Overexpression of Cholesterol 7α-Hydroxylase (CYP7A) in Mice Lacking the Low Density Lipoprotein (LDL) Receptor Gene

LDL TRANSPORT AND PLASMA LDL CONCENTRATIONS ARE REDUCED*

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This study was undertaken to determine the effect of transient overexpression of hepatic cholesterol 7α-hydroxylase on low density lipoprotein (LDL) cholesterol transport in mice lacking LDL receptors (LDL receptor−/−). Primary overexpression of hepatic 7α-hydroxylase in LDL receptor−/− mice was accompanied by a dose-dependent decrease in the rate of LDL cholesterol appearance in plasma (whole body LDL cholesterol transport) and a corresponding reduction in circulating LDL cholesterol levels. The increase in hepatic 7α-hydroxylase activity necessary to achieve a 50% reduction in plasma LDL cholesterol concentrations was ~10-fold. In comparison, cholestyramine increased hepatic 7α-hydroxylase activity ~3-fold and reduced plasma LDL cholesterol concentrations by 17%. This study demonstrates that augmentation of hepatic 7α-hydroxylase expression is an effective strategy for lowering plasma LDL concentrations even in animals with a genetic absence of LDL receptors.

Conversion of cholesterol to bile salts is the principal regulated pathway whereby cholesterol is removed from the body. The initial and rate-limiting enzyme in the major bile salt biosynthetic pathway is hepatic cholesterol 7α-hydroxylase (1). Hepatic cholesterol 7α-hydroxylase is regulated at the transcriptional level in response to bile salts fluxing through the liver in the enterohepatic circulation (2–5). Bile salt sequestrants such as cholestyramine bind bile salts in the intestinal lumen, thereby preventing their reabsorption and decreasing the return of bile salts to the liver. Loss of bile salts from the enterohepatic circulation results in derepression of hepatic 7α-hydroxylase expression and an increase in the synthesis of bile salts from cholesterol (6–9). Interventions that accelerate the conversion of cholesterol to bile salts reduce plasma LDL1 concentrations and prevent coronary events (10–12). The efficacy of these interventions is postulated to result from a reduction in the availability of unesterified cholesterol within hepatocytes. Depletion of unesterified cholesterol within the hepatocyte triggers a compensatory increase in de novo cholesterol synthesis and induction of the LDL receptor pathway, the latter leading to enhanced clearance of LDL from plasma (7, 13–15). Although induction of hepatic LDL receptor activity has been emphasized as the major mechanism responsible for the cholesterol-lowering effects of bile salt sequestrants, a recent study suggests that these agents may also reduce the rate of LDL cholesterol entry into plasma (16).

Humans (and animals) who genetically lack LDL receptors are characterized by massive elevations of circulating LDL levels, accelerated atherosclerosis, and premature coronary heart disease (17). In the absence of functional LDL receptors, the clearance of LDL from plasma is reduced, and the conversion of VLDL to LDL is increased. In Watanabe heritable hyperlipidemic (WHHL) rabbits, the absence of functional LDL receptors leads to a ~20-fold elevation of the plasma LDL concentration that is the result of a 60–75% reduction in the rate of LDL clearance from plasma coupled with a 2–5-fold increase in the rate of LDL entry into the plasma space (whole body LDL transport) (18, 19). In the mouse, targeted disruption of the LDL receptor gene leads to a 14-fold increase in the plasma concentration of LDL that is due to an 88% reduction in the rate of LDL clearance from plasma coupled with a 70% increase in whole body LDL transport (20).

In a recent study, we transiently overexpressed an exogenous 7α-hydroxylase gene in hamsters using adenovirus-mediated gene transfer and determined the effects on hepatic sterol balance and LDL transport (21). This demonstrated that primary overexpression of hepatic 7α-hydroxylase markedly lowered plasma LDL concentrations in animals fed control or Western-type diets. Notably, the reduction in plasma LDL levels was due, in large part, to a decrease in the rate of LDL cholesterol entry into the plasma space (whole body LDL transport). This observation raised the possibility that enhancing hepatic 7α-hydroxylase activity might also be effective in lowering plasma LDL concentrations in animals lacking LDL receptors. The present study characterizes the response to hepatic 7α-hydroxylase overexpression in mice with targeted disruption of the LDL receptor gene. The results of this study indicate that enhancement of hepatic 7α-hydroxylase expression is an effective strategy for lowering plasma LDL concentrations not only in animals with diet-induced hypercholesterolemia, but also in animals that genetically lack LDL receptors.

MATERIALS AND METHODS

Animals and Diets—All studies were performed in female mice with targeted disruption of the LDL receptor gene (22). All animals were housed in colony cages (five animals/cage) in a room with light cycling (12 h of light and 12 h of dark) and controlled temperature and humidity. All measurements were made at the mid-dark phase of the light cycle. The animals were maintained on a low fat (50 mg/g of diet), low
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cholesterol (0.23 mg/g of diet) cereal-based diet (Wayne Lab Blox, Allied Mills, Chicago, IL).

Recombinant Viruses—The recombinant adenoviruses AdCMV7α (carrying a gene encoding rat 7α-hydroxylase), AdCMVlac (carrying a gene encoding firefly luciferase), and AdCMVγ (carrying the Escherichia coli lacZ gene) have been described previously (21, 23). Large-scale production of recombinant adenovirus was performed as described (24) by infecting confluent monolayers of 293 cells grown in 15-cm tissue culture plates with primary stock at a multiplicity of infection of 0.1–1.0. Infected monolayers were lysed with Nonidet P-40 when >90% of the cells showed cytopathic changes, and recombinant virus was purified by precipitation with polyethylene glycol 8000, centrifugation on a discontinuous CsCl density gradient, and desalting by chromatography on Sepharose CL-4B. Purified virus eluting in the void volume was collected, snap-frozen in liquid N₂, and stored at −80 °C until used.

Hepatic 7α-Hydroxylase Activity—Hepatic 7α-hydroxylase activity was determined using an HPLC/photometric assay that quantifies the mass of 7α-hydroxycholesterol formed from endogenous microsomal cholesterol after enzymatic conversion to 7α-hydroxycholesten-3-one using cholesterol oxidase (25).

Determination of Hepatic LDL Uptake Rates in Vivo—Plasma was obtained from LDL receptor−/− donor mice. The LDL was isolated from plasma by preparative ultracentrifugation in the density range of 1.020–1.055 g/ml and labeled with 131I or 125I-tyramine cellulose as described previously (26). Rates of hepatic LDL uptake were measured using primed infusions of 125I-tyramine cellulose-labeled LDL. The infusions of 125I-tyramine cellulose-labeled mouse LDL were continued for 4 h, at which time each animal was administered a bolus of 131I-tyramine cellulose-labeled LDL as a marker of plasma volume and killed 10 min later by exsanguination through the inferior vena cava. Tissue samples along with aliquots of plasma were assayed for radioactivity in a γ-counter (Packard Instrument Co.). The tissue spaces achieved by the labeled LDL at 10 min (131I dpm/g of tissue divided by 131I dpm/μl of plasma) and at 4 h (125I dpm/g of tissue divided by 125I dpm/μl of plasma) were then calculated and have the units of μl/g.

The increase in tissue space over the 4-h experimental period equals the rate of radiolabeled LDL movement into each organ and is expressed as μl of plasma cleared of its LDL content per h g of tissue or per whole organ. Clearance values were multiplied by the plasma LDL concentration to obtain the absolute rates of LDL uptake.

VLDL ApoB Turnover—VLDL (d < 1.006 g/ml) was isolated from fasted rabbits by preparative ultracentrifugation and radioiodinated using iodine monochloride (27). VLDL turnover studies were performed in mice as described by Ishibashi et al. (22).

Determination of Hepatic Cholesterol Synthesis Rates—Rates of hepatic cholesterol synthesis were measured in vivo using [3H]water. As described previously (28), the animals were administered ~20 μCi of [3H]water intravenously and then returned to individual cages under a fume hood. One h after the injection of [3H]water, the animals were anesthetized and exsanguinated through the inferior vena cava. Aliquots of plasma were taken for the determination of body water specific activity and a sample of liver was taken for the isolation of diglycerides and precipitable sterols. Rates of sterol synthesis are expressed as nmol of [3H]water incorporated into diglyceride-precipitable sterols/μg of liver.

Determination of mRNA Levels—Hepatic 7α-hydroxylase and glycerolaldehyde-3-phosphate dehydrogenase (used as an invariant control) mRNA levels were determined by nuclease protection as described previously (29). Probes were synthesized using 0.5 μM [32P]dCTP and 1 μM (mouse 7α-hydroxylase), 5 μM (rat 7α-hydroxylase), or 300 μM (mouse glycerolaldehyde-3-phosphate dehydrogenase) unlabeled dCTP.

Samples of liver were homogenized in guanidinium thiocyanate, and the RNA was isolated by the method of Chomczynski and Sacchi (30). Total RNA (40 μg) was hybridized with the 32P-labeled cDNA probes simultaneously at 42 °C overnight. Unhybridized probe, present in excess relative to the amount of specific mRNA, was then digested with 40 units of mung bean nuclease (Life Technologies, Inc.). The mRNA-protected 32P-labeled probes were separated on 7 % urea, 6% polyacrylamide gels together with 32P-labeled MspI-digested pBR322 size standards. The radioactivity in each band, as well as background radioactivity, was quantified using an isotopic imaging system (AMBIS, Inc., San Diego, CA). The level of glycerolaldehyde-3-phosphate dehydrogenase mRNA did not vary among the various experimental groups and was used to correct for any procedural losses.

Determination of Bile Salt Pool Size and Composition—The liver, gallbladder, and small bowel were removed en block from LDL receptor−/− mice that had been administered AdCMV7α or control virus. The tissues and their contents were placed in a 400-ml beaker with ~200 ml of ethanol and (trace amounts of tauros24,14C)cholic acid as an internal standard and refluxed for 4 h. After extraction with diethyl ether, bile acids in the ethanolic extract were separated and quantified by HPLC.

RESULTS

To examine the role of hepatic 7α-hydroxylase in controlling plasma LDL concentrations in animals lacking LDL receptors, we first determined the effect of agents known to suppress (cholate) or up-regulate (cholestyramine) 7α-hydroxylase expression. LDL receptor−/− mice were fed a low cholesterol diet supplemented with cholate (0.3%) or cholestyramine (3%) for 4 weeks. Each value represents the mean ± S.D. for data obtained from five animals.

Determination of Liver and Plasma Cholesterol Distribution—Liver cholesterol was quantified by capillary gas-liquid chromatography. The cholesterol distribution in plasma was determined by FPLC using a Superose 6 column (Sigma). Two-ml fractions were collected and assayed for cholesterol using an enzymatic kit (Sigma).

Statistical Analysis—The data are presented as means ± S.D. To test for differences among the dietary regimens, one-way analysis of variance was performed. Significant results were further analyzed using the Tukey multiple comparison procedure.

We next investigated the effect of overexpressing an exogenous 7α-hydroxylase gene on plasma lipoprotein concentrations in mice lacking LDL receptors. LDL receptor−/− mice were administered, by intravenous injection, recombinant adenovirus expressing rat 7α-hydroxylase from the cytomegalovirus promoter (AdCMV7α) or equivalent doses of control virus (recombinant adenoviruses expressing either the firefly luciferase gene or the E. coli lacZ gene from the cytomegalovirus promoter). Preliminary time course studies showed that plasma LDL concentrations progressively declined for 2–3 days.
after the administration of AdCMV\(\alpha\)-7a-hydroxylase, remained relatively constant for 4–5 days, and then returned to preinjection values over the next 7–10 days; neither of the control viruses altered plasma LDL cholesterol concentrations over this time frame. Therefore, all subsequent studies were performed 3 days after the injection of recombinant adenovirus. Any animal that lost weight during this 3-day period of time was not studied.

The relationship between hepatic \(\alpha\)-7a-hydroxylase activity and plasma LDL concentrations in mice lacking LDL receptors is illustrated in Fig. 3. LDL receptor \(\alpha\)-7a-hydroxylase activity was administered 1 or 2 \(\times\) 10\(^9\) pfu of AdCMV\(\alpha\) or equivalent doses of AdCMVluc. Three days later, the animals were killed and used for the determination of hepatic \(\alpha\)-7a-hydroxylase expression and plasma lipoprotein concentrations. AdCMV\(\alpha\) increased hepatic \(\alpha\)-7a-hydroxylase activity and reduced plasma LDL cholesterol concentrations in a dose-dependent manner. The increase in hepatic \(\alpha\)-7a-hydroxylase activity necessary to achieve a 50% reduction in plasma LDL concentrations was \(~\)10-fold. By comparison, 3% cholestyramine increased hepatic \(\alpha\)-7a-hydroxylase activity 3-fold and reduced plasma LDL cholesterol concentrations by 17%.

Fig. 4 shows the effect of \(\alpha\)-7a-hydroxylase gene transfer on the lipoprotein distribution of plasma cholesterol in mice as determined by FPLC. LDL receptor \(\alpha\)-7a-mice were administered 1 \(\times\) 10\(^9\) pfu of AdCMV\(\alpha\) or the same dose of control virus (AdCMVluc). Three days later, the animals were killed after a 6-h fast, and equal volumes of plasma were pooled and injected onto a Superose 6 column. Primary overexpression of hepatic \(\alpha\)-7a-hydroxylase markedly reduced the amount of cholesterol carried in low density lipoprotein (92% reduction in VLDL and 53% reduction in IDL/LDL); high density lipoprotein cholesterol was also reduced (19%), but to a lesser extent. Lipoproteins in fractions corresponding to VLDL (tubes 1–6) and IDL/LDL (tubes 8–18) were separated on precast 1% agarose gels and stained with Fat Red 7B (31). Overexpression of hepatic \(\alpha\)-7a-hydroxylase markedly reduced the amount of pre-\(\beta\)-migrating lipoproteins in FPLC fractions corresponding to VLDL and \(\beta\)- and pre-\(\beta\)-migrating lipoproteins in FPLC fractions corresponding to IDL/LDL (data not shown).

A change in the plasma concentration of LDL may be due to a change in the rate of LDL entry into the plasma space or to a change in the rate of LDL clearance by one or more tissues of the body. To determine the mechanism responsible for the fall in plasma LDL concentrations associated with \(\alpha\)-7a-hydroxylase gene transfer, we performed LDL transport studies using \(^{125}\)I-tyramine cellubiose-labeled homologous LDL. LDL receptor \(\alpha\)-mice were administered 1 \(\times\) 10\(^9\) pfu of AdCMV\(\alpha\) or the same dose of control virus (AdCMVluc). Three days later, LDL transport rates were determined as described under “Materials and Methods.” As shown in Table I, rates of LDL clearance by the liver, extrahepatic tissues, and whole body were not altered by primary overexpression of hepatic \(\alpha\)-7a-hydroxylase. Multiplication of the tissue clearance rates by the concentration of LDL cholesterol in plasma yields the mass of LDL cholesterol transported by the liver, extrahepatic tissues, and whole body, and these values are also shown in Table I. Because \(\alpha\)-7a-hydroxylase gene transfer reduced plasma LDL cholesterol concentrations but had no effect on LDL clearance rates, the absolute rates of LDL cholesterol uptake fell in proportion to the decrease in plasma LDL cholesterol concentrations. At steady state, the
rate of LDL cholesterol uptake by all tissues of the body must equal the rate of LDL cholesterol entry into the plasma space (whole body LDL cholesterol transport). Overexpression of hepatic 7α-hydroxylase reduced the rate of whole body LDL cholesterol uptake by ∼50% (from 27 to 14 μg/h/100 g of body weight), but had no effect on whole body LDL cholesterol clearance, indicating that the fall in plasma LDL cholesterol concentrations was due entirely to a decrease in the rate of LDL cholesterol entering the plasma space.

We also examined the effect of 7α-hydroxylase gene transfer on rates of VLDL apoB clearance from plasma. LDL receptor−/− mice were administered 1 × 10^9 pfu of AdCMV7α or the same dose of control virus (AdCMVluc). Three days later, each animal was injected intravenously with [125I]-labeled apoB was measured by isopropyl alcohol precipitation (32), and the radioactivity present at each time point was expressed relative to the radioactivity present 2 min after the injection of radiolabeled VLDL. As shown in Fig. 5, the rate of disappearance of [125I]-VLDL from plasma did not differ between the AdCMV7α or the same dose of control virus (AdCMVluc). Three days later, each animal was injected intravenously with [125I]-labeled VLDL, and blood was collected from the retro-orbital sinus at 2 min and at 1, 2, 4, and 8 h. The plasma content of [125I]-labeled apoB was measured by isopropyl alcohol precipitation (32), and the radioactivity present at each time point was expressed relative to the radioactivity present 2 min after the injection of radiolabeled VLDL. As shown in Fig. 5, the rate of disappearance of [125I]-VLDL from plasma did not differ between the AdCMV7α and AdCMVluc-treated mice.

To further characterize the compensatory response to primary overexpression of hepatic 7α-hydroxylase, we measured hepatic cholesterol levels and hepatic cholesterol synthesis rates in LDL receptor−/− mice administered AdCMV7α or control virus. LDL receptor−/− mice were administered 1 × 10^9 pfu of AdCMV7α or control virus (AdCMVluc). Three days later, the animals were administered 20 mCi of [3H]water and killed 1 h later for the determination of hepatic cholesterol levels and hepatic cholesterol synthesis rates. As shown in Table II, rates of hepatic cholesterol synthesis were up-regulated ∼5-fold in animals administered AdCMV7α, whereas control virus had no significant effect. The concentration of unesterified and unesterified cholesterol in the liver was not significantly altered in animals administered AdCMV7α. As also shown in Table II, cholestyramine increased the rate of hepatic cholesterol synthesis ∼3-fold, whereas cholate suppressed hepatic cholesterol synthesis by 75%.

To investigate the effect of primary overexpression of hepatic 7α-hydroxylase on the enterohepatic pool of bile salts, we determined the size and composition of the bile salt pool in LDL receptor−/− mice administered AdCMV7α. LDL receptor−/− mice were administered 1 × 10^9 pfu of AdCMV7α. Three days later, the animals were killed, and the amount and type of bile salts present in the liver, gallbladder, and small intestine were determined. For comparative purposes, bile salt pool size and composition were also determined in animals fed cholestyramine (3%) or cholate (0.3%). As shown in Table III, administration of AdCMV7α expanded the size of the bile salt pool by 39%, but had little effect on the composition. The size of the bile salt pool was also increased in mice fed cholate, and in these animals, the pool was enriched with cholate.

The effect of primary overexpression of an exogenous 7α-hydroxylase gene on expression of the endogenous gene was next examined. LDL receptor−/− mice were administered 1 × 10^9 pfu of AdCMV7α or control virus (AdCMVluc). Three days later, the animals were killed, and samples of liver were taken for the determination of 7α-hydroxylase mRNA levels using a nuclease protection assay with probes specific for the exogenous (rat) or endogenous (mouse) genes. Preliminary nuclease protection experiments showed that the mouse probe did not yield a protected band when hybridized with rat RNA, and the rat probe did not yield a protected band when hybridized with mouse RNA (data not shown). As illustrated in Fig. 6, expression of the transgene had relatively little effect on expression of the endogenous gene. The endogenous gene was suppressed by only ∼30% even under conditions in which the transgene was expressed at levels sufficient to raise 7α-hydroxylase activity 10-fold. This observation is in contrast to the hamster, in which overexpression of an exogenous 7α-hydroxylase gene reciprocally suppressed expression of the endogenous gene (5).

A final set of experiments was undertaken to determine if expansion or depletion of the enterohepatic pool of bile salts had any effect on 7α-hydroxylase activity or plasma LDL cholesterol levels in animals overexpressing an exogenous 7α-hydroxylase gene. LDL receptor−/− mice were maintained on a low cholesterol control diet or the same diet supplemented with cholate (0.3%) or cholestyramine (3%). After 4 weeks, the animals were administered 1 × 10^9 pfu of AdCMV7α and killed 3 days later for the determination of hepatic 7α-hydroxylase expression and plasma LDL cholesterol concentrations. As shown in Fig. 7, administration of AdCMV7α was associated with an ∼11-fold increase in hepatic 7α-hydroxylase activity. In animals administered AdCMV7α, hepatic 7α-hydroxylase activity was the same whether the animals were consuming the control diet or diets supplemented with cholate or cholestyramine. Nuclease protection assays demonstrated that mRNA encoding the endogenous (mouse) 7α-hydroxylase was completely suppressed (undetectable) in animals ingesting the cholate diet. As also shown in Fig. 7, administration of Ad-

**Table II**

| Hepatic cholesterol content | Unesterified | Esterified |
|-----------------------------|-------------|-----------|
| Hepatic cholesterol synthesis | mg/g        | nmol/h/g  |
| Control                      | 1.5 ± 0.2   | 0.42 ± 0.05 |
| AdCMV7α                     | 1.6 ± 0.3   | 0.37 ± 0.06 |
| Cholestyramine              | 1.6 ± 0.2   | 0.38 ± 0.04 |
| Cholate                      | 1.7 ± 0.3   | 0.92 ± 0.09 |

*Differs from the control value (p < 0.05).*
Digestion were separated by denaturing polyacrylamide gel electrophoresis. Fragments protected from mung bean nuclease were also made in animals that had been fed 3% cholestyramine or 0.3% cholate for 4 weeks.

Up-regulation of hepatic LDL receptor activity, in turn, reduces cardiovascular mortality (12, 33). The proposed mechanism is depletion of unesterified cholesterol within hepatocytes, resulting in a compensatory increase in hepatic 7a-hydroxylase activity and plasma LDL cholesterol concentrations (7, 13–15). Up-regulation of hepatic LDL receptor activity, in turn, results in enhanced clearance of LDL from plasma by receptor-dependent and receptor-independent pathways. In this study, primary overexpression of hepatic 7a-hydroxylase in LDL receptor−/− mice markedly reduced the rate of LDL cholesterol entry into plasma. In contrast, overexpression of hepatic 7α-hydroxylase had no effect on the rate of LDL clearance in individual tissues or the whole body. These observations indicate that major reductions in LDL concentrations can be achieved through mechanisms independent of LDL receptor induction.

The concentration of LDL in plasma is determined by the rate at which LDL enters the plasma space relative to the rate at which LDL is cleared from plasma by receptor-dependent or receptor-independent pathways. In this study, primary overexpression of hepatic 7a-hydroxylase in LDL receptor−/− mice markedly reduced the rate of LDL cholesterol entry into plasma. In contrast, overexpression of hepatic 7α-hydroxylase had no effect on the rate of LDL clearance in individual tissues or the whole body. These observations are consistent with a recent study indicating that most of the LDL cholesterol-lowering effect of bile salt sequestrants can be attributed to a reduction in the rate of LDL cholesterol entry into plasma (16). Enhanced conversion of cholesterol to bile salts presumably decreases the content of unesterified cholesterol within hepatocytes, resulting in a compensatory increase in de novo cholesterol synthesis as discussed below. How depletion of hepatic cholesterol leads to a decrease in LDL cholesterol entry into plasma is less clear. A reduction in the rate of LDL cholesterol appearance in plasma could be the result of decreased hepatic secretion of apoB100-containing VLDL, a decrease in the proportion of apoB100-containing VLDL that is converted to LDL, or a decrease in the direct secretion of LDL from the liver. Overexpression of hepatic 7α-hydroxylase was associated with a marked reduction in plasma VLDL concentrations, but no change in the rate of VLDL disappearance from plasma, suggesting that decreased hepatic secretion of apoB100-containing VLDL contributed to the decrease in LDL cholesterol appearance in plasma. Whether or not the liver directly releases LDL

![Fig. 7. Effect of cholate or cholestyramine on hepatic 7α-hydroxylase activity and plasma LDL cholesterol concentrations in LDL receptor−/− mice constitutively expressing an exogenous 7α-hydroxylase gene.](image)

### Table III

| Total | MCA | CA | CDCA | DCA |
|-------|-----|----|------|-----|
| Control | 49 ± 7 | 32 | 63 | 3 | 2 |
| AdCMV7α | 68 ± 10b | 36 | 58 | 5 | 1 |
| Cholestyramine | 31 ± 7b | 27 | 69 | 2 | 2 |
| Cholate | 84 ± 15b | 2 | 89 | 1 | 8 |

*α* MCA, muricholic acid; *α* cholesterol; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid.

*β* Significantly different (*p* < 0.05) from the control value.

**DISCUSSION**

Clinical interventions that accelerate the conversion of cholesterol to bile salts lower plasma LDL concentrations and reduce cardiovascular mortality (12, 33). The proposed mechanism is depletion of unesterified cholesterol within hepatocytes, leading to compensatory increases in 3-hydroxy-3-methylglutaryl-CoA reductase and LDL receptor activities (7, 13–15). Up-regulation of hepatic LDL receptor activity, in turn, results in enhanced clearance of LDL from plasma. We previously showed that primary overexpression of hepatic 7α-hydroxylase, the rate-limiting enzyme in the bile salt biosynthetic pathway, markedly lowered plasma LDL concentrations in hamsters fed control or Western-type diets (21). Moreover, the reduction in plasma LDL concentrations was largely the result of a decrease in the rate of LDL entry into the plasma space (whole body LDL transport), suggesting that enhancing hepatic 7α-hydroxylase activity might also be effective in lowering plasma LDL concentrations in animals lacking LDL receptors. The present study demonstrates that direct augmentation of

![Image](image)

**FIG. 6. Nuclease protection analysis of cholesterol 7α-hydroxylase expression in mice infected with AdCMV7α or control virus.** Hepatic mRNA was prepared from LDL receptor−/− mice 3 days after the administration of 1 or 2 × 10⁹ pfu of AdCMV7α or 2 × 10⁹ pfu of control virus (AdCMVluc). Total RNA (40 μg) was hybridized with ²²P-labeled single-stranded cDNA probes encoding rat 7α-hydroxylase, mouse 7α-hydroxylase, and mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Fragments protected from mung bean nuclease digestion were separated by denaturing polyacrylamide gel electrophoresis and autoradiographed. *nt*, nucleotides.

![Image](image)

**TABLE III**

**Effect of 7α-hydroxylase gene transfer on the size and composition of the bile salt pool**

Animals (six/group) were administered 1 × 10⁷ pfu of AdCMV7α. Three days later, the amount and type of bile salts present in the liver, gallbladder, and small bowel were determined as described under “Materials and Methods.” For comparative purposes, these measurements were also made in animals that had been fed 3% cholestyramine or 0.3% cholate for 4 weeks.

- **Control** | 49 ± 7 | 32 | 63 | 3 | 2 |
- **AdCMV7α** | 68 ± 10b | 36 | 58 | 5 | 1 |
- **Cholestyramine** | 31 ± 7b | 27 | 69 | 2 | 2 |
- **Cholate** | 84 ± 15b | 2 | 89 | 1 | 8 |

*α* MCA, muricholic acid; *α* cholesterol; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid.

*β* Significantly different (*p* < 0.05) from the control value.
into the circulation is disputed (16, 36–39). Lipoprotein turnover studies of apoB100-containing particles indicate that more LDL enters the plasma space than can be accounted for by the metabolism of VLDL (16, 38, 39). Whether this difference is due to the direct release of LDL from the liver or to the release of a rapidly metabolized VLDL precursor has not been resolved. In any event, recent reports suggest that interruption of the enterohepatic circulation (by ileal bypass or bile salt seques-
trants) decreases the rate of LDL entry into plasma by reducing the direct secretion of LDL by the liver (16, 38, 39).

The effect of 7α-hydroxylase gene transfer was compared with the bile salt sequestrant cholestyramine. Cholestyramine increased 7α-hydroxylase activity 3-fold and modestly lowered plasma LDL concentrations (by ~17%), consistent with previous studies in which bile salt sequestrants were used in animals or humans lacking LDL receptors. This study suggests that the modest hypcholesterolemic effect of currently available bile salt sequestrants is related to the limited capacity of these agents to interfere with bile salt absorption and thereby increase hepatic 7α-hydroxylase activity and bile salt synthesis. More impressive malabsorption of bile salts can be achieved with ileal bypass or complete biliary diversion, and when these procedures are performed in WHHL rabbits, plasma cholesterol concentrations fall by ~40% (34, 35). However, marked depletion of the enterohepatic bile salt pool is associated with a number of undesirable side effects that make these procedures less than ideal as therapy for clinical hypercholesterolemia (12, 40).

Augmentation of hepatic 7α-hydroxylase activity did not increase the rate of hepatic LDL clearance even in animals in which hepatic 7α-hydroxylase activity was increased >10-fold and plasma LDL concentrations had fallen by >50%. This suggests that, in the absence of the LDL receptor pathway, other receptors capable of binding LDL are not up-regulated or derepressed in response to depletion of hepatic cholesterol. Hepatic LDL uptake in WHHL rabbits and LDL receptor−/− mice appears to occur entirely via receptor-independent mechanisms (19, 20). Apparently, the role of the LDL receptor is not subsumed by other lipoprotein transporters (LDL receptor-related protein, very low density lipoprotein receptor, and scavenger receptor type BI), at least under the conditions studied.

Overexpression of hepatic 7α-hydroxylase for 3 days increased the size of the bile salt pool by ~40%. It may take longer than 3 days for the size of the bile salt pool to plateau at a new steady-state level where the rate of bile salt excretion in the feces equals the rate of bile salt synthesis in the liver. However, the transient nature of 7α-hydroxylase overexpression precluded extended time course studies or studies of fecal bile salt excretion. If prolonged overexpression of 7α-hydroxylase can be achieved, either in transgenic animals or with improved somatic cell gene transfer techniques, it will be important to compare changes in hepatic 7α-hydroxylase activity with changes in the dynamics of the enterohepatic circulation and fecal bile salt excretion. It is possible that massive and persistent overexpression of hepatic 7α-hydroxylase could increase bile salt synthesis enough to cause diarrhea. However, in this study, mice consuming ~70 µmol of cholate/day, which exceeds by more than 10-fold the amount of bile salts normally excreted in the feces (41), did not have diarrhea. Moreover, the size of the bile salt pool in the cholate-fed animals increased by only ~70%. The quantity of bile salts in the enterohepatic circulation is regulated by a number of factors including the activity of the ileal bile salt transporter (42). Recent studies suggest that expansion of the enterohepatic bile salt pool results in down-regulation of the ileal bile salt transporter in mice (43) and other rodents (44). Regulation of the ileal bile salt transporter by luminal bile salts would tend to prevent marked expansion of the enterohepatic pool of bile salts in response to overexpression of hepatic 7α-hydroxylase or bile salt feeding (43, 44).

Replacement of LDL receptors by adenovirus-mediated gene transfer has been shown to normalize plasma LDL concentrations in LDL receptor−/− mice and WHHL rabbits (23, 45, 46). The reduction in plasma LDL concentrations is transient, however, due to an immune response mounted against the adenovirus vector. In addition, animals completely void of LDL receptor protein expression (such as LDL receptor−/− mice) generate both humoral and cellular immune responses specific for the therapeutic transgene product (47). This problem may limit the efficacy of replacement gene therapy for genetic deficien-
cy states where expression of the mutated gene product is completely absent. One strategy for circumventing the destructive immune response that is generated against the replace-
gene product is to overexpress alternative proteins that result in similar metabolic consequences. This strategy was recently demonstrated using transfer of the VLDL receptor gene to lower LDL concentrations in LDL receptor−/− mice (47). The present study indicates that direct augmentation of hepatic 7α-hydroxylase expression can lower plasma LDL cholesterol concentrations in animals with a genetic absence of LDL receptors. The magnitude of the hypcholesterolemic ef-
fact despite the marked compensatory increase in hepatic cho-
esterol synthesis suggests that the combination of 7α-hydroxylase gene transfer and an inhibitor of hepatic cholesterol synthesis might have considerable efficacy in lowering plasma LDL cholesterol concentrations in individuals lacking LDL rece-
ptors. Such a strategy might prove useful in patients with familial hypercholesterolemia who are completely devoid of residual LDL receptor expression if stable gene transfer be-
comes practical in humans.

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