway. SCP-2 is widely distributed in the cells and appears to deliver cholesterol in multiple directions (see Discussion) in the maintenance of intracellular cholesterol homeostasis.

play a major role in bile acid synthesis via either the neutral or the alternative pathway.

The effect of replenishment of mitochondrial cholesterol on rates of bile acid synthesis in hepatocytes has not been tested. If StAR transports cholesterol only from the outer to the inner mitochondrial membrane, other mechanisms must exist to replenish cholesterol from intracellular sites (lysosome, lipid droplets, plasma membranes, etc.) to the outer mitochondrial membrane. SCP-2 has been proposed to fulfill such a role (18, 27). Previous observations have shown that SCP-2 is promiscuous in enhancing mitochondrial membrane cholesterol transfer regardless of whether the mitochondria were isolated from steroidogenic or non-steroidogenic cells (11). In the present study, we tested the hypothesis by using co-overexpression of StAR and SCP-2 genes and comparing it to co-overexpression of StAR and CYP27A1 or MLN64, attempting to replenish available cholesterol in the mitochondrial membrane for bile acid synthesis. As shown in Fig. 1, StAR protein expression was not affected by co-overexpression. Interestingly, unlike MLN64, the combination of StAR/SCP-2 did not affect the StAR-up-regulated bile acid synthesis. These results suggest the possibility of reverse cholesterol transport from mitochondria to other subcellular organelles rather than from other subcellular organelles to mitochondria in the situation of the StAR/SCP-2 combination. In agreement with our results, Gallegos et al. (11) reported that the initial rate of spontaneous sterol transfer from lysosomal membranes to mitochondrial membrane isolated from SCP-2-expressing cells was 4-fold slower than from SCP-2-deficient cells. Moreover, ste-

rol transport from outer mitochondrial membranes to lysosomal membranes was much faster and exhibited an initial rate that was 23-fold faster than in the forward direction from lysosomal membranes to mitochondrial membranes (11). Therefore, SCP-2 may not further enhance cholesterol transport from other organelles to mitochondria when StAR protein is overexpressed in the cells.

Thus, what kind of cholesterol transport protein can replenish mitochondrial cholesterol when StAR or StAR-like protein depletes cholesterol for bile acid synthesis through the alternative pathway is still unclear. It is possible that pro-StAR protein binds cholesterol and brings it from an endoplasmic reticulum pool directly to the outer membrane of the mitochondria, where pro-StAR is processed to mature StAR protein (molten protein). The mature StAR protein can then transport cholesterol from the outer to the inner membrane of mitochondria for bile acid synthesis. The possible role of StAR, MLN-64, and SCP-2 in cellular cholesterol movement is presented in Fig. 7.

In summary, the three different individually overexpressed cholesterol binding proteins were capable of increasing the rate of bile acid synthesis through the alternative pathway. However, bile acid synthesis was most greatly affected by StAR, a protein capable of increasing the amount of cholesterol reaching the inner membrane of the mitochondria. MLN64, although capable of increasing the rates of bile acid synthesis after its overexpression, did so at a much lower level. Surprisingly, StAR- and SCP-2-related transport activities were inhibited by coexpression with MLN64. These results suggest that the intracel-

lular trafficking of cholesterol is complex and that each cholesterol binding protein contributes in its own way to intracellular cholesterol movement.¹¹

This work was supported by grants from the Veterans Administration, the National Institutes of Health (P01 DK-38030), and a Jeffress Research Grant. S.R. is a recipient of the American Liver Foundation Scholar Award. Dr. Jane Glick provided the SCP-2 adenoviral construct; Dr. Jeff Billheimer provided antibody against SCP-2; Dr. J. F. Strauss provided STAR cDNA and antibody. Human hepatocytes were provided by the Liver Tissue Procurement and Distribution System (N01 DK-92310).

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