Transgenic Expression of Cholesterol-7-α-Hydroxylase Prevents Atherosclerosis in C57BL/6J Mice

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Abstract—C57BL/6J mice are susceptible to atherosclerosis when fed a diet consisting of fat, cholesterol, and taurocholate. The susceptibility to diet-induced atherosclerosis is linked to a reduction in plasma high density lipoprotein (HDL). Diet-induced reduction of plasma HDL shows a physiological and a genetic correlation with repression of cholesterol-7-α-hydroxylase, the liver-specific enzyme that regulates the conversion of cholesterol into bile acids. To examine the hypothesis that the repression of cholesterol-7-α-hydroxylase is responsible for initiating the metabolic alterations leading to the formation of atherosclerosis and gallstones, we determined whether constitutive transgenic expression of cholesterol-7-α-hydroxylase in C57BL/6J mice would confer resistance to these 2 common human diseases. When fed the atherogenic diet, nontransgenic littermates, but not cholesterol-7-α-hydroxylase transgenic mice, accumulated cholesterol and cholesterol esters in their livers and plasma. Although the atherogenic diet caused a marked decrease in plasma HDL cholesterol in nontransgenic mice, HDL levels in transgenic mice remained relatively unchanged. Moreover, the ability of cholesterol-7-α-hydroxylase transgenic mice to maintain cholesterol and lipoprotein homeostasis completely prevented the formation of atherosclerosis and gallstones. These data establish the integral role that cholesterol-7-α-hydroxylase has in maintaining hepatic cholesterol homeostasis and, thus, in the susceptibility to the formation of gallstones and atherosclerosis. (Arterioscler Thromb Vasc Biol. 2002;22:121-126.)

Key Words: atherosclerosis • bile acids • cholesterol-7-α-hydroxylase • gallstones • lipoproteins

The formation of bile acids from cholesterol accounts for the majority of cholesterol that is metabolized and removed from the body of mammals.1 This pathway is tightly regulated by expression of the liver-specific enzyme cholesterol-7-α-hydroxylase (CYP7A1).2 In some species, the consumption of cholesterol in amounts that exceed nutritional requirements results in a homeostatic response that includes the induction of CYP7A1 expression and increased synthesis of bile acids.2-4 Conversely, hamsters5 and mice lacking the oxysterol receptor, liver-X-receptor-α,6 display an inability to induce CYP7A1 in response to dietary cholesterol and a marked susceptibility to cholesterol accumulation in plasma lipoproteins and liver. These findings suggest that modulation of the cholesterol/bile acid synthetic pathways contribute to the maintenance of cholesterol homeostasis. A severely reduced rate of bile acid synthesis and/or the expression of CYP7A1 is associated with 2 common human diseases: cholesterol gallstone disease (cholelithiasis)7,8 and atherosclerosis.9

The inbred mouse strain C57BL/6J develops dyslipidemia, gallstones, and atherosclerosis when fed a diet containing fat, cholesterol, and a bile acid (either cholate or taurocholate).3,10–14 In response to this atherogenic diet, C57BL/6J mice display a reduced expression of CYP7A1 mRNA that is due to negative-feedback inhibition by the dietary taurocholate. CYP7A1 repression was found to be associated with dyslipidemia characterized by an accumulation of plasma VLDLs, IDLs, and LDLs and a reduction in HDLs.3,10–14 Diet-induced reduction in plasma HDL cholesterol is one of the major changes linked to atherosclerosis in C57BL/6J mice.3,10–12

The phenotype of the inbred mouse strain C3H/HeJ is distinct from that of C57BL/6J mice; in response to the atherogenic diet, the C3H/HeJ strain displays no transcriptional repression of CYP7A1,12–15 no decrease in plasma HDL,12,16 and no formation of gallstones or atherosclerosis.12,16 Two separate, but complementary, studies suggest that diet-induced repression of CYP7A1 is linked to reduced HDL levels, gallstone disease, and atherosclerosis: in one study, diet-induced changes in plasma HDL levels vary as a linear function of diet-induced changes in CYP7A1 mRNA levels11; in the other study, 2 genetic loci that associate CYP7A1 regulation with HDL levels and with gallstones have been identified from progeny obtained from an intercross between C57BL/6J and C3H/HeJ mice.13 We have developed C57BL/6J mice that constitutively express a...
CYP7A1 transgene to examine the hypothesis that repression of CYP7A1 is responsible for initiating the metabolic alterations leading to the formation of atherosclerosis and gallstones.

Methods

Mice and Diets

The CYP7A1 transgenic mice were produced by using a liver-specific expression transgenic vector and bred as described.\textsuperscript{15} N5 generation mice were used. Female mice aged 8 to 12 weeks were used, unless noted otherwise. Mice were kept in a room having a light cycle from 6:00 AM to 6:00 PM. Mice were fed the designated diet ad libitum and had unrestricted access to drinking water. Transgenic mice were bred hemizygous for the transgene, and nontransgenic litters were used as controls. Mice were fed a chow diet (No. 5015 Harlan-Teklad) or the atherogenic diet, consisting of 12.5% fat, 1.25% cholesterol, and 0.5% taurocholic acid (No. 88051 Harlan-Teklad) for 8 to 20 weeks, as indicated.

Plasma Lipids

Mice were fasted overnight after the indicated feeding regimen. Mice were anesthetized by using isoflurane for retro-orbital phlebotomy. Aliquots of plasma were subjected to lipid (cholesterol, cholesterol ester, and triglyceride) analysis by using commercially available enzymatic kits and calibration standards (Sigma Chemical Co), as described.\textsuperscript{3} At least 4 mice were used per group.

CYP7A1 Enzyme Activity

Hepatic microsomes (n=4) were isolated at the mid-dark period of the light/dark cycle for the atherogenic diet studies. The enzyme activity of CYP7A1 was determined by high-performance liquid chromatography with the use of 4\textsuperscript{14}C]cholesterol.\textsuperscript{3,14} The atherogenic diet was fed to male mice (for 24 weeks) and female mice (for 15 weeks). The gallbladder was excised, and its contents were rinsed with 95% ethanol, centrifuged, and dried at 37°C. The gallstones were rinsed into a microcentrifuge tube. The gallstones were rinsed with ethanol and centrifuged, and then quantification of gallstones.

Northern Analysis

Mice were fasted overnight after the indicated feeding regimen. Mice were anesthetized with isoflurane for retro-orbital phlebotomy. Aliquots of plasma were subjected to lipid (cholesterol, cholesterol ester, and triglyceride) analysis by using commercially available enzymatic kits and calibration standards (Sigma Chemical Co), as described.\textsuperscript{3} At least 4 mice were used per group.

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Plasma Lipoprotein Fractionation and Analysis

Fasting plasma from 4 mice per group was pooled and separated by fast-performance liquid chromatography (FPLC) with the use of 2 Superose-6 columns (Pharmacia) in series. Fractions were assayed for total cholesterol as described.\textsuperscript{15} Weight gain was similar in CYP7A1 transgenic and nontransgenic littermates on either the chow diet or the taurocholate-containing atherogenic diet (Figure 1A). Livers from CYP7A1 transgenic C57Bl/6J mice fed a chow diet displayed no adverse effects from the \textsuperscript{5} fold increase in CYP7A1 enzyme activity.\textsuperscript{15} Weight gain was similar in CYP7A1 transgenic and nontransgenic littermates on either the chow diet or the taurocholate-containing atherogenic diet (Figure 1A). Livers from CYP7A1 transgenic C57Bl/6J mice fed a chow diet\textsuperscript{15} displayed a \textsuperscript{>20-fold increase in CYP7A1 mRNA expression (Figure 1A). CYP7A1 mRNA expression was decreased by 70% in nontransgenic mice fed the atherogenic diet, whereas CYP7A1 transgenic mice fed the atherogenic diet displayed no decrease (Figure 1A). These data demonstrate that the liver-specific enhancer from the human apoE gene,\textsuperscript{19} which was used as the promoter for the CYP7A1 transgene, was unaffected by the taurocholate-containing atherogenic diet. Accordingly, compared with their nontransgenic littermates, CYP7A1 transgenic mice fed the atherogenic diet displayed a \textsuperscript{7-fold greater microsomal CYP7A1 enzyme activity (Figure 1B).}

Bile Acid Pool Size and Composition

Mice (n=4) were fed the atherogenic diet for 8 weeks. Bile was extracted from the gallbladders and analyzed by high-performance liquid chromatography\textsuperscript{14} with the use of ultraviolet detection at 210 nm, as described.\textsuperscript{15}

Statistical Analysis

Results are given as mean±SD. Statistical analysis was determined by using the Student t test. Values of P\textless0.05 were considered to be significant.

Results

Transgenic Expression of CYP7A1 in C57BL/6J Mice

Previous studies have shown that CYP7A1 transgenic mice fed a chow diet display no adverse effects from the \textsuperscript{5} fold increase in CYP7A1 enzyme activity.\textsuperscript{15} Weight gain was similar in CYP7A1 transgenic and nontransgenic littermates on either the chow diet or the taurocholate-containing atherogenic diet (Figure 1A). Livers from CYP7A1 transgenic C57BL/6J mice fed a chow diet\textsuperscript{15} displayed a \textsuperscript{>20-fold increase in CYP7A1 mRNA expression (Figure 1A). CYP7A1 mRNA expression was decreased by 70% in nontransgenic mice fed the atherogenic diet, whereas CYP7A1 transgenic mice fed the atherogenic diet displayed no decrease (Figure 1A). These data demonstrate that the liver-specific enhancer from the human apoE gene,\textsuperscript{19} which was used as the promoter for the CYP7A1 transgene, was unaffected by the taurocholate-containing atherogenic diet. Accordingly, compared with their nontransgenic littermates, CYP7A1 transgenic mice fed the atherogenic diet displayed a \textsuperscript{7-fold greater microsomal CYP7A1 enzyme activity (Figure 1B).}

Expression of the CYP7A1 Transgene Maintains Cholesterol Homeostasis

Previous studies have shown that compared with nontransgenic littermates on the chow diet, CYP7A1 transgenic mice on the chow diet display a \textsuperscript{50} reduction in plasma cholesterol levels from lipoprotein particles containing apoB (ie,
VLDL, IDL, and LDL). In the present study, the atherogenic diet caused a significant 3-fold increase in plasma VLDL, IDL, and LDL cholesterol in nontransgenic C57BL/6J mice (Figure 2A). Compared with nontransgenic mice, CYP7A1 transgenic mice displayed reduced plasma levels of VLDL, IDL, and LDL cholesterol (Figure 2A) on either diet. Although in CYP7A1 transgenic mice, the atherogenic diet, compared with the chow diet, increased plasma VLDL, IDL, and LDL cholesterol by 2-fold, the levels were not significantly different from those of chow-fed nontransgenic mice (Figure 2A). Thus, expression of the CYP7A1 transgene prevented diet-induced accumulation of plasma VLDL, IDL, and LDL cholesterol.

Although the atherogenic diet reduced plasma HDL cholesterol levels by 60% in nontransgenic mice, HDL levels in CYP7A1 transgenic mice were only slightly affected (Figure 2B). This shows for the first time a causal link between the diet-dependent repression of CYP7A1 and reduction in plasma HDL cholesterol. We also analyzed the plasma lipoprotein cholesterol levels by FPLC (Figure 2C). Cholesterol was quantified in each designated fraction. Solid diamonds indicate Non-Tg mice fed the Athero diet; open squares, CYP7A1-Tg mice fed the Athero diet. The elution of plasma VLDL, IDL, and HDL is indicated. D, Plasma concentrations of triglycerides. Values are the mean ± SD. *P < 0.01 for CYP7A1-Tg vs Non-Tg mice.

Non-Tg mice showed none of these diet-induced changes (Figure 2C). However, the atherogenic diet caused a 50% decrease in plasma triglyceride levels in nontransgenic mice but had no effect on triglyceride levels in CYP7A1 transgenic mice (Figure 2D).

Marked differences in the appearance of the livers between the 2 groups of mice fed the atherogenic diet were evident (Figure 3A). The livers of nontransgenic mice appeared white/pink in color, whereas livers of CYP7A1 transgenic mice were dark red and indistinguishable from the livers of chow-fed mice. Quantification of hepatic cholesterol and cholesterol esters by gas-liquid chromatography showed that, when fed the atherogenic diet, livers of nontransgenic mice accumulated significantly more free (16-fold) and esterified (55-fold) cholesterol (Figure 3B) than CYP7A1 transgenic mice.

Expression of the CYP7A1 Transgene Increases mRNA Expression of the LDL Receptor and Cholesterol Biosynthetic Enzymes

It is well established that under most physiological conditions, the hepatic expression of cholesterol biosynthetic enzymes and the LDL receptor vary in parallel with the expression of CYP7A1. Additional studies have shown that the transgenic expression of CYP7A1, in cultured cells and in livers of chow-fed C57BL/6J mice, induces the expression of the LDL receptor, 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase), and squalene synthase. These mRNA increases were associated with an increase in mature sterol response element–binding protein-1. Therefore, we examined how the atherogenic diet affected the expression these genes. Northern blot analysis of hepatic mRNA from mice fed the atherogenic diet showed that the CYP7A1 transgenic mice expressed greater (4-fold) levels of mRNAs encoding the LDL receptor, HMG-CoA reductase, and squalene synthase than did the nontransgenic mice (Figure 3C). These data suggest that decreased hepatic accumulation of cholesterol and cholesterol esters in CYP7A1 transgenic mice fed the atherogenic diet is not caused by decreased expression of LDL receptors or cholesterol biosynthetic enzymes.
Expression of the CYP7A1 Transgene Prevents Gallstone Formation

When fed the bile acid–containing atherogenic diet, C57BL/6J mice develop cholesterol gallstones. Recent studies examining the susceptibility of different inbred mouse strains suggest that gallstone formation is correlated with dietary repression of CYP7A1. The CYP7A1 transgenic mice provided an opportunity to examine whether CYP7A1 repression is required for the formation of gallstones in C57BL/6J mice.

We have reported that the bile acid pool of CYP7A1 transgenic mice on a chow diet is twice that of nontransgenic littermates on a chow diet; this response is partially due to a 10-fold increase in taurochenodeoxycholate. The atherogenic diet did not further increase the bile acid pool of CYP7A1 transgenic mice, whereas it doubled that of nontransgenic mice. Thus, the bile acid pool size of both transgenic and nontransgenic mice becomes similar when fed the diet. Compared with bile from nontransgenic mice, bile from CYP7A1 transgenic mice contained more free cholesterol (Figure 4A). Compared with bile from nontransgenic mice, bile from CYP7A1 transgenic mice contained slightly more taurochenodeoxycholate (P<0.01) and smaller, but not significant, differences in other bile acids (Figure 4B). In addition, bile from nontransgenic mice contained 1.5-fold more free cholesterol than did bile from CYP7A1 transgenic mice (Figure 4C). No cholesterol ester was detected in bile from either group.

Gallbladders from all nontransgenic mice contained insoluble material identified to be cholesterol (ie, gas-liquid chromatography analysis demonstrated that >99.5% consisted of unesterified cholesterol, ie, gallstones). Moreover, although the gallbladders of all nontransgenic mice contained significant amounts of gallstones, virtually no gallstones were detected in the gallbladders of CYP7A1 transgenic mice.

Expression of the CYP7A1 Transgene Prevents Atherosclerosis

Because male C57BL/6J mice are less susceptible to diet-induced atherosclerosis than are female mice, they were fed the atherogenic diet for 24 weeks, whereas female mice were fed the diet for 15 weeks. Atherosclerotic lesions in the proximal aorta were clearly present in female (Figure 6A) and male (Figure 6B) nontransgenic mice. In marked contrast, CYP7A1 transgenic female (Figure 6A) and male (Figure 6B) mice displayed no detectable amounts of atherosclerotic lesions. These data demonstrate that augmented transgenic expression of CYP7A1 completely blocked the formation of atherosclerotic lesions.

Discussion

Our findings emphasize the importance of cholesterol homeostasis in the formation of 2 common diseases associated with cholesterol accumulation in bile (gallstones) and in cells residing within the arterial wall (atherosclerosis). As a result of augmented transgenic expression of CYP7A1, C57BL/6J transgenic mice displayed a complete resistance to the formation of gallstones and atherosclerosis. Our findings provide compelling evidence supporting the hypothesis that the cholesterol–bile acid biosynthetic pathway provides a therapeutic target that, in the context of C57BL/6J mice, is capable of preventing both these diseases.

Cholesterol precipitates in bile when its concentration relative to bile acids and phospholipids becomes excessive. Analysis of C57BL/6J mice indicates that the atherogenic diet induces the cholesterol gallstone formation by disproportionately increasing the secretion into bile of cholesterol relative to bile acids and phospholipids. The present study showed that preventing the diet-induced repression of CYP7A1 via constitutive transgenic expression of CYP7A1 completely blocked gallstone formation (Figure 5). Similarly, CYP7A1 transgene expression was also associated
with the prevention of hepatic accumulation of cholesterol and cholesterol esters (Figure 3). Although we did not measure biliary cholesterol excretion, previous findings have demonstrated that gallstone susceptibility in inbred mice is associated with the hypersecretion of biliary cholesterol. Thus, it is reasonable to propose that transgenic CYP7A1 expression blocked this “hypersecretion” by decreasing hepatic cholesterol accumulation (Figure 3). The finding that gallbladder bile obtained from CYP7A1 transgenic mice, compared with that obtained from nontransgenic mice, contained 50% less cholesterol (Figure 4C) is consistent with this proposal. The characteristics associated with the resistance of CYP7A1 transgenic mice to gallstone formation are consistent with those described for patients having gallstones (ie, reduced activity of CYP7A1 and greater levels of hepatic cholesterol).\(^5\)

There are 2 separate processes responsible for the susceptibility of C57BL/6J mice to diet-induced atherosclerosis: (1) dyslipidemia, which includes increased plasma VLDL, IDL, and LDL cholesterol and reduced HDL cholesterol, and (2) sensitivity of the cells within the liver to oxidatively modified LDL. In the absence of dyslipidemia, C57BL/6J mice are resistant to diet-induced atherosclerosis. Conversely, even in the presence of severe dyslipidemia, inflammation-resistant mice display resistance to atherosclerosis.\(^7\,33\)

Maintenance of hepatic cholesterol homeostasis can explain the resistance of CYP7A1 transgenic mice to diet-induced dyslipidemia (ie, hypercholesterolemia [Figure 2A] and hyperalphalipoproteinemia [Figure 2B]). The content of cholesterol ester relative to triglyceride in the liver determines the content of cholesterol ester relative to triglyceride in the core of nascent VLDL particles.\(^34\) Plasma VLDL from CYP7A1 transgenic mice contained markedly less cholesterol ester relative to triglycerides than did plasma VLDL from nontransgenic mice (Figure 2C), suggesting that reduction of cholesterol esters in the livers of CYP7A1 transgenic mice (Figure 3) led to plasma VLDL containing relatively less cholesterol esters. The secretion of VLDL containing less cholesterol esters and the increased hepatic expression of LDL receptor mRNA (Figure 3C) can account for the resistance of CYP7A1 transgenic mice to diet-induced hypercholesterolemia. Furthermore, because CYP7A1 transgenic mice also secrete more triglyceride-rich VLDL particles, which are rapidly cleared from plasma, it is likely that more surface phospholipids will be available to form HDL particles.\(^36\)

Our results showing that constitutive high-level expression of CYP7A1 is protective against hypercholesterolemia are not inconsistent with reports showing that genetic deletion of CYP7A1 (ie, CYP7A1\(^{-/-}\) mice) does not cause hypercholesterolemia.\(^18\) CYP7A1\(^{-/-}\) mice are resistant to hypercholesterolemia because they have a markedly reduced bile acid pool size that prevents efficient absorption of cholesterol. In marked contrast, when fed the atherogenic diet, CYP7A1 transgenic mice have a bile acid pool size that is the same as that in their nontransgenic littermates (Figure 4A); when fed a chow diet, CYP7A1 transgenic mice have a bile acid pool that is 2 times that of the nontransgenic mice.\(^15\) Also consistent with our conclusion that expression of the CYP7A1 transgene prevents hypercholesterolemia is the finding that liver-X-receptor-\(\alpha\)-deficient mice are more susceptible to diet-induced hypercholesterolemia because they lack the ability to induce the expression of CYP7A1.\(^6\)

The present study shows for the first time that augmented expression of CYP7A1 prevents the changes in plasma lipoproteins that are necessary to initiate the atherogenic process in C57BL/6J mice (Figure 6). The prevention of diet-induced dyslipidemia displayed by CYP7A1 transgenic mice may prevent the formation of proatherogenic lipid signals (eg, oxidized LDL)\(^19\) by (1) decreasing the amount of plasma LDL available to from oxidized LDL and (2) maintaining plasma levels of paraoxonase-1-containing HDL.\(^36\) Because CYP7A1 also decreases the amount of cholesterol available to form oxysterols, which may promote atherogen-
esis, its ability to reduce atherosclerosis lesion formation may involve multiple sites of intervention.

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