

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

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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. (*Arterioscler Thromb Vasc Biol.* 2006;26:301-306.)

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 PON1¹⁴ or CYP7A1²⁰ reduced atherosclerotic lesion formation in susceptible C57BL/6 mice. Trans-

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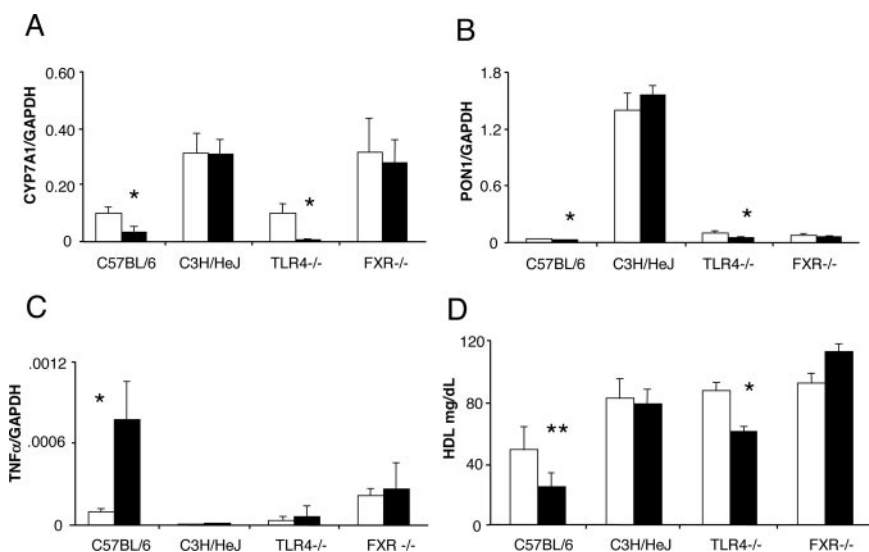


Figure 1. Response of C57BL/6, C3H/HeJ, C57BL/6 TLR4^{-/-}, and C57BL/6 FXR^{-/-} mice to dietary bile acid. Mice were fed either a chow diet (□) or an atherogenic diet containing 0.5% taurocholate (■) for 2 weeks, except FXR^{-/-} mice, which were fed for 5 days. Hepatic mRNA expression of CYP7A1 (A), PON1 (B), TNF- α (C), and HDL levels (D) are shown as the mean \pm SD for 6 mice per group. * P <0.0005, ** P <0.02 indicate differences between chow and atherogenic diet.

genic expression of PON1 reduced atherosclerotic lesion formation via the production of plasma HDL that protected low-density lipoprotein from oxidation.⁴ Transgenic expression of CYP7A1 reduced atherosclerotic lesion formation by preventing diet-induced hypercholesterolemia and reduced plasma HDL levels.²⁰ 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 deleted²¹ were a gift from Dr Peter Tobias (Scripps Research Institute). The generation of mice lacking FGFR4 has been described.^{22,23} C57BL/6 mice lacking FXR²⁴ 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 isoflurane and bled. Plasma total cholesterol and HDL cholesterol were determined.²⁰

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 μ g of RNA using the iScript cDNA synthesis kit (BioRad).

HepG2 Experiments

HepG2 cells, cultured as described,²⁵ 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 μ g) 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 \pm 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.²⁶ Because inflammatory cytokines TNF- α and interleukin 1, induced by bile acid activation of macrophages, block hepatic expression of CYP7A1,²⁵ 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*^{-/-} knockout mice²⁷ were fed either chow or the bile acid-containing atherogenic diet.²⁰ 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.

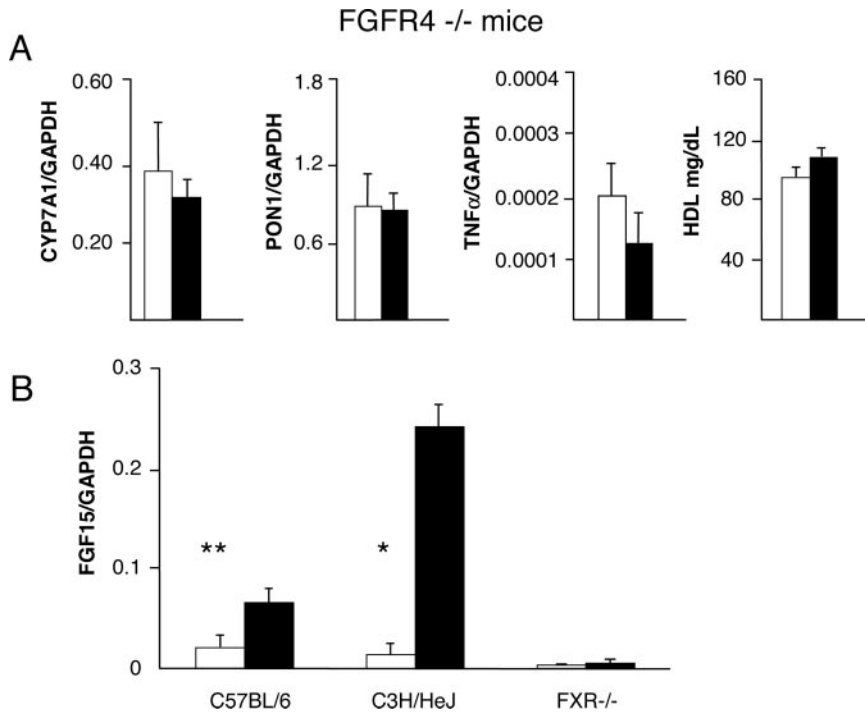


Figure 2. FGFR4 is required for the reduction in hepatic expression of CYP7A1, PON1, and plasma HDL cholesterol levels caused by dietary bile acid. Mice were fed either a chow diet (□) or an atherogenic diet containing 0.5% taurocholate (■) for 2 weeks, except FXR^{-/-} mice, which were fed for 5 days. (A) Hepatic mRNA expression of CYP7A1, PON1, TNF- α , and HDL levels are shown as the mean \pm SD for 6 mice per group. (B) Ileal expression of FGF15 in C57BL/6, C3H/HeJ, and FXR^{-/-} mice are shown as the mean \pm SD for 6 mice per group. * P < 0.0005, ** P < 0.04 indicate differences between chow and atherogenic diet.

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 (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^{-/-} 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.^{23,35} Additional studies show that dietary bile acids activate FGFR4 and, as a result, the expression of CYP7A1 mRNA is reduced via a mechanism that appears to not require FXR-inducible small heterodimer partner or c-Jun N-terminal kinase.³⁵ Expression of a mutant form of FGFR4, thought to cause constitutive activation of the tyrosine kinase domain in mice lacking the endogenous FGFR4, resulted

in constitutive repression of CYP7A1.³⁵ We examined the regulation of PON1 expression in mice lacking FGFR4 (Figure 2A). FGFR4^{-/-} mice exhibited a phenotype similar to C3H/HeJ and FXR^{-/-} mice, that is, resistance to bile acid repression of CYP7A1 and PON1 mRNA (Figure 2A). In addition, mice lacking FGFR4 also failed to display the induction of hepatic TNF- α mRNA expression and decrease in HDL cholesterol, which occur in response to dietary bile acids (Figure 2A). These findings indicate that activation of FGFR4 signaling reduces expression of both CYP7A1 and PON1, as well plasma HDL cholesterol levels via a mechanism independent of TNF- α .

Murine FGF15 and its human homologue FGF19 are one of many FGF ligands that bind to and activate FGFR4 signaling.^{33,34,36} FGF15 gene expression in the ileum has been shown to be bile acid inducible via activation of FXR and has been proposed to be responsible for FGFR4-mediated repression of CYP7A1 in response to bile acids.^{23,33} We examined the ability of the individual groups of mice to display an induction of FGF15 mRNA by the bile acid-containing atherogenic diet (Figure 2B). Our findings show that the bile acid-containing atherogenic diet resulted in enhanced expression of FGF15 mRNA in all of the mice (including bile acid-resistant C3H/HeJ mice) examined except those lacking FXR (Figure 2B). Our findings support those suggesting that FXR mediates the induction of FGF15 mRNA by bile acids. Because C3H/HeJ mice displayed an induction of FGF15 mRNA similar to the induction displayed by C57BL/6 mice (Figure 2B), impaired FXR induction of FGF15 is not responsible for differences in response to the atherogenic diet.

FGF19 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

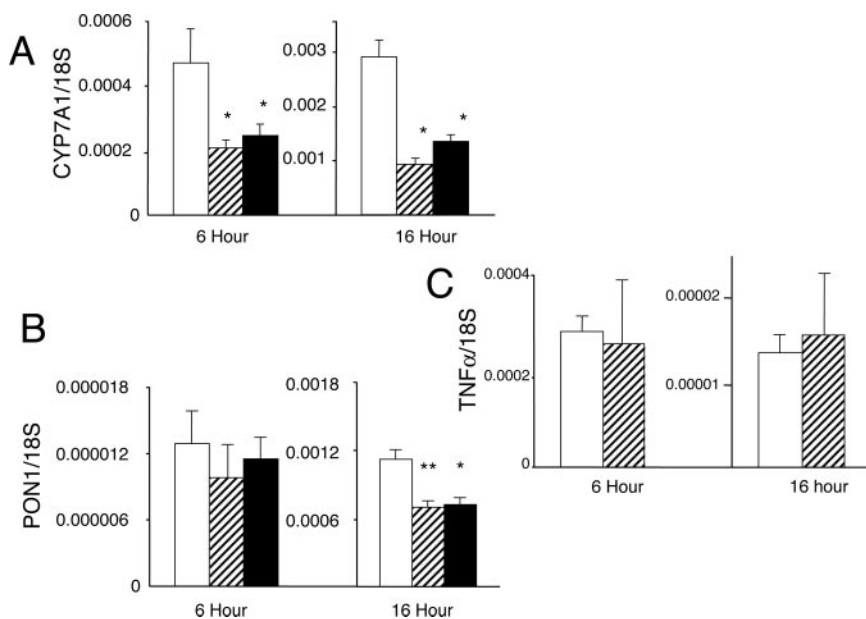


Figure 3. FGF19 decreases the expression of CYP7A1 and PON1 mRNA by HepG2 cells without increasing TNF- α mRNA. HepG2 cells were incubated with either 160 ng/mL FGF19 (hatched), 10 ng/mL TNF- α (black), or no treatment (white) for time indicated. mRNA expression of CYP7A1 (A), PON1 (B), and TNF- α (C) relative to 18S RNA was determined by real-time PCR and is shown as the mean \pm SD of triplicate dishes. * $P < 0.0005$, ** $P < 0.02$ indicate differences between no treatment and respective condition.

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.

FGFR4 mRNA, But Not Protein, Is Elevated in the Livers of C3H/HeJ Mice

We examined the hepatic content of FGFR4 mRNA and protein in the livers of C57BL/6 and C3H/HeJ mice fed chow or the bile acid-containing atherogenic diet. When fed either diet, FGFR4 mRNA relative to GAPDH was significantly (5-fold) greater in C3H/HeJ mice compared with C57BL/6 mice (Figure IIA). When fed the chow diet, the amount of immunodetectable FGFR4 protein in the livers of C57BL/6 was significantly ($\approx 20\%$; $P < 0.006$) higher than that of C3H/HeJ mice (Figure IIB). However, when fed the bile acid-containing diet, the amount of FGFR4 protein in livers from C57BL/6 mice decreased (-23% ; $P < 0.025$; Figure IIC), whereas this decrease did not occur in the livers of resistant C3H/HeJ mice. Thus, when fed the bile acid-containing diet, the amount of FGFR4 protein in the livers of C57BL/6 mice became similar to those of C3H/HeJ mice. Thus, the inability of C3H/HeJ mice to display taurocholate repression of PON-1 and CYP7A1 mRNAs was not because of a lack of induction of FGF15 but, rather, signaling events distal to FGF15-FGFR4 association.

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 More recent analysis indicates that factors other than the PON1 gene are important determinants of plasma PON1 activity.⁵ Our findings provide evidence indicating that bile acids repress PON1 mRNA expression via FXR activation of ileal FGF15, which subsequently acts on hepatic FGFR4 causing decreased hepatic PON1 mRNA expression. Our findings additionally show that the inability of atherosclerosis-resistant 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.

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 of murine FGF15,^{33,34} caused repression of both PON1 and CYP7A1 (Figure 3). Perhaps the most parsimonious interpretation of the combined data are that via ligand activation of FXR, bile acids induce ileal expression of FGF15, which, through FGFR4-dependent signaling, caused decreased expression of both PON1 and CYP7A1. In previous studies, a diet containing 2% cholic acid, of which the bile acid content is 4-fold greater than the diet used in this 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 II). 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, 2005).

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 signif-

icant 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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