Antiatherosclerotic effect of farnesoid X receptor

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Mencarelli A, Renga B, Distrutti E, Fiorucci S. Antiatherosclerotic effect of farnesoid X receptor. Am J Physiol Heart Circ Physiol 296: H272–H281, 2009. First published November 21, 2008; doi:10.1152/ajpheart.01075.2008.—The farnesoid X receptor (FXR) is a member of the nuclear receptor superfamily that functions as an endogenous sensor for bile acids and regulates cholesterol and fatty acid metabolism. The effect of FXR activation on aortic plaque formation was assessed by feeding apolipoprotein E-deficient (ApoE−/−) mice with the synthetic FXR ligand INT-747, a cheno-deoxycholic acid derivative, at doses of 3 and 10 mg·kg−1·day−1, or with rosiglitazone, a peroxisome proliferator-activated receptor-γ ligand, at the dose of 10 mg·kg−1·day−1 for 12 wk. Administration of INT-747 reduced formation of aortic plaque area by 95% (P < 0.01), and a similar antiplaque activity was exerted by administration of rosiglitazone. INT-747 administration to ApoE−/− mice reduced aortic expression of IL-1β, IL-6, and CD11b mRNA, while it upregulated the expression of FXR and its target gene, the small heterodimer partner (SHP). FXR activation reduced the liver expression of sterol regulatory element binding protein 1c, resulting in reduced triglyceride and cholesterol content in the liver and amelioration of hyperlipidemia. FXR expression, mRNA and protein, was detected in human macrophages and macrophage cell lines. FXR activation by natural and synthetic ligands in these cell types attenuated IL-1β, IL-6, and TNF-α gene induction in response to Toll-like receptor 4 activation by LPS. Using spleen monocytes from wild-type and FXR−/− mice, we demonstrated that FXR gene ablation exacerbates IL-6 and TNF-α generation by LPS-stimulated macrophages. FXR was also able to reduce cholesterol uptake on macrophages by regulation of CD36 and ABCA1 expression. We found that FXR and SHP are expressed in the aorta and macrophages and that FXR ligands might have utility in prevention and treatment of atherosclerotic lesions.

THE FARNESOID X RECEPTOR (FXR) is a member of the nuclear receptor superfamily of ligand-activated transcription factors that regulates cholesterol and fatty acid metabolism and functions. FXR is an endogenous sensor for bile acids, with cheno-deoxycholic acid (CDCA) being the most potent endogenous ligand (21, 30, 35, 37, 47). In the liver, FXR ligands induce the activation of the small heterodimer partner (SHP), an atypical nuclear receptor that lacks a DNA-binding domain and mediates some of the metabolic activities of FXR (10, 28). A SHP-dependent inhibition of P-450 cholesterol 7α-hydroxylase (CYP7A1), which is central to the synthesis of bile acids from cholesterol, and of sterol regulatory element binding protein 1c (SREBP-1c), a master gene involved in the regulation of hepatic fatty acid and triglyceride biosynthesis, has been demonstrated to mediate some of the metabolic effects of FXR, leading to a decrease in hepatic triglyceride storage and very low-density lipoprotein (VLDL) production (48). FXR also plays a role in lipoprotein metabolism since both the phospholipid transfer protein, which is essential for phospholipids transfer from VLDL to high-density lipoprotein (HDL), and the apolipoprotein CII (ApoCII), an obligate cofactor for lipoprotein lipase, are FXR-regulated genes (19, 28, 44). In addition, FXR ligands inhibit the expression of apolipoprotein A-I (ApoA-I), the major apolipoprotein component of HDL (7). Further supporting a regulatory role on lipid homeostasis, FXR-null mice fed a high-cholesterol diet (1% cholesterol) were distinguished from wild-type mice by elevated serum bile acid, cholesterol, and triglycerides, increased hepatic cholesterol and triglycerides, and a proatherogenic serum lipoprotein profile (42). In addition, FXR and ApoE double-knockout mice fed a high-cholesterol diet show an increased tendency toward development of atherosclerotic lesions in comparison with single-knockout mice, further suggesting that FXR activation might have a mechanistic implication in protecting against atherosclerosis (13).

Atherosclerosis, a major cause of morbidity and mortality in Western society, is a chronic disease characterized by subendothelial accumulation of atherogenic lipoproteins, extracellular matrix components, and inflammatory cells (26). Activation of inflammatory genes in the vessel wall, with subsequent adhesion, chemoattraction, subendothelial migration, retention, and activation of inflammatory and immune cells such as monocytes and T cells, is believed to play a critical role in the initiation, progression, and destabilization of atherosclerotic plaques (26). Macrophages are central to the development of atherosclerotic lesions because of their ability to take up modified lipoproteins and to release inflammatory mediators (6). Peroxisome proliferator-activated receptors (PPARs) and liver X receptors (LXRs), two metabolic nuclear receptors that regulate fatty acid and cholesterol homeostasis (5, 18), exert counterregulatory effects on macrophages, and their antiatherosclerotic activity is mainly ascribed to their ability to regulate macrophage biology (17, 25). Whether FXR ligands have the potential to protect against atherosclerosis and to modulate the innate immunity of the cell is unknown. Here, we report that human macrophages express FXR, mRNA and protein, and that FXR activation by natural and synthetic ligands attenuates expression of inflammatory genes induced by Toll-like receptor 4 (TLR-4) activation. In addition, we provide evidence that in vivo activation of FXR with 6-ethyl-CDCA (INT-747) (36), a synthetic FXR ligand, reverses the development of atherosclerotic plaques in apolipoprotein E-deficient (ApoE−/−) mice through regulation of lipid metabolism, macrophage cholesterol uptake and efflux, and inflammation. This finding paves the way for the development of a potent FXR ligand to treat atherosclerotic disorders.
MATERIAL AND METHODS

Animals. ApoE\(^{-/-}\) male mice and C57BL/6J male mice were from Jackson Laboratory (Ann Harbor, MI). All studies were approved by the Animal Study Committee of the University of Perugia (Italy). Mice were maintained in a temperature-controlled facility with a 12:12-h light-dark cycle and were given free access to food and water. Ten-week-old ApoE\(^{-/-}\) mice were randomized into four groups: group 1 received no treatment; groups 2 and 3 were administered INT-747 (8, 36) (3 and 10 mg/kg), respectively; and group 4 was administered rosiglitazone (20 mg/kg). All drugs were administered 5 days a week for 12 wk by gavage. During the experiment, mice were weighed once a week. At the end of the experiment, animals were fasted 12 h and anesthetized with pentobarbital sodium before harvesting of blood for subsequent lipid measurements and tissues for RNA isolation, lipid measurement, and histology. Serum and liver content of total cholesterol, triglyceride, HDL, LDL, and VLDL was determined.

RESULTS

FXR-null (FXR\(^{-/-}\)) mice were provided by F. Gonzales (National Institutes of Health, Bethesda, MD). FXR\(^{-/-}\) and wild-type mice were on the same mixed background (C57BL/6N × 129/SVJ, backcross × C57BL/6N). All studies were approved by the Animal Study Committee of the University of Perugia (Italy). Mice were maintained in a temperature-controlled facility with a 12:12-h light-dark cycle and were given free access to food and water. At the end of the experiment, animals were fasted 12 h and anesthetized with pentobarbital sodium before harvesting of the spleen for subsequent monocyte isolation. Monocytes were obtained by means of a magnetic cell separation isolation system as previously described using mouse CD11b Microbeads (Milteny Biotech).

Quantification of atherosclerotic lesions and liver histology. Quantification of early atherosclerotic fatty streak lesions was done by evaluating the lesion size in the aortic sinus as described previously by others (34). Briefly, the heart and upper section of the aorta were removed from the animals, cleaned of peripheral fat under a dissecting microscope, and sectioned parallel to the atra leaflets. The upper section was embedded in Tissue-Tek OCT (Miles, Elkhart, IN) in a cryostat mold and snap frozen in nitrogen. Ten sections (10 \(\mu\)m thick) throughout the aortic sinus (400 \(\mu\)m), each separated by 30-\(\mu\)m space, were taken for analysis. The distal portion of the aortic sinus was recognized by the three valve cusps that are the junctions of the aorta to the heart. Sections were evaluated for fatty streak lesions after staining with hematoxylin and eosin. The mean lesion area was quantified from 10 digitally captured sections per mice using a BX60.
microscope (Olympus, Rome, Italy) and digitalized using a SPOT-2 camera (Diagnostic Instruments, Sterling Heights, MI) with a resolution of 1,315 × 1,033 pixels. Area measurements (expressed in pixels) were done using the free software ImageJ 1.33a (W. Rasband, National Institutes of Health). Additional sections were used for neutral lipid staining with Oil Red O and CD11b immunohistochemistry using a rat anti-mouse CD11b polyclonal antibody (BD PharMingen, San Diego, CA). In a second set of experiments, protection exerted by in vivo treatment with INT-747 and rosiglitazone (same protocol) was assessed by Sudan IV staining of aortic plaques on the whole aorta (en face analysis). For en face analysis, aortas, including the ascending arch, thoracic, and abdominal segments, were dissected, gently cleaned of the adventitia, and stained with Sudan IV. The surface lesion area was quantified as described above. For liver histology, samples of the right and left liver lobes (10–15 mg each) taken from each animal were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

Real-time PCR analysis. Quantification of gene expression in tissues was performed by quantitative real-time polymerase chain reaction (qRT-PCR) as described previously (14). The relative gene expression of each gene was expressed as 2−ΔΔCt (where C is cycle threshold). The following primers were used for analysis: murine (m)SREBP-1c: gataaagaggagctcaggtagtagttgatcagcttgcttgg; mFXR-CoA-dehydrogenase (mLCAD): gcatcaacatgcgcagagaaa and tcgcaatatagggcaggtttcaagatcgcaatgg andctccttggtgctccactagc; murine long-chain acyltransferase 1 (mCPT1): catgtcaagccagacgaaga and tggtaggagagcaggggacaggcaaat and ttcacaaacacctccccttc; murine carnitine palmitoyltransferase 1 homologue 1 (mABCG1): gaagtggcatcaggggagta and aaagaaacgggttcacatcg; mABCA1: and gagccatctaggcaatctcg; murine ATP-binding cassette transporter G1 (mABCG1): cacgttccttgaagat; murine LDL receptor (mLDLR): tcctggagatgtgatggaca; murine glutaryl-CoA-reductase (mHMGCoAR): ccgaattgtatgtggcactg and ggtggtggatgggaagctgtcta and acatcatcgagggtgaaagg; murine hydroximetylglutarate dehydrogenase (mHMGCoA): acaagagtggcttgctggtt and gcacctgccactcatgctcagcttcagagtgtcagatta and cgtccggcacaaatcctg; mSHP: tctcttcttccgccctatca and mPNP: gactactctgcatgcttcttg; mIL-1α: ccgaatgctgaggaagatgc and tcgtatatcactgtcttcattcacggtctgat; hIL-1α: aggagacttgcctggtgaaa and caggggtggttattgcatct; hSHP: cctactgatgtgtcgaa; hTNF-α: gggctggtggtaaggcgagtg; hIL-6: ggacaagctgaggaagatgc and tcgtatatcactgtcttcattcacggtctgat; hIL-6: tcacagcagca- catcaacaa and tgtcctcatcctcgaaggtc; mIL-6: ccggagaggagacttcacag and ccgaatgctgaggaagatgc; mIL-1β: cccaatgagtaggctggaga and tctggacccattccttcttg; mIL-1β: tcgcctctgcgctaga and tgtcctcatcctcgaaggtc; mIL-1β: ccgaatgctgaggaagatgc and tcgtatatcactgtcttcattcacggtctgat; hIL-1β: ggacaatgctgaggaagatgc and tcgtatatcactgtcttcattcacggtctgat; hIL-1β: ccgaatgctgaggaagatgc and tcgtatatcactgtcttcattcacggtctgat; INT-747 3 mg/Kg

Isolation of human peripheral blood mononuclear cells. Human peripheral blood mononuclear cells (PBMCs) from healthy individual donors were obtained from the Blood Bank Service of Perugia University Hospital. PBMCs were isolated by density gradient centrifugation through a Ficoll-Hypaque gradient (Pharmacia Biotech, Uppsala, Sweden). Monocytes were isolated by negative selection using magnetic cell sorting according to the manufacturer’s instructions (Miltenyi Biotec, Milan, Italy). Assessment of FXR expression

on purified monocytes was carried out by Western blot analysis and qualitative RT-PCR according to a previously published method.

Immunohistochemistry. The cells were spread on poly-L-lysine-coated slides at a concentration of 500,000 cells/ml (150 μl/slide) by using a cytospin. The slides were subsequently fixed in ethanol-acetone (1:1 vol/vol) for 10 min at −20°C and processed for immunocytochemistry. The specimens were incubated 30 min at 94–98°C in buffer citrate; then, after a wash in Tris-buffered saline-Tween (10% rabbit serum), primary antibody (rabbit anti-human; Santa Cruz) was added for 30 min at room temperature. For the secondary steps, we used Histostain-Plus Kit (DAB, Rabbit, Zymed).

Macrophage isolation for ABCA1 and ABCG1 expression. For macrophage isolation, groups of 8- to 12-wk-old female mice were used. On day 0, mice were injected intraperitoneally with 2 ml of 3% thioglycolate, and peritoneal macrophages were collected on day 4. After centrifugation at 1,000 g for 5 min, cells were harvested and resuspended into RPMI 1640 medium containing 10% fetal bovine serum and 100 U/ml penicillin-streptomycin. Three to five million cells were plated onto 100-mm petri dishes. The nonadherent cells were removed, and the culture media were changed after 4-h incubation at 37°C. Peritoneal-derived macrophages were obtained from wild-type and ApoE−/− mice. Macrophages were treated with INT-747 (10 μM) for 18 h.

Flow cytometry. Blood-derived macrophages were collected from wild-type and ApoE−/− mice (n = 4/genotype) as described in Macrophage isolation for ABCA1 and ABCG1 expression. The cells were stained by anti-CD36-PE antibody (2 μg/half-million cells; Santa Cruz) and analyzed by a flow cytometer (Beckman Coulter).
FXR activation protects against atherosclerosis development in ApoE \(^{-/-}\) mice. Treating ApoE \(^{-/-}\) mice with INT-747 (both doses) and rosiglitazone had no effect on body weight or mortality of the mice (data not shown). ApoE \(^{-/-}\) mice administered the FXR and PPAR-\(\gamma\) ligand demonstrated a significant attenuation of aortic plaque formation in comparison with untreated mice. The plaque area was reduced by INT-747 treatment in a dose-dependent manner (Fig. 1, B, E, H, K, N, P, Q, and R). At the 10 mg/kg dose, INT-747 almost completely abrogated plaque formation, resulting in \(\approx 95.0\%\) reduction of plaque size as measured by morphometric analysis in comparison with untreated ApoE \(^{-/-}\) mice (\(P < 0.01\)). Analysis of Oil Red O staining and Sudan IV staining of whole aortas (Figs. 1 and 2), two measures of neutral lipid content in the aorta, demonstrated that INT-747 attenuated plaque lipid accumulation. In addition, INT-747 (10 mg/kg) significantly reduced CD11b immunostaining on atherosclerotic plaques (Fig. 1, C, F, I, L, and O). Treating mice with the PPAR-\(\gamma\) ligand rosiglitazone (20 mg/kg) effectively reduced plaque formation (Figs. 1 and 2), lipid accumulation (Oil Red O and Sudan IV staining), and CD11b immunostaining in aortic plaques. The effect exerted by 10 mg/kg INT-747 was comparable to that of 20 mg/kg rosiglitazone (Fig. 1).

To further investigate the mechanism through which FXR exerts its antiatherosclerotic effects, we then measured the expression of genes involved in inflammation and lipid homeostasis in the aorta. We found that expression of the two isoforms of FXR, \(\alpha\) and \(\beta\), was significantly enhanced in ApoE \(^{-/-}\) mice in comparison with wild-type naive mice (\(\approx 4\)-fold induction). Furthermore, a robust induction of FXR-\(\beta\) expression was strongly decreased (\(P < 0.05\)). Exposure of ApoE \(^{-/-}\) macrophages to the INT-747 caused a robust induction of ABCA1 mRNA expression, suggesting that FXR induces cholesterol export from macrophages.

**Statistical analysis.** All values are expressed as means \(\pm SE\). The analysis of variance with Bonferroni correction for multiple comparisons (Graphpad Software, San Diego, CA) was used to assess significant statistical difference between groups.

**RESULTS**

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mRNA, an ≈150-fold increase (≈21-fold induction in comparison with ApoE<sup>−/−</sup>), was observed in mice administered INT-747 (10 mg/kg) but not rosiglitazone (Fig. 2A). INT-747 administration resulted in a dose-dependent induction of SHP expression (≈100-fold). In contrast, PPAR-γ mRNA expression was abrogated in ApoE<sup>−/−</sup> aorta (≈90% reduction). INT-747 (both doses) and rosiglitazone were equally effective in restoring PPAR-γ mRNA expression in the aorta (Fig. 2A).

Analysis of proinflammatory mediators in the aorta revealed that expression of CD11b, MCP-1, IL-6, TNF-α, and IL-1β mRNAs was highly induced in ApoE<sup>−/−</sup> mice (P < 0.01 vs. naïve). This gene pattern was altered by INT-747 in a dose-dependent manner (Fig. 3B). Thus, at the 10 mg/kg dose, INT-747 lowered CD11b, IL-6, TNF-α, and IL-1β mRNA expression (P < 0.01 vs. ApoE<sup>−/−</sup>). A similar pattern was observed in ApoE<sup>−/−</sup> mice treated with rosiglitazone, which also reduced MCP-1 (Fig. 2B; P < 0.01 vs. ApoE<sup>−/−</sup>).

FXR is a counterregulatory gene in macrophages. Since the above mentioned data demonstrate that FXR activation regulates the expression of inflammatory genes in the aorta and that macrophages are an essential cell type involved in plaque formation (26), we then investigated whether FXR is expressed by and modulates the effector functions of macrophages in vitro. Cell sorting of human PBMCs by specific cell lineage markers gives rise to cell populations that were 90–95% pure. By RT-PCR analysis, expression of FXR-α and -β, the two isoforms of FXR, was detected in CD11b<sup>+</sup> cells, monocytes (Fig. 3A). These data were confirmed by Western blot analysis, using an FXR antibody directed against a common epitope expressed on both FXR subtypes (Fig. 3A) and by immunohistochemistry staining of CD11b<sup>+</sup> cells with an anti-FXR antibody (Fig. 3B). As shown in Fig. 3B, immunohistochemistry staining of human CD11b<sup>+</sup> cells gives rise to a characteristic pattern of nuclear localization, similar to that observed in HepG2 cells, a hepatocarcinoma cell line that expresses FXR (data not shown). Altogether, these data establish that human macrophages express FXR.

To establish whether macrophage’s FXR was functionally active, we then investigated whether exposure to natural and synthetic FXR ligands modulates one of the key effector function of activated macrophages, i.e., cytokine generation in response to TLR-4 activation. As shown in Fig. 3C, both INT-747 and CDCA reversed induction of proinflammatory mediators caused by TLR-4 activation. SHP, a known FXR target, is also expressed by human macrophages as shown by RT-PCR. We found that exposure to INT-747 induced SHP over the same range of concentrations that inhibit TNF-α mRNA expression (Fig. 3D). To confirm that these effects were mediated by FXR, we then tested whether INT-747 inhibits cytokine generation in spleen-derived macrophages obtained from wild-type and FXR<sup>−/−</sup> mice. The qRT-PCR analysis (3 h after LPS stimulation) confirmed that INT-747 effectively inhibited IL-6 and TNF-α mRNA expression induced by LPS in wild-type mice. This inhibitory effect was lost in FXR<sup>−/−</sup> macrophages. In addition, exposure of FXR<sup>−/−</sup> macrophages to LPS resulted in enhanced expression, both mRNA and cytokine levels, of IL-6 and TNF-α in comparison with macrophages isolated from wild-type mice (Fig. 4).

FXR regulates the expression of cholesterol transporters in macrophages. The accumulation of cholesterol in macrophage foam cells is a central event in atherogenesis; in fact, these cells express several scavenger receptors that are capable of taking up oxidized LDL as CD36. Interestingly, in vivo treatment with INT-747 caused a robust reduction of CD36 expression on CD11b<sup>+</sup> cells (Fig. 3E).

![Image](http://ajpheart.physiology.org/)

Fig. 4. FXR gene ablation exacerbates cytokine production and abrogates inhibition of INT-747 on cytokine generation (mRNA and protein levels) induced by Toll-like receptor (TLR)-4 ligation. A: IL-6 mRNA levels. B: TNF-α mRNA levels. C: IL-6 supernatant concentration. D: TNF-α supernatant concentration. Spleen-derived macrophages were obtained from wild-type and FXR<sup>−/−</sup> mice. Macrophages were treated with LPS (1 µg/ml) alone or in combination with LPS and INT-747 (1 µM) for 18 h. 2-ddCt, 2<sup>−ΔΔCt</sup>. Data are shown as means ± SE of 4 mice. *P < 0.05 vs. medium; **P < 0.05 vs. LPS alone; ΨP < 0.05 vs. wild-type mice.
Since ABCA1 and ABCG1 regulate cellular cholesterol efflux from macrophages, we then investigated whether these transporters were regulated by FXR. We found that ABCG1 mRNA expression was increased in macrophages obtained from ApoE<sup>−/−</sup> mice compared with wild-type mice, while ABCA1 mRNA expression was strongly decreased (n = 4; P < 0.05). Exposure of ApoE<sup>−/−</sup> macrophages to the INT-747 caused a robust induction of ABCA1 mRNA expression, suggesting that FXR induces cholesterol export from macrophages (Fig. 3F). Modulation of ABCA1 expression might explain the reduction of HDL observed in mice administered INT-747 (see Fig. 5).

**FXR activation attenuates hyperlipidemia in ApoE<sup>−/−</sup> mice.** Blood cholesterol levels and LDL fraction were significantly higher in ApoE<sup>−/−</sup> mice compared with naive mice (Fig. 5, A and B; P < 0.01). Twelve-week administration of INT-747 (Fig. 5, A, C, and D) resulted in a dose-dependent reduction of total cholesterol levels (289.7 ± 17.0 mg/dl vs. 416.1 ± 17.4 mg/dl; P < 0.01) as well as HDL (39.7 ± 5.5 mg/dl vs. 84.4 ± 5.6; P < 0.01) and VLDL fraction (109.4 ± 13.20 vs. 157.0 ± 10.5; P < 0.05). In contrast, rosiglitazone treatment failed to reduce serum cholesterol levels nor did it have any effect on HDL and VLDL (Fig. 5A and Fig. 6, B and D). Triglyceride levels in ApoE<sup>−/−</sup> mice were similar to control mice and were unaffected by INT-747 and rosiglitazone administration (Fig. 5E). Finally, INT-747 and rosiglitazone treatment had no effect on plasma aspartate amino transferase and alanine amino transferase levels (data not shown).

Liver histology and liver lipid content is ameliorated by FXR activation. Liver cholesterol and triglyceride contents were markedly increased in ApoE<sup>−/−</sup> mice in comparison with control mice (Fig. 7; P < 0.01). Administering ApoE<sup>−/−</sup> mice with INT-747 reduced both liver cholesterol and triglyceride content (Fig. 6, A and B; P < 0.01 vs. untreated mice). INT-747 (10 mg/kg) reduced liver cholesterol and triglyceride levels by ~50% (Fig. 6, A and B; P < 0.01 vs. ApoE<sup>−/−</sup>). A similar beneficial effect on liver lipid content was observed in ApoE<sup>−/−</sup> mice treated with rosiglitazone (P < 0.01 vs. ApoE<sup>−/−</sup>). Reduction of liver lipids in INT-747-treated mice correlated with a significant improvement of fatty liver as shown by liver histology (Fig. 6C).

**FXR activation induces a fatty liver protective gene pattern.** SREBP-1c (Fig. 7) is the key regulatory gene involved in modulation of de novo biosynthesis of triglycerides from acetyl-CoA through a pathway that involves AceCS, AceCC, FAS, and SCD in the liver (41). Previous studies have shown that FXR activation exerts a SHP-dependent negative regulatory effect on SREBP-1c expression in the liver (49). Sterol regulatory element binding protein 2 (SREBP-2) is involved in the de novo biosynthesis of cholesterol through a pathway that involves HMGCoAS and HMGCoAR (15). Analysis of ApoE<sup>−/−</sup> mice showed a 2.5- to 5-fold induction of SREBP-1c and SREBP-2 in comparison with naive mice (P < 0.01). Chronic administration of INT-747 caused a dose-dependent reduction of liver SREBP-1c mRNA expression, which correlated with a strong reduction of AceCS, AceCC, FAS, and SCD (Fig. 7A; P < 0.05 vs. ApoE<sup>−/−</sup>). In vivo activation of FXR also resulted in downregulation of liver expression of the SREBP-2 as well as SREBP-2-regulated genes HMGCoAS and HMGCoAR (Fig. 7B; P < 0.05 vs. ApoE<sup>−/−</sup>). A similar pattern of regulation was observed in mice administered rosiglitazone (Fig. 7, A and B; P < 0.05 vs. ApoE<sup>−/−</sup>). We then investigated whether FXR activation modulates the expression of genes involved in fatty acid β-oxidation and found that liver expression of MCAD, LCAD, CPT, and ME mRNA was significantly enhanced in ApoE<sup>−/−</sup> mice in comparison with naive mice (Fig. 7C; P < 0.01). The expression of these genes was only partially altered by the FXR ligand with the exception of ME (Fig. 7; P < 0.05 vs. ApoE<sup>−/−</sup>). Analysis of lipoprotein transporters demonstrated that liver expression...
of the LDLR, the ABCA1, and the ABCG5 mRNAs was slightly altered in comparison with naïve mice. As shown in Fig. 7D, the expression of these genes was attenuated by INT-747 but not by rosiglitazone (P < 0.01 vs. ApoE−/−).

Liver expression of FXR, SHP, LRH-1, and PPAR-γ mRNA was downregulated in ApoE−/− mice in comparison with naïve mice (P < 0.01). This pattern was partially reversed by INT-747, which significantly increased FXR and SHP (P < 0.05 vs. ApoE−/−). In contrast, LXR mRNA expression was upregulated in ApoE−/− mice and its expression was reduced by INT-747 (10 mg/kg). CYP7A1, which is a LXR target gene, was also reduced by INT-747 treatment (Fig. 7B; P < 0.05 vs. ApoE−/−). Rosiglitazone treatment strongly upregulated the expression of PPAR-γ mRNA (Fig. 7E).

DISCUSSION

Here, we have provided evidence that in vivo administration of INT-747, a synthetic FXR ligand (8, 37), protects against atherosclerosis development in ApoE−/− mice. FXR is a nuclear receptor that functions as a bile acid sensor and plays a regulatory role in cholesterol, triglyceride, and lipoprotein metabolism (37). FXR−/− mice express a proatherogenic lipoprotein profile, although they do not spontaneously develop atherosclerosis (13). However, FXR and ApoE double-knockout mice fed a high-cholesterol diet show increased tendency toward development of atherosclerotic lesions than single-knockout mice (13). Atherosclerosis induced by ApoE gene deletion is a well-established model of atherosclerosis (33): at the age of 20 wk, ApoE−/− mice show extensive atherosclerotic lesions with foam cells and induction of proinflammatory mediators, IL-6, and IL-1β, among others, in the aortic wall (1). Here, we demonstrated that administering ApoE−/− mice with a synthetic FXR ligand reduced the extent of atherosclerotic plaques in a dose-dependent manner, resulting in a robust attenuation of aortic plaque formation with the higher dose. This was confirmed in two different experimental settings using the histological analysis of cross-sectional sections of ApoE−/− aortas as well as the en face analysis of whole aorta. This protective effect was comparable to that exerted by rosiglitazone, a PPAR-γ ligand that also protected against atherosclerotic plaque formation in this model.

One striking observation of the present study was the demonstration that serum cholesterol, HDL, and VLDL levels were reduced by long-term administration of INT-747. In addition, 12-wk administration of INT-747 was associated with a downregulation of liver expression of SREBP-1c and genes, AceCC, AceCC, FAS, and SCD, which mediate the triglyceride biosynthetic pathway, without affecting the β-oxidation pathway. Concomitantly, we observed induction of liver expression of FXR and SHP mRNAs by INT-747. Since the lipid-lowering effect of natural and synthetic FXR ligands is compromised in SHP−/− mice, our data represent further evidence that an FXR-SHP regulatory cascade modulates SREBP-1c expression/activity in the liver (41, 49). In addition, INT-747 admin-

![Fig. 6. FXR activation reduces liver cholesterol and triglyceride (TG) content and ameliorates fatty liver in ApoE−/− mice. A and B: liver cholesterol content (A) and liver triglyceride content (B) in naïve mice, untreated ApoE−/− mice, and ApoE−/− mice treated with INT-747 (3 and 10 mg/kg) and rosiglitazone (20 mg/kg). Data are shown as means ± SE of 5–9 mice per group and are expressed as µg/mg total protein. *P < 0.01 ApoE−/− vs. wild-type mice. **P < 0.01 vs. untreated ApoE−/−. C: histologic analysis of liver sections obtained from naïve mice, untreated ApoE−/− mice, and ApoE−/− mice treated with INT-747 (3 and 10 mg/kg) and rosiglitazone (20 mg/kg). Hematoxylin and eosin staining. Original magnification, ×10.](http://ajpheart.physiology.org/10.1152/ajpheart.00020.2008)
cholesterol accumulation, this does not occur in ApoE
CYP7A1 could result in reduced bile acid synthesis and liver
negative regulatory cascade (12). Although the inhibition of
of cholesterol to bile acids is modulated by an FXR-SHP-LXR
mRNA (Fig. 7). Previous studies have shown that conversion
amelioration of fatty liver histology.
into reduced liver triglyceride and cholesterol content and
way (Fig. 7). The above mentioned regulatory effects translate
which play a critical role in the cholesterol biosynthetic path-
administration reduced liver expression of SREBP-2 and HMGCoAR,
which play a critical role in the cholesterol biosynthetic pathway (Fig. 7). The above mentioned regulatory effects translate into reduced liver triglyceride and cholesterol content and amelioration of fatty liver histology.
Additionally, INT-747 reduced liver expression of CYP7A1 mRNA (Fig. 7). Previous studies have shown that conversion of cholesterol to bile acids is modulated by an FXR-SHP-LXR negative regulatory cascade (12). Although the inhibition of CYP7A1 could result in reduced bile acid synthesis and liver cholesterol accumulation, this does not occur in ApoE–/– mice exposed to INT-747. This might be explained by taking into account that INT-747 directly reduces the de novo synthesis of cholesterol in the liver. In addition, it is known that while CYP7A1 catalyses the key, rate-limiting step of the classical, major pathway of the overall bile salt synthesis, cholesterol disposal through bile acid excretion might occur through an alternative pathway that involves CYP27A1, mitochondrial sterol 27-hydroxylase (9, 38), which is FXR independent.

The expression of nuclear receptors in the aorta is regulated in this mouse model of metabolic disorder. Indeed, while FXR and SHP expression was markedly upregulated, the opposite was observed for PPAR-γ. The pathogenetic relevance of this observation is unclear, but it is noteworthy that FXR gene ablation in ApoE–/– mice increases the tendency toward the development of atherosclerotic lesions in animals fed a high-fat diet (13). Thus the overexpression of FXR in ApoE–/– aortic plaque lesions could be interpreted as an escape mechanism aimed to counterbalance the metabolic/inflammatory derangement that takes place in this model. The finding that chronic administration of FXR ligand further increases FXR and SHP gene expression might have therefore mechanistic relevance on the ability of INT-747 to protect against atherosclerotic plaque formation.

Confirming previous studies (26), we demonstrated that aortic expression of proinflammatory mediators (CD11b, MCP-1, IL-6, and IL-1β) is increased in ApoE–/– mice. This increase, which is thought to play a mechanistic role in atherosclerotic plaque formation and progression, was partially reversed by long-term administration of INT-747, while it was totally reversed by rosiglitazone, which is thought to exert antiplaque activities mainly by modulating plaque’s macrophages (25).

One important observation of this study was the demonstration that in addition to its lipid-lowering effect, FXR exerts counterregulatory activities on macrophages. Thus, not only have we shown that FXR, mRNA and protein, is expressed in human macrophages and confirmed this finding by anti-FXR immunostaining, but we have also shown that both natural and synthetic FXR ligands attenuate macrophage generation of IL-1β, IL-6, and TNF-α in response to TLR-4 activation with LPS and that inhibition exerted by INT-747 is lost in spleen macrophages obtained from FXR–/– mice. By using FXR-null mice we have also provided evidence that FXR gene ablation results in a proinflammatory phenotype of spleen-derived macrophages that react to bacterial LPS with an enhanced generation of IL-6 and TNF-α in comparison with wild-type mice.
Although we have not investigated the effect of INT-747 in FXR\(^{-/-}\) mice because they do not develop atherosclerosis spontaneously, the lack of effect of INT-747 on FXR\(^{-/-}\) macrophages highlights the fact that modulation of IL-6 and TNF-\(\alpha\) by this ligand requires an intact FXR. The modulatory effect exerted in vitro was maintained in ApoE\(^{-/-}\) mice. INT-747 administration to these mice resulted in a significant downregulation of IL-6 and IL-1\(\beta\) mRNA expression in aortic plaques, suggesting that macrophage modulation is part of the antiatherosclerotic activity of INT-747.

Previous studies investigating the expression of FXR in macrophages have given rise to conflicting results (11, 40). Here we provide evidence that human and mouse macrophages express a functionally active FXR and that in vivo treatment with INT-747 causes a negative regulation of marker of activation on macrophages. Macrophages play an essential role in atherogenesis (26). Gene deletion and bone marrow transplantation experiments have provided evidence that macrophage scavenger receptor A and CD36 exert important functions in mediating uptake of oxidized LDL and promoting the development of atherosclerotic lesions (3, 27). We found that in vivo treatment with INT-747 caused a robust reduction of CD36 expression on circulating macrophages. Because CD36 is essential for the uptake of oxidized LDL and foam cell formation, we speculate that downregulation of this receptor might contribute to the antiatherogenic activity of INT-747 (16, 23, 31, 32).

The accumulation of cholesterol in macrophage foam cells is a central event in atherogenesis. Several ATP-binding cassette (ABC) transporters are involved in cholesterol homeostasis in macrophages (43). ABCG1 and ABCA1 play an important role in the removal of excess cholesterol from macrophages. ABCA1 mediates the efflux of cholesterol and phosphatidylcholine to ApoA-I, which forms pre-\(\beta\)-HDL (24, 44). Mutations of ABCA1 are responsible for a genetic disease, the Tangier disease, characterized by severe HDL deficiency (2, 4, 39). ABCG1 mediates the efflux of cholesterol to pre-\(\beta\)-HDL and HDL but not to the lipid-free ApoA-I (46, 48). We found that expression of ABCA1 mRNA expression was significantly reduced in ApoE\(^{-/-}\) macrophages and that exposure to INT-747 reverted this pattern, suggesting that FXR could interfere in foam cell formation by inducing cholesterol efflux from macrophages.

We have observed that FXR activation reduces HDL levels in ApoE\(^{-/-}\) mice. Despite that several mechanisms might account for this effect, it has been demonstrated previously that FXR induces the expression of the hepatic scavenger receptor B1 (SR-B1). SR-B1 exerts a critical role in regulating HDL uptake by liver and peripheral tissues (22). Because activation of this receptor helps to remove cholesterol from circulation by increasing the clearance of plasmatic HDL, it is tempting to speculate that reduction of HDL levels documented in INT-747-treated mice was mediated by activation of this receptor (22). In further support of this view, FXR-deficient mice show high plasma levels of HDL and cholesterol as well as a reduced rate of plasma HDL cholesterol ester clearance (22, 42).

In conclusion, we have provided evidence that FXR activation exerts antiatherosclerotic effects and that these effects derive from a combined modulation of liver lipid metabolism, macrophage cholesterol uptake and efflux, and inflammation. By demonstrating that FXR is expressed by and regulates effector activities of human macrophage, our data support the notion that FXR functions as a breaking signal in cells of innate immunity. These data suggest that FXR ligands may have utility in the treatment of atherosclerotic disorders.

GRANTS

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