Bile Acids Decrease Hepatic Paraoxonase 1 Expression and Plasma High-Density Lipoprotein Levels via FXR-Mediated Signaling of FGFR4

Alejandra Gutierrez, Eric P. Ratliff, Allen M. Andres, Xinqiang Huang, Wallace L. McKeehan, Roger A. Davis

Objective—The purpose of this research was to determine how dietary bile acids repress hepatic expression of paraoxonase 1 (PON1).

Methods and Results—C57BL/6 mice and C3H/HeJ mice, having different susceptibilities to atherosclerosis, were fed a chow diet and an atherogenic diet containing taurocholate. Compared with the more atherosclerosis-susceptible C57BL/6 mice, C3H/HeJ mice display resistance to dietary bile acid repression of hepatic PON1 mRNA and decreased high-density lipoprotein cholesterol. Whereas knockout of toll receptor 4 did not affect response to taurocholate, deletion of either FXR or FGFR4 blocked taurocholate repression of PON1 and CYP7A1. FGF19, an activator of FGFR4 expressed in human ileum, decreased expression of both PON1 and CYP7A1 expression by human hepatoma cells. In all of the mice studied, dietary taurocholate increased ileal expression of FGF15, a FXR-inducible murine homologue of human FGF19.

Conclusions—Hepatic PON1 and CYP7A1 mRNA expression is repressed by bile acids via FXR-mediated induction of FGF15. Thus, the inability of C3H/HeJ mice to display taurocholate repression of PON1 and CYP7A1 mRNAs was not because of a lack of induction of FGF15 but rather signaling events distal to FGF15-FGFR4 association.

Key Words: paraoxonase1 ■ HDL ■ FXR ■ FGF15 ■ FGFR4

Multiple case-controlled studies of humans show an inverse relationship among the plasma activity of paraoxonase 1 (PON1), the formation of atherosclerotic lesions, and myocardial infarction.1–6 Thus, plasma levels of PON1 accurately predict susceptibility to atherosclerosis.

PON1 is mainly expressed in the liver7 and displays the unusual characteristic of being secreted with an intact N-terminal signal sequence.8 PON1 exhibits multiple enzyme activities including acting as an organophosphate esterase, a carboxyl esterase,9,10 a lactonase,11,12 and a phospholipase A2.13,14 The latter activity has been proposed to play an important role in inactivating the proatherogenic inflammatory lipids produced by the oxidative modification of low-density lipoprotein.15 Most of the PON1 present in plasma is associated with high-density lipoprotein (HDL), which may explain the well-established atheroprotective effect of HDL.13,16,17 The antiinflammatory properties of HDL are dependent, at least in part, on the presence of PON1.13 Dissociation of PON1 from HDL causes the HDL particle to become atherogenic.13

Studies using inbred strains of mice showed that a cholic acid–containing atherogenic diet reduced the hepatic expression of both PON1 and CYP7A1 mRNAs and plasma HDL cholesterol levels in atherosclerosis-susceptible C57BL/6 mice but not in atherosclerosis-resistant C3H/HeJ mice.18 Additional analysis of a subset of recombinant inbred strains derived from the B6 and C3H parental strains showed that the ability of the cholic acid–containing atherogenic diet to decrease hepatic PON1 mRNA expression segregated with aortic lesion development.18 In a similar subset of recombinant progeny from B6 and C3H parental strains, HDL levels were linked to 3 individual genetic loci also linked to the hepatic expression of CYP7A1.19 Thus, the ability of the cholic acid–containing atherogenic diet to reduce hepatic expression of both PON1 and CYP7A1 correlated with both plasma HDL levels and atherosclerosis lesion formation.

The atheroprotective effects of PON1 and CYP7A1 were additionally demonstrated by studies showing that transgenic expression of either PON14 or CYP7A120 reduced atherosclerotic lesion formation in susceptible C57BL/6 mice. Trans-
genic expression of PON1 reduced atherosclerotic lesion formation via the production of plasma HDL that protected low-density lipoprotein from oxidation. Transgenic expression of CYP7A1 reduced atherosclerotic lesion formation by preventing diet-induced hypercholesterolemia and reduced plasma HDL levels. The goal of this research was to elucidate the mechanism through which atherogenic bile acid–containing diets reduce hepatic expression of PON1 in atherosclerosis-susceptible C57BL/6 mice.

Methods

Mice and Diets

Male mice were housed in a room with a 12-hour light cycle. C57BL/6 mice with both alleles of the TLR4 deleted were a gift from Dr Peter Tobias (Scripps Research Institute). The generation of mice lacking FGFR4 has been described. C57BL/6 mice lacking FXR were a gift from Dr Frank Gonzalez (National Cancer Institute). The mice were fed either a chow (no. 5015 Harlan Teklad) or an atherogenic diet composed of chow and 20% olive oil, 2% cholesterol, and 0.5% taurocholate. FXR null animals were fed the experimental diets for 5 days because of increased mortality when fed the atherogenic diet. During the feeding periods, there were no apparent changes in appetite and/or body weight. Mice were euthanized at mid-dark.

Plasma Lipids

Mice were anesthetized with isofluorane and bled. Plasma total cholesterol and HDL cholesterol were determined.

Liver and Ileum RNA Isolation

After euthanization, livers and intestines were removed and flash frozen. A length of 5 cm from the cecum was used for the ileum. RNA was isolated from frozen tissue using the Versagene RNA Tissue kit (Gentra Systems). cDNA was made from 4 μg of RNA using the iScript cDNA synthesis kit (BioRad).

HepG2 Experiments

HepG2 cells, cultured as described, were treated with FGF19 (160 ng/mL; a gift from Genentech) or 10 ng/mL tumor necrosis factor (TNF-α) (R&D Systems) and harvested as indicated.

Real-Time SybrGreen PCR Analysis

Quantitative real-time PCR, using SYBR Green, was performed on an IQ-Cycler (BioRad) using primer sequences and annealing temperatures described in Figure I (available online at http://atvb.ahajournals.org).

Western Blot Detection of FGFR4

Livers samples (50 μg) were separated by SDS-PAGE electrophoresis and electroblotted onto PVDF membranes. A goat anti-mouse FGFR4 antibody 1:1000 (R&D systems) was used for detection. Samples were quantitated to their respective tubulin controls using ImageJ software (National Institutes of Health). Please see Figure II for more detail (available online at http://atvb.ahajournals.org).

Statistical Analysis

Statistical analysis between the groups were performed using the Student t test (double tailed, unpaired). All of the values are reported as mean±SD.

Results

TLR4 Function Is Not Involved in the Resistance of C3H/HeJ Mice to Diet-Induced Repression of Hepatic PON1 and CYP7A1 and Decreased HDL Cholesterol Levels

C3H/HeJ mice lack functional TLR4 and, thus, lipopolysaccharide signaling. Because inflammatory cytokines TNF-α and interleukin 1, induced by bile acid activation of macrophages, block hepatic expression of CYP7A1, functionally defective TLR4 might explain the inability of C3H/HeJ mice to display bile acid induction of inflammatory cytokines and repression of CYP7A1. To examine this possibility C57BL/6 tlr4 mice were fed either chow or the bile acid–containing atherogenic diet. Compared with C57BL/6, C3H/HeJ mice show a resistance to diet-induced repression of hepatic CYP7A1 (Figure 1A) and PON1 (Figure 1B). Induction of TNF-α (Figure 1C), and the associated decrease in HDL cholesterol levels (Figure 1D). Moreover, the lack of functional TLR4 did not affect the ability of the bile acid–containing atherogenic diet to cause similar changes in C57BL/6 mice (Figure 1A through 1D). Thus, the inability of atherosclerosis-resistant C3H/HeJ mice to respond to the bile acid–containing atherogenic diet does not require a functionally active TLR4.
FXR Is Required for Diet-Induced Repression of Hepatic PON1 and CYP7A1 mRNA Levels and Decreased HDL Cholesterol Levels

One of the major pathways through which bile acids affect gene expression is by binding to the nuclear receptor FXR.28–30 We examined the response of C57BL/6 mice lacking functional FXR nuclear receptors24 to the bile acid–containing atherogenic diet. Because these mice display increased hepatotoxicity to bile acids, the mice were fed the bile acid diet for only 5 days, allowing all of the mice to remain healthy throughout the experiment. In the absence of functional FXR nuclear receptors, livers of C57BL/6 mice display no repression of CYP7A1 (Figure 1A) or PON1 (Figure 1B), lack the induction of TNF-α/H9251 (Figure 1C), and the mice display no significant decrease in HDL cholesterol levels (Figure 1D) in response to the bile acid–containing atherogenic diet. Previous studies have shown that FXR/H11002 mice exhibit increased HDL cholesterol levels.31 Our findings indicate that FXR is essential for mediating dietary bile acid repression of both CYP7A1 and PON1 and the associated decrease in HDL cholesterol.

FGFR4 Links Hepatic Expression of CYP7A1, PON1, and HDL Cholesterol Levels to the Bile Acid–Containing Atherogenic Diet

FGFR4, containing a tyrosine kinase domain, can be activated by a number of ligands, including FXR-inducible FGF15 (mouse) and FGF19 (human).23,32–35 Mice lacking FGFR4 express unusually high levels of CYP7A1/H11002, and the mice display no significant decrease in HDL cholesterol levels (Figure 1D) in response to the bile acid–containing atherogenic diet. Previous studies have shown that Fxr/H11002–/– mice exhibit increased HDL cholesterol levels.35 Our findings indicate that FXR is essential for mediating dietary bile acid repression of both CYP7A1 and PON1 and the associated decrease in HDL cholesterol.

FGFR4 Decreases the mRNA Expression of CYP7A1 and PON1 by HepG2 Cells

We examined the regulation of PON1 mRNA expression by human hepatoma HepG2 cells. HepG2 cells were treated with
recombinant FGF19, the human homologue of FGF15 and an agonist of FGFR4.34 Cells were also treated with TNF-α, which reduces CYP7A1 expression.25 Within 6 hours, both FGF19 and TNF-α reduced CYP7A1 mRNA expression but did not affect the expression of PON1 mRNA (Figure 3). However, after 16 hours, the expression of both CYP7A1 and PON1 mRNA were significantly reduced by either FGF19 or TNF-α (Figure 3). The extremely short half-life of CYP7A1 mRNA37 may explain the more rapid decrease in CYP7A1 mRNA compared with PON1 mRNA. These combined data indicate that FGF15 (mouse) and FGF19 (HepG2 cells) decreased hepatic expression of both CYP7A1 and PON1 via a mechanism dependent on FGFR4.

Discussion

There is an agreement in results of studies of humans and experimental animals showing that the activity of plasma PON1 varies inversely with the development of atherosclerosis lesions.1–6 Additional studies showing that alteration of plasma activity of PON1 through manipulation of its genetic expression results in inverse changes in atherosclerosis lesion formation, indicating that PON1 plays a causal role.3,4,18,38 Some epidemiological studies have shown correlations between the enzyme activities of PON1 and genetic polymorphisms.39

Dietary Bile Acids Repress PON1 and CYP7A1 by 2 Mechanisms: Activation of Inflammatory Cytokines (eg, TNF-α) and Activation of FGFR4

Previous studies show that cholic acid is the dietary component in the atherogenic diet responsible for decreased hepatic expression of CYP7A1 and PON1.18,25,40 Activation of FXR28–30 and increased expression and secretion of inflammatory cytokines (eg, TNF-α)25,41 are 2 of the mechanisms through which bile acids alter gene expression. Our studies showing that genetic deletion of TLR4 has no effect on bile acid repression of either CYP7A1 or PON1 (Figure 1) clearly indicate that the lipopolysaccharide signaling receptor TLR4 is not required for bile acid repression of both genes. These findings corroborate gene mapping studies indicating that the loci responsible for resistance of C3H/HeJ mice to bile acid repression of CYP7A1 do not include the tlr4 locus on chromosome 4.19 In addition, whereas dietary bile acids increased hepatic expression of TNF-α in the livers of C57BL/6 mice (Figure 1C), this did not occur in C57BL/6
mice lacking TLR4; yet, they displayed repression of CYP7A1 and PON1.

**Bile Acid Activation of FXR Induces FGF15 and Represses CYP7A1 and PON1 via FGFR4 Signaling**

When fed the bile acid–containing diet, C57BL/6 mice lacking FXR24 showed neither a repression of PON1 and CYP7A1 nor an induction of the inflammatory cytokine TNF-α (Figure 1). These data clearly show that FXR is required for both bile acid repression of PON1 and CYP7A1. Several lines of evidence indicate that the FXR requirement involves induction of FGF15 by the ileum: (1) in the absence of FGFR4, dietary bile acids neither repressed PON1 nor CYP7A1 (Figure 2A); (2) in all of the mice studied except those lacking FXR, dietary bile acids induce the ileal expression of FGF15 (Figure 2B); and (3) in human hepatoma HepG2 cells, the addition of FGF19, the assumed homologue in humans, represses CYP7A1 and PON1 (Figure 2C). In previous studies, a diet containing 2% cholic acid, of which the bile acid content is 4-fold greater than the diet used in the present study, was shown to decrease CYP7A1 mRNA expression in FGFR4-deficient mice.23 This higher diet content of cholate may have been sufficient to cause liver inflammation and induction of TNF-α, a potent inhibitor of CYP7A1 mRNA expression.25

Our findings additionally indicate that the inability of atherosclerosis-resistant C3H/HeJ mice to respond to bile acid feeding is not caused by a lack of FXR-dependent induction of ileal FGF15 (Figure 2) or the hepatic content of FGFR4 protein (Figure 2D). The combined data suggest that the inability of C3H/HeJ mice to display taurocholate repression of PON1 and CYP7A1 mRNAs is caused by signaling events that occur distal to FGF15-FGFR4 association. Impaired signaling of the FGFR4 can explain the pleiotropic resistance of C3H/HeJ mice displayed in the inflammatory response of liver and vascular wall cells to atherogenic stimuli.42,43 FGFR signaling has a significant influence on arterial wall cell growth, differentiation, and susceptibility to atherosclerosis.44,45 One of several gene loci responsible for the resistance of C3H/HeJ mice to diet-induced atherosclerosis is located close to and may include FGFR4 (A.J. Lusis, unpublished data).

An interesting aspect of our findings is that reduction in HDL cholesterol levels, one of the major factors responsible for the susceptibility of mice to diet-induced atherosclerosis,42,43,46 correlates with repression of PON1 and CYP7A1 (Figures 1 and 2). Constitutive expression of a CYP7A1 transgene in C57BL/6 mice prevented the decrease in HDL cholesterol and atherosclerotic lesion formation that occurs in response to the bile acid–containing atherogenic diet.25 Transgenic overexpression of PON1 is associated with the protection of HDL from oxidative stress.4,47 Our combined findings suggest that regulation of hepatic expression of PON1 and CYP7A1 by FGFR4 signaling can have a significant influence on HDL cholesterol levels and susceptibility to atherosclerosis. The finding that FXR-mediated induction of ileal production of FGF15 is an important factor controlling hepatic PON1 and CYP7A1 expression reveals how a response of the intestine to diet may influence hepatic gene expression and susceptibility to atherosclerosis.

**Acknowledgments**

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**References**


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Figure I. Primers and annealing temperatures used for Real-Time PCR. Primer sequences and annealing temperatures are indicated. Primer sequences were checked for specificity using the NCBI BLASTn search program. PCR conditions were 95°C 3 min, followed by 40 cycles of 95°C 10 sec, annealing 45 sec, and 72°C 30 sec. PON1 PCR was performed three times with two different sets of primers which both gave the same results. PCR analysis were run in triplicate and melt curve analyses of PCR products were shown to produce a single DNA duplex. Internal standards were checked to confirm that they did not vary between the various conditions used.
Figure II. Livers from C3H/HeJ mice express more FGFR4 mRNA but less FGFR4 immunoreactive protein. Mice were fed either a chow diet (open bars) or an atherogenic diet containing 0.5% taurocholate (filled bars) for two weeks. (A) Hepatic expression of FGFR4 mRNA relative to GAPDH are shown as the mean +/- S.D. for 6 mice per group. * P< 0.02 significant increase compared to C57BL/6 mice. (B-D) FGFR4 protein levels. Livers (100 mg) were homogenized in buffer containing 50 mM HEPES (pH 7.4), 10 mM EDTA, 100 mM sodium fluoride, 100 mM sodium pyrophosphate, 10 mM sodium vanadate, 2 mM PMSF, 10 µg/mL aprotinin, and 1% Triton X-100. 50 µg of protein from each sample was added to SDS electrophoresis sample buffer and separated using a 4-12% Tris-glycine gradient gel, then electroblotted onto PVDF membranes. Immunodetection was performed following incubation with goat anti-mouse FGFR4 antibody 1:1000 (R & D systems) and rabbit anti-goat secondary antibody 1:2500 (Kirkengaard & Perry Lab) using the ECL Plus Western Blotting Detection Reagents (Amersham). Samples were quantitated to their respective tubulin loading controls using ImageJ software (NIH). Samples from FGFR4 deficient mice failed to produce an immunoreactive band indicating the specificity of the FGFR4 antibody, ie. FGFR4 deficient liver samples were used as negative controls (data not shown). Values for each sample (FGFR4: α-tubulin) represent the mean +/- S.D. for 3 separate mice. * P<0.005, ** P< 0.02 indicate differences between chow and atherogenic diet.
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**Figure I**
Figure II