Expression of Sterol 27-Hydroxylase (CYP27A1) Enhances Cholesterol Efflux*

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Cholesterol efflux from CHOP cells transfected with sterol 27-hydroxylase (CYP27A1) was compared with non-transfected and mock-transfected cells. Transfection caused expression of CYP27A1, formation of 27-hydroxycholesterol, and inhibition of cholesterol biosynthesis. Transfection enhanced cholesterol efflux to apolipoprotein A-I or human plasma by 2–3-fold but did not affect the efflux in the absence of acceptor. The analysis of released sterols revealed that 27-hydroxycholesterol represented only a small proportion of sterols, most of which was non-oxidized cholesterol. Time course and dose dependence studies showed that expression of CYP27A1 in CHOP cells mostly affected the efflux of the “fast” cholesterol pool, and relatively more cholesterol was released with low concentrations of an acceptor. Precubination of non-transfected cells with exogenous 27-hydroxycholesterol (10−9 and 10−7 M) led to the stimulation of cholesterol efflux by 24–60%. Expression of CYP27A1 in CHOP cells did not affect ABCA1 expression and abundance of ABCA1 protein. Thus, introduction of CYP27A1 into cells stimulates cholesterol efflux and therefore may increase protection against atherosclerosis.

The reverse cholesterol transport pathway removes excess cholesterol from extrahepatic tissues including vessel wall, thus preventing development of atherosclerosis. The first and most likely rate-limiting step of reverse cholesterol transport is cholesterol efflux, the transfer of cholesterol from cells to acceptors in plasma. Two key pathways of cholesterol efflux are currently known. The first involves lipidation of lipid-free or lipid-poor apolipoprotein A-I (apoA-I), and is most likely mediated by the ABCA1 transporter (for review see Ref. 1). The second involves transfer of cholesterol from plasma membrane caveolae to lipidated apoA-I or mature high density lipoprotein (2) and may be mediated by scavenger receptor B1. Induction of ABCA1 (3), scavenger receptor B1 (4), and caveolin (5) results in a stimulation of cholesterol efflux. Cholesterol efflux may also be stimulated by inducing, or introducing into cells, elements of a pathway capable of cholesterol catabolism. Bjorkhem and co-workers (6, 7) demonstrated that limited oxidation of cholesterol in human macrophages may represent such a mechanism; however, its contribution to overall cholesterol efflux remains unclear. In this work we demonstrate for the first time that expression of transfected sterol 27-hydroxylase (CYP27A1) enhances efflux of non-oxidized cholesterol from cultured cells.

MATERIALS AND METHODS

Cells—CHOP-C4 cells (8) were grown in RPMI 1640 medium containing 10% fetal bovine serum, 2 mM l-glutamine, penicillin/streptomycin (50 units/ml), and 0.2 mg/ml G418. The day before transfection, cells were plated in 12-well plates at a density of 0.6 × 105 cells per well. Transfection was performed with DEAE-dextran as described previously (9), using 200 ng of plasmid DNA (CYP27A1 in pcDNA1 or pcDNA1 alone) per well. The efficiency of transfection checked with β-galactosidase reporter gene was 80% or better. CYP27A1 was isolated from a human kidney cDNA library during studies of progesterone metabolism using a screening protocol described previously (9) for the isolation of 11βHSD2. The CYP27A1 clone was sequenced in its entirety and found to contain the full-length coding region with a three-nucleotide 5′ untranslated region and full-length polyadenylated 3′ sequence. There were no nonsense mutations.

Cholesterol Acceptors—Blood from healthy normolipidemic (plasma total cholesterol values ranging from 3.4 to 5.0 mmol/liter) volunteers was collected in saline containing streptokinase (Sigma; final concentration 150 units/ml), and plasma was isolated by repeated centrifugation for 15 min at 1500 × g at 4 °C. Plasma samples were not pooled but rather used individually, which, in part, explains variations in cholesterol efflux levels. Apolipoprotein A-I was isolated as described previously (10).

Cholesterol Efflux—Transfected, non-transfected, and mock-transfected CHOP cells were grown to 80% confluence prior to experiments. The cultures were 100% confluent by the time of incubation with cholesterol acceptors. Two methods were used to label cellular cholesterol. Metabolic labeling was conducted by incubating cells in serum-free medium with [1-14C]acetate (Amersham Biosciences), specific radioactivity 1.81 GBq/mmol, final radioactivity 0.8 MBq/ml for 24 h at 37 °C in a CO2 incubator. Alternatively, cells were incubated in serum-containing medium with [1,2,6,13H]cholesterol (Amersham Biosciences; specific radioactivity 1.81 TBq/mmol, final radioactivity 0.2 MBq/ml) for 24 h at 37 °C. The CYP27A1 clone was sequenced in its entirety and found to contain the full-length coding region with a three-nucleotide 5′ untranslated region and full-length polyadenylated 3′ sequence. There were no nonsense mutations.

Cholesterol Efflux Analysis by TLC—Lipids were extracted with 3 volumes of ethyl acetate and separated using TLC (chloroform/ethyl acetate 4:1) (v/v) (11). TLC plates containing labeled lipids were exposed to a phosphorimaging plate and analyzed on the Bioimager BAS-1000 (Fuji), and

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§ The abbreviations used are: apoA-I, apolipoprotein A-I; GCMS, gas chromatography mass spectrometry; GPDH, glyceraldehyde-3-phosphate dehydrogenase.
Lipid extracts were dried under nitrogen and then treated with bis-(2-methoxyethyl)trifluoroacetamide (50 μl) and pyridine (50 μl) at 60 °C for 20 min to form trimethylsilyl derivatives. For analysis of cell cholesterol, an internal standard of cholesterol (2.5 μg) was added to each sample prior to GCMS analysis. Samples were analyzed by full scan electron impact GCMS using an Agilent 5973 GCMS fitted with a 30-m × 0.25-mm inner diameter HP-5MS column with helium carrier gas at a flow rate of 1 ml/min. The initial column temperature was 165 °C, increased at 20 degrees/min to 280 °C, and then held. 27-Hydroxycholesterol was identified by comparison to an authentic standard, which showed a molecular ion at 546 m/z for the bis-TMS ether. Other characteristic ions included 456 (M – 90), 441 (M – 90), 417 (M – 90), and 39 (M – 90). Reverse Transcriptase PCR and Northern Blotting—Total RNA was extracted from CHOP cells following a modification of the guanidinium thiocyanate method (13). The RNA concentration was determined by measuring the absorbance at 260 nm. Reverse transcription was carried out in 20 μl containing 20 μM Tris-HCl, pH 8.4, 50 mM KCl, 5 mM MgCl₂, 10 mM dithiothreitol, 40 units of RNase inhibitor, and 50 units of Superscript II reverse transcriptase (Invitrogen). Briefly, 2 μg of total RNA were incubated for 5 min at 65 °C with 0.5 μg of oligo(dT)₁₂₋₁₅, and 0.5 μg dNTPs and placed on ice for 1 min. The remaining components of the reaction were added and incubated for 1 h at 42 °C. The reaction was terminated at 70 °C for 15 min, and RNA was digested with 2 units of RNase H for 20 min at 37 °C. PCR was performed in a total volume of 50 μl containing 10 μM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM (GAPDH) or 5 mM (CYP27A1) MgCl₂, 200 μM dNTPS, 100 ng of the appropriate forward and reverse primer CYP27A1 forward, GCAAGCTCTTATACC GGAT, CYP27A1 reverse, GCA AACTAGGGGCGCGTTGCAGCTC TCTGCGAAGG; GAPDH forward, AC GCGCAATTCACCGCGACGTCA; GAPDH reverse, CATT GGGGTAGGGAACACGGAAG; 2 μl of reverse transcribed cDNA, and 1 unit of Taq polymerase (Invitrogen). The reaction was amplified with a DNA thermal cycler (PerkinElmer Life Sciences) for 35 cycles. The amplification profile involved denaturation at 94 °C for 15 s, primer annealing at 55 °C for 30 s, and elongation at 72 °C for 1 min. 10 μl of each PCR reaction was mixed with 2 μl of 6-fold concentrated loading buffer and loaded on a 1% agarose gel containing ethidium bromide. Electrophoresis was carried out with a constant voltage for 1 h. The sequences of the fragments amplified by PCR were confirmed by DNA sequencing.

For Northern blotting, RNA was separated on a 1% agarose gel, transferred to a nylon membrane, and probed with 32P-labeled mouse ABCA1 cDNA (14) (gift of Dr. G. Chimini) and 32P-labeled GAPDH cDNA as an internal standard. The membrane was exposed to a phosphorimaging plate and analyzed on the Bioimager BAS-1000 (Fuji). Activity of CYP27A1 was first assessed by analyzing sterol synthesis in transfected cells following metabolic labeling with [14C]acetate. The amount of newly synthesized non-oxidized cholesterol in transfected cells was decreased by more than 50% compared with mock-transfected cells (Fig. 2A). The amount of oxidized cholesterol and cholesterol esters was exceedingly low compared with the amount of newly synthesized cholesterol (see below), it is unlikely that cholesterol metabolism is responsible for decreased amounts of newly synthesized cholesterol. The inhibition of cholesterol biosynthesis is a more likely explanation. This finding is consistent with the report by Esterman et al. (15) who showed previously that 27-hydroxycholesterol inhibits cholesterol biosynthesis in Chinese hamster ovary cells. The amount of newly synthesized 27-hydroxycholesterol found in transfected cells was 2.5-fold higher than in mock-transfected cells (Fig. 2B). However, the overall amount of 27-hydroxycholesterol formed was low consisting of only 1.5 and 0.6% for transfected and mock-transfected cells, respectively.

The activity of CYP27A1 was further assessed following the labeling of cellular cholesterol pool with [3H]cholesterol. No 27-hydroxycholesterol was found in mock-transfected cells (Table II) whereas 2.2% of [3H]cholesterol was converted into 27-hydroxycholesterol in transfected cells. The majority of 27-hydroxycholesterol formed in transfected cells was released into medium following 2 h of incubation with 5% human serum (Table II). An unidentified cholesterol metabolite accounting for 3% of total radioactive lipids was found in the medium of

**RESULTS**

Expression of CYP27A1 in CHOP Cells—CHOP cells were transiently transfected with CYP27A1 as described under “Materials and Methods.” Expression of CYP27A1 was analyzed by reverse transcriptase PCR, and abundance of CYP27A1 protein was analyzed by Western blot. When CYP27A1 mRNA content was analyzed, a strong signal was detected in transfected cells whereas no signal was detected in mock-transfected cells (Fig. 1A). GAPDH was used as internal standard, and a fragment of 561 bp of identical intensity was found in both transfected and mock-transfected cells (not shown). Expression of CYP27A1 tagged with a Myc epitope was also analyzed with Western blot using an anti-Myc antibody. The presence of a band migrating at 58 kDa was detected in transfected but not in mock-transfected cells, consistent with the size of CYP27A1 (Fig. 1B). The antibody cross-reacted with an unknown protein migrating at 35 kDa, but this band was of the same intensity in mock-transfected and CYP27A1-transfected cells (Fig. 1B).

**CYP27A1 Activity in CHOP Cells**—The presence of 27-hydroxycholesterol was analyzed in transfected and mock-transfected cells using gas chromatography mass spectrometry. About 0.8% of total sterols in transfected cells were found in the 27-hydroxycholesterol peak; no 27-hydroxycholesterol was found in the mock-transfected cells (Table I). In HepG2 cells, used as a positive control as they contain CYP27A1 as part of bile acid synthesis pathway, 0.16% of sterols was found in the 27-hydroxycholesterol peak.

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Table I

Quantification of 27-hydroxycholesterol in transfected and mock-transfected cells

| Cell type/transfection       | Cholesterol (μg/dish) | 27-Hydroxycholesterol |
|------------------------------|-----------------------|-----------------------|
| CHOP-Mock                    | 2.6                   | ND                    |
| CHOP-CYP27                   | 5.4                   | 0.043                 |
| HepG2                        | 9.6                   | 0.015                 |

Figure 2. Biosynthesis of cholesterol (A) and 27-hydroxycholesterol (B) in cells transfected with CYP27A1. CHOP cells were transfected with CYP27A1 or mock-transfected with pcDNA1 and cultured for 48 h. One day after transfection, cells were incubated for 24 h in serum-free medium containing [1-14C]acetate. Sterols from cells were extracted, separated with TLC as described under “Materials and Methods,” and counted. Means ± S.D. of quadruplicate determinations are shown. *, p < 0.01 (versus mock-transfected cells).

Figure 3. Efflux of newly synthesized cholesterol (A) and 27-hydroxycholesterol (B) from cells transfected with CYP27A1. CHOP cells were transfected with CYP27A1 or mock-transfected and cultured for 48 h. One day after transfection, cells were incubated for 24 h in serum-free medium containing [1-14C]acetate. Cells were then washed and incubated for 2 h with 5% human plasma. Sterols from both cells and medium were extracted and separated by TLC as described under “Materials and Methods.” Background values (i.e., the efflux to the medium alone) were subtracted. Means ± S.D. of quadruplicate determinations are shown. *, p < 0.01 (versus mock-transfected cells).

Table II

Conversion of [3H]cholesterol in transfected and mock-transfected cells

CHOP cells transfected with CYP27A1 or mock-transfected were labeled with [3H]cholesterol as described under “Materials and Methods.” Cells were then incubated with human plasma (final concentration 5%), for 2 h at 37 °C in a CO2 incubator. Medium was collected, cells were harvested, and lipids from cells and medium were extracted and separated by TLC as described under “Materials and Methods.” Values are percentages of total radioactivity (cells plus medium) extracted to the organic phase. ND, not detected.

| Cell type/transfection       | Free cholesterol (%) | Cholesteryl esters (%) | 27-Hydroxycholesterol (%) | Unidentified lipids (%) |
|------------------------------|----------------------|------------------------|---------------------------|-------------------------|
| CHOP-Mock                    | 11.9                 | 0.9                    | ND                        | ND                      |
| CHOP-CYP27                   | 74.4                 | 12.8                   | 2.2                       | 2.8                     |

Figure 4. Cholesterol efflux to human plasma and lipid-free apolipoprotein A-I. CHOP cells transfected with CYP27A1, mock-transfected, and non-transfected were labeled with [3H]cholesterol as described under “Materials and Methods.” Cells were then incubated with human plasma (final concentration 5%), human lipid-free apoA-I (final concentration 30 μg/ml), or serum-free medium alone for 2 h at 37 °C in a CO2 incubator. Medium was collected, cells were washed, and the amount of radioactivity in the cells and medium was determined by liquid scintillation spectrometry. Cholesterol efflux is expressed as the percentage of labeled cholesterol moved from cells to medium (i.e., radioactivity in the medium/radioactivity in the medium + radioactivity in the cells). Means ± S.D. of quadruplicate determinations are shown. *, p < 0.001 versus mock-transfected and non-transfected cells.

CHOP cells transfected with CYP27A1. A small amount of cholesteryl esters was also found in the cells and in the medium of both transfected and mock-transfected cells. The amount of cholesterol and cholesteryl esters in the medium was higher, whereas the amount of cholesterol and cholesteryl esters in the cells was lower in transfected cells compared with mock-transfected cells (Table II). Thus, expression of CYP27A1 in transfected cells was confirmed by detecting CYP27A1 mRNA and protein and measuring enzyme activity (assessed by the formation of 27-hydroxycholesterol) and its biological effect (inhibition of cholesterol biosynthesis and stimulation of cholesterol efflux).

Effect of CYP27A1 on Cholesterol Efflux—When cells metabolically labeled with [3H]acetate were exposed to 5% human plasma for 2 h, significantly more cholesterol and 27-hydroxycholesterol was released from transfected cells compared with mock-transfected cells (Fig. 3, A and B). It should be noted that because the amount of newly synthesized 27-hydroxycholesterol in the cells was considerably lower than the amount of newly synthesized cholesterol, the contribution of 27-hydroxycholesterol to overall sterol efflux was less than 3%.

To further study the effect of transfection with CYP27A1 on cholesterol efflux, cellular cholesterol was labeled with [3H]cholesterol, and cells were incubated in the presence or absence of whole human plasma or lipid-free human apoA-I. The level of cholesterol efflux from cells labeled with [3H]cholesterol was considerably lower than after metabolic labeling. We speculate that this may be because of preferential distribution of newly synthesized cholesterol to pools more readily accessible to the efflux in rapidly dividing CHOP cells (e.g., plasma membrane). Differences in cholesterol efflux from different cellular pools have been observed previously (16, 17). Transfection of cells with CYP27A1 resulted in a 3-fold increase in cholesterol efflux to whole plasma and a doubling of the efflux to apoA-I compared with both non-transfected and mock-transfected cells (Fig. 4). This difference was not because of differences in the amount of labeled cholesterol loaded into cells, which was 437 ± 87 versus 342 ± 150 dpm/μg cell protein for transfected and non-transfected cells, respectively. Cholesterol efflux to
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FIG. 5. Time course (A) and dose dependence (B) of cholesterol efflux to human plasma. CHOP cells transfectected with CYP27A1 or mock-transfectected were labeled with [3H]cholesterol as described under "Materials and Methods." Cells were then incubated with human plasma added at a final concentration of 5% (A) or at the indicated concentrations (B) or serum-free medium alone for the indicated periods of time (A) or for 2 h (B) at 37 °C in a CO2 incubator. Medium was then collected, cells were washed, and the amount of radioactivity in the cells and medium was determined in a β-counter. Cholesterol efflux is expressed as the percentage of labeled cholesterol moved from cells to medium (i.e. radioactivity in the medium/radioactivity in the cells). Background values (i.e. the efflux to the medium alone) were subtracted. Means ± S.D. of triplicate determinations are shown.

The time course of cholesterol efflux to human plasma is shown in Fig. 5A. As a single band migrating at the expected size (9.5 kb) by Northern blot analysis when using mouse ABCA1 cDNA as a probe (Fig. 7A). Therefore expression of ABCA1 mRNA in mock-transfectected cells was greater during the fast phase (2.5–3-fold stimulation within the first 20 min) compared with the slow phase (40–80% stimulation at 1–2 h). However, the effect of transfection on the absolute amount of cholesterol released was greater in the slow phase. The effect of plasma concentration on cholesterol efflux is shown in Fig. 5B. Relatively more cholesterol was released from transfected cells at low concentrations of plasma, and the difference between transfected and non-transfectected cells gradually disappeared as the concentration of plasma increased.

Effect of 27-Hydroxycholesterol on Cholesterol Efflux—To ascertain whether the effect of transfection with CYP27A1 can be reproduced with exogenous 27-hydroxycholesterol, non-transfectected cells were preincubated for 18 h at 37 °C with 10−9 or 10−7 M 27-hydroxycholesterol added into the incubation medium. Assuming that this incubation results in nearly equilibration between extracellular and intracellular 27-hydroxycholesterol, the concentrations of 27-hydroxycholesterol used are within the range of its expected concentration in cells transfected with CYP27A1 (Table I). Preincubation of cells with 27-hydroxycholesterol led to stimulation of cholesterol efflux by 24 and 60%, respectively (Fig. 6).

Effect of CYP27A1 on Expression of ABCA1—27-Hydroxycholesterol and possibly another product of CYP27A1, cholestenoic acid, may be ligands of the liver X-activated receptor, regulating a number of genes involved in lipid metabolism including ABCA1 (19). Therefore expression of ABCA1 was evaluated by Northern blot in transfected and mock-transfectected cells to determine the effect of CYP27A1. ABCA1 mRNA was identified as a single band migrating at the expected size (9.5 kb) by Northern blot analysis when using mouse ABCA1 cDNA as a probe (Fig. 7A). The expression of ABCA1 in transfected cells was slightly less than in mock-transfectected cells; the ratio of ABCA1 mRNA to GAPDH mRNA was 0.84 and 1.36 for transfected and mock-transfectected cells, respectively (Fig. 7A). The
abundance of ABCA1 protein in transfected and mock-transfected cells was analyzed using Western blot (Fig. 7B). The size of CHOP cell ABCA1 was found to be over 200 kDa, which is similar to the calculated molecular mass of 248 kDa for the human protein (20). When the same amount of cellular protein was loaded, no significant difference in ABCA1 abundance was found between transfected and mock-transfected cells.

**DISCUSSION**

Oxidation of cholesterol in liver is the major pathway of cholesterol catabolism (21) resulting in formation of water-soluble bile acids and their subsequent excretion. It was assumed that liver is the only tissue capable of this process. Bjorkhem et al. (6) first suggested that this mechanism may not be unique for liver but may also be present in macrophages and endothelial cells, contributing therefore to the protection against cholesterol accumulation in the vessel wall (7, 22, 23). According to this hypothesis the more hydrophilic 27-hydroxycholesterol and cholestenoic acid may be released from cells more readily (22). Cholestenoic acid may also be released to albumin instead of high density lipoprotein (24). This hypothesis was supported by findings that a genetic CYP27A1 deficiency in humans (cerebrotendinous xanthomatosis) is associated with lipid deposition in connective tissue and an increased risk of cardiovascular disease (6, 25), classic symptoms of deficiency of reverse cholesterol transport. In CYP27A1 knockout mice, however, most abnormalities were localized in the liver with little effect of the gene deletion on cholesterol homeostasis in extrahepatic tissues (26). Furthermore, overexpression of CYP27A1 in transgenic mice did not result in major changes in lipoprotein metabolism (27). These differences might be a result of profound dissimilarities in lipoprotein metabolism in mice and humans and may also reflect the need to distinguish between roles of CYP27A1 in liver and nonhepatic cells. We addressed this issue by overexpression of CYP27A1 in an *in vitro* system and measured cholesterol efflux.

The major finding of this work is that transfection of cells with sterol 27-hydroxylase stimulates the efflux of non-oxidized cholesterol. The stimulation of cholesterol efflux could be explained by two mechanisms. First, the appearance of sterol 27-hydroxylase in the plasma membrane may facilitate the release of cholesterol and/or 27-hydroxycholesterol to an acceptor in plasma. Second, 27-hydroxycholesterol or its derivative cholestenoic acid, may be a ligand of the liver X-activated receptor, which regulates a number of genes involved in cholesterol and cholestenoic acid may be released to cells.

According to this hypothesis the more hydrophilic 27-hydroxycholesterol may facilitate the release of cholesterol and may also be present in macrophages although to a lesser degree.

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