Farnesoid X Receptor Ligands Inhibit Vascular Smooth Muscle Cell Inflammation and Migration

Yoyo T.Y. Li, Karen E. Swales, Gareth J. Thomas, Timothy D. Warner, David Bishop-Bailey

Objective—The farnesoid X receptor/bile acid receptor (FXR; NR1H4) is a ligand-activated transcription factor that regulates bile acid and lipid homeostasis, and is highly expressed in enterohepatic tissue. FXR is also expressed in vascular tissue. We have investigated whether FXR regulates inflammation and migration in vascular smooth muscle cells.

Methods and Results—The FXR target gene, small heterodimer partner (SHP), was induced in vascular smooth muscle cells after treatment with synthetic FXR ligands, GW4064, or 6α-ethyl-chenodeoxycholic acid. FXR ligands induced smooth muscle cell death and downregulated interleukin (IL)-1β–induced inducible nitric oxide synthase and cyclooxygenase-2 expression. In addition, FXR ligands suppressed smooth muscle cell migration stimulated by platelet-derived growth factor-BB. Reporter gene assays showed that FXR ligands activated an FXR reporter gene and suppressed IL-1β–induced nuclear factor (NF)-κB activation and iNOS in a manner that required functional FXR and SHP.

Conclusion—Our observations suggest that a FXR-SHP pathway may be a novel therapeutic target for vascular inflammation, remodeling, and atherosclerotic plaque stability. (Arterioscler Thromb Vasc Biol. 2007;27:2606-2611.)

Key Words: FXR ■ vascular smooth muscle ■ iNOS ■ COX-2 ■ cell migration

Atherosclerosis is considered a progressive inflammatory disease characterized by the accumulation of lipids and fibrous elements in the large arteries. This view is supported by the massive expression of activated inflammatory cells such as macrophages and T lymphocytes in atherosclerotic plaques. The levels of synthetic enzymes for inflammatory cells such as macrophages and T lymphocytes in atherosclerotic plaques are supported by the massive expression of activated inflammatory cells in the large arteries. This view is supported by the massive expression of activated inflammatory cells such as macrophages and T lymphocytes in atherosclerotic plaques.1 The levels of synthetic enzymes for inflammatory cells such as macrophages and T lymphocytes in atherosclerotic plaques are supported by the massive expression of activated inflammatory cells such as macrophages and T lymphocytes in atherosclerotic plaques.2,3 The levels of these and other proinflammatory mediators and enzymes are controlled by the activation of proinflammatory transcription factor signaling pathways, such as nuclear factor (NF)κB and activator protein (AP)-1.4,5 In addition to inflammation, inappropriate vascular remodeling has been reported to underlie the pathogenesis of atherosclerosis.6 VSMC proliferation and migration as well as extracellular matrix remodeling are important in atherosclerosis.7,8 These events are mediated by various cytokines and growth factors, and also depend on the degradation of extracellular matrix by proteinases such as matrix metalloproteinases.

The farnesoid X receptor/bile acid receptor (FXR; NR1H4) is a member of the nuclear receptor superfamily of ligand-activated transcription factors that binds and acts as a heterodimer with retinoid X receptors, and is highly expressed in liver, kidney, adrenals, and intestine.9 FXR expression was recently found in vascular tissue especially in the VSMCs, where its activation led to apoptosis.10 FXR can be activated by high levels of farnesol, but is now recognized as a bile acid receptor with ligands including chenodeoxycholic acid (CDCA) and deoxycholic acid (DCA).11 Synthetic FXR ligands have also been identified, such as GW4064 and 6α-ethyl-chenodeoxycholic acid (6ECDCA).12,13 FXR activation leads to induction of an orphan nuclear receptor, small heterodimer partner (SHP), that mediates some of the inhibitory effects of FXR ligands on bile acid and lipid metabolism.14,15

Here we report that FXR activation leads to a downregulation of the proinflammatory enzymes iNOS and COX-2, as well as cell migration in VSMCs. FXR may therefore be a novel therapeutic target through which to reduce vascular inflammation, remodeling, and atherosclerotic plaque stability.

Methods

Materials and Reagents
Polyclonal antibodies to iNOS (INO22-S) and FXR (sc-13063) were purchased from Autogen Bioclear. Anti–COX-2 polyclonal antibody (Cay-160106) and 1400W were purchased from Axxora.
IL-1β was from R&D Systems. pNF-κB-Luc was from BD Biosciences Clontech. FXR-responsive IR-1 reporter gene, pcDNA-rFXR, and pcDNA dominant-negative-(DN)-rFXR were generous gifts from Dr Tom Kocarek (Wayne State University). All RT-PCR reagents were obtained from Promega. NovaFECTOR was from Vennova. Platelet-derived growth factor (PDGF)-BB was from Calbiochem. Rat specific siRNA to SHP and control siRNA were from Ambion Applied Biosystems. 6ECDCA and GW4064 were kindly donated by Dr Roberto Pellicciari (U. Perugia) and Dr Eric Niesor (ILEX Corp), respectively. All other reagents were from Sigma.

Cell Culture
Rat aortic smooth muscle cells (RASMCs; WKY3m-22), HepG2, and HEK293 cells were grown and maintained as previously described.16 Human aortic vascular smooth muscle cells were cultured according to the supplier’s instructions (Promocell).

iNOS Activity and Measurement of RASMC Death
FXR ligands, 6ECDCA (0.1 to 30 μmol/L), GW4064 (0.1 to 30 μmol/L), or vehicle control(s) were added 1 hour prior to incubation with IL-1β (0.01 to 10 ng/mL, 24 hour). In some experiments, the selective iNOS inhibitor, 1400W (10 μmol/L) was included 1 hour before IL-1β addition, or cells were preincubated with control or SHP specific siRNA using NovaFECTOR 24 hours before drug treatment. The cellular supernatants were then removed from each well for nitrite analysis.17 Total accumulated nitrite was determined spectrophotometrically (OD630) using the Griess assay.18 Cell viability was measured by the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 0.2 mg/mL) assay,19 as previously described.20

Semi-Quantitative Reverse Transcription–Polymerase Chain Reaction
RT-PCR was performed as using standard techniques. Primers for rat FXR and SHP10 were as previously described. Rat β-actin (452 bp) was chosen as a housekeeping gene internal control.21 In parallel reactions where reverse transcriptase or cDNA was omitted, no bands were visible (data not shown). Quantification of the relative intensity of each band was determined and analyzed using ImageJ (http://rsb.info.nih.gov/ij/).

Western Blot Analysis
Protein was extracted from RASMCs and Western Blot analysis was essentially as previously described using rabbit anti-iNOS (1:1000), anti-COX-2 (1:1000), anti-FXR (1:500) polyclonal antibodies, or a mouse monoclonal anti-β-actin (1:1000) antibody as a loading control. HepG2 protein was used as a positive control for FXR protein expression.

Transient Transfection and Reporter Gene Assays
Transfection and luciferase reporter gene assays were performed as previously described.16 In HEK293 cells the IR-1 luciferase reporter gene23 or pNF-κB-Luc was cotransfected using NovaFECTOR, with pcDNA-rFXR in the presence or absence of pcDNA dominant-negative-(DN)-rFXR, or SHP-PCMV-SPORT6 (Geneservice), with pcDNA3.1 as control. In RASMCs, the miNOSpG2Basic (a kind gift from Dr Mark A. Perrella, Brigham and Women’s Hospital, Harvard, Boston) was cotransfected using Lipofectamine2000 (Invitrogen) with SHP-PCMV-SPORT6 or pcDNA3.1 control plasmid. 16 hours posttransfection, cells were transfected with 6ECDCA or IL-1β (10 ng/mL). In some experiments 6ECDCA was added 1 hour before the addition of IL-1β. After 18 hours, cells were lysed for measurement of luciferase activity and protein concentration.

Cell Migration Assays
Smooth muscle cell migration across growth factor reduced Matrigel coated Transwell filters (8μ pore size, Transwell Corning), was essentially as previously described,24 using either IL-1β (10 ng/mL) or PDGF-BB (25 ng/mL) as the stimulant for migration.

Results
FXR Is Functionally Expressed in RASMCs
FXR protein (Figure 1a) and mRNA (Figure 1b) were detected in RASMCs. Consistent with alternative FXR activators,10 activation of RASMCs by either of the selective FXR ligands, GW4064 or 6ECDCA, induced expression of the FXR target gene, SHP (Figure 1c), and RASMC death (Figure 1d).

FXR Ligands Inhibit IL-1β–Induced iNOS Activity in RASMCs
Incubation of cultured RASMC with IL-1β (0.01 to 10 ng/mL) for 24 hours resulted in a marked increase in NO synthesis determined by nitrite accumulation using the Griess assay (Figure 2). Both basal and IL-1β–induced nitrite release were strongly inhibited by the selective iNOS inhibitor, 1400W (Figure 2a). Incubation of RASMCs with GW4064 (Figure 2b), 6ECDCA (Figure 2c), or CDCA (supplemental Figure 1a, available online at http://atvb.ahajournals.org) inhibited both basal and IL-1β–induced nitrite release in a concentration-dependant manner. The synthetic ligand GW4064 was 3-fold more potent than the semisynthetic ligand 6ECDCA, consistent with their relative potencies on FXR. Western blot analysis showed that both basal and IL-1β–induced iNOS protein was inhibited by coinubation with either GW4064 (3 μmol/L, a concentration that did not significantly affect RASMC viability), 6ECDCA (30 μmol/L, Figure 2d), or CDCA (supplemental Figure 1). Similarly, GW4064 and 6ECDCA also inhibited the induction of COX-2 protein by IL-1β in RASMCs (Figure 2d).
FXR Ligands Downregulate RASMC Migration

PDGF-BB (25 ng/mL) but not IL-1β (10 ng/mL) stimulated RASMC migration (Figure 3). Cell migration, basally and in the presence of IL-1β or stimulated by PDGF-BB was abolished in the presence of 6ECDCA (30 μmol/L; Figure 3).

FXR Ligands Inhibit Human Aortic Vascular Smooth Muscle Cell Responses

FXR mRNA was present in human aortic vascular smooth muscle cells (HASMCs; Figure 4a). 6ECDCA induced SHP expression (Figure 4b), inhibited IL-1β-induced COX-2 expression (Figure 4c and 4d), and abolished PDGF-BB induced cell migration (Figure 4d).

NF-κB Activation by IL-1β Is Reduced by FXR and SHP

In HEK293 cells, 6ECDCA activated the IR-1 FXR reporter gene in the presence, but not absence of FXR. DN-FXR strongly inhibited 6ECDCA-induced FXR activation (Figure 5a). Incubation of HEK293 cells with IL-1β (10 ng/mL) induced NF-κB luciferase activity in transfected cells (Figure 5b). IL-1β-induced NF-κB reporter gene activation was reduced to baseline by 6ECDCA only when cells were transfected with FXR. 6ECDCA had no inhibitory effects on IL-1β-induced NF-κB activation in cells without FXR or in the presence of FXR cotransfected with DN-FXR. The inhibitory effect of FXR activation on IL-1β-induced NF-κB activity in HEK293 cells was mimicked by cotransfection of SHP but not control plasmid pcDNA (Figure 6a). Overexpression of SHP in RASMCs also suppressed IL-1β-induced iNOS reporter gene activation compared with control plasmid pcDNA (Figure 6b). Moreover, siRNA knockdown of SHP in RASMCs (Figure 6c), abolished the ability of 6ECDCA to inhibit IL-1β-induced iNOS activity (nitrite formation; Figure 6d).

Discussion

Here we show FXR activation inhibits vascular smooth muscle inflammatory responses and migration. We previously reported that FXR is expressed and induced apoptosis in VSMCs.10 These newer selective FXR ligands, 6ECDCA and GW4064, similarly induced the FXR target gene SHP and cause VSMC death at higher concentrations, consistent with their relative potencies on FXR.11,25,26 Although we find in other systems that high concentrations of GW4064 induce caspase-3–dependent apoptosis via FXR27 to look at the effects of FXR on VSMC activation, “nonapoptotic” concentrations of these FXR ligands were used.

IL-1β and PDGF-BB are well established activators of VSMCs. The induction of iNOS and COX-2 by IL-1β were both inhibited by FXR ligands. Consistent with known VSMC responses, PDGF-BB but not IL-1β induced VSMC migration.28 RASMC migration under basal, IL-1β, or PDGF-BB–stimulated conditions was abolished by 6ECDCA. In slight contrast, PDGF-BB–stimulated but not basal HASMC migration was inhibited by 6ECDCA. This difference most likely reflects that the RASMC cell line is already partially active in culture (as seen by low levels of basally expressed iNOS and COX-2). By contrast, in hepatic stellate cells29 FXR ligands induce matrix metalloproteinase (MMP)-2 activity by suppressing (TIMP)-1 and -2, whereas they induce vascular endothelial cell migration by inducing MMP-9 expression.30 The effects of FXR on cell movement or MMPs are therefore highly tissue-specific.

As FXR ligands inhibit distinct proinflammatory pathways, it is likely that a common mechanism(s) exists for such inhibition, similar to those found with related nuclear
receptors. The peroxisome proliferator-activated receptors, for example, inhibit vascular inflammation by a variety of mechanisms including induction of the NFκB inhibitor (IκBα), inhibition of c-Jun or c-Fos binding to AP-1, and by direct interactions with AP-1, NFκB, and STAT signaling pathways via competition for essential cofactors. NFκB can regulate the expression of iNOS and COX-2. Moreover, FXR has previously been shown to inhibit both AP-1 and NFκB activation in endothelial cells. In HEK293 cells the FXR ligand 6ECDCA only induced an FXR-responsive reporter gene activity, or inhibited IL-1β-induced NFκB activity in the presence of active FXR. SHP overexpression in HEK293 cells or RASMCs suppresses IL-1β-induced NFκB or iNOS reporter gene activation, respectively. In contrast, specific siRNA knockdown of SHP in RASMC removed the ability of FXR activation to inhibit iNOS activity.

FXR knockout mice have severe dyslipidaemia and when crossed with apolipoprotein E (apoE) knockouts produce male offspring with enhanced atherosclerotic lesion formation as well as increased mortality when fed with a high-fat Western style diet, as might be expected. In contrast, male FXR+/−/low-density lipoprotein receptor (Ldr)/−/− double knockout mice and female FXR+/−/apoE+/− double-null mice fed with similar diets have reduced atherosclerotic lesion formation, despite a proatherosclerotic lipid profile. Although these reports have different and yet to be fully explained results, our current findings of FXR having an antiinflammatory influence on RASMCs are consistent with both of the male double-null mice where hepatic inflammatory genes such as tumor necrosis factor-α were upregulated thereby promoting hepatic inflammation.

FXR is expressed in human and rat VSMCs and endothelial cells. The majority of published studies so far have been unable to establish expression or a direct role of FXR in inflammatory cells such as monocytes. An antiinflammatory profile would in theory be of benefit in atherosclerosis. However, a high smooth muscle cell/inflammatory cell ratio correlates with stable atherosclerotic lesions. If the antiproliferative or antiinflammatory effects of FXR were solely limited to smooth muscle cells and not inflammatory cells, atherosclerotic plaques might then become unbalanced and actually more prone to rupture. The local vascular roles of FXR in the atherosclerotic lesions of these complex models have yet to be ascertained. FXR ligands attributable to their metabolic effects have also been suggested as novel therapeutics for dyslipidemia and diabetes, established cardiovascular risk factors. The effect of FXR activation on athero-

**Figure 3.** FXR ligands reduce PDGF-BB–induced RASMC migration. RASMC migration induced by either IL-1β (10 ng/mL) or PDGF-BB (25 ng/mL) compared with basal migration in the presence or absence of the FXR ligand 6ECDCA (6E; 30 μmol/L) at 72 hours. Data are mean±SEM of results from 4 experiments. *P<0.05 between FXR ligand and respective control. †P<0.05 between control and PDGF-BB.

**Figure 4.** FXR expression and activation in primary human aortic vascular smooth muscle cells (HASMCs). a, RT-PCR for FXR in 2 separate HASMC cultures (HepG2 as positive control). b, 6ECDCA (6E; 30 μmol/L) induces SHP, in HASMCs as detected by RT-PCR. Data are representative of n=4 experiments. Representative blot (c) and densitometry (d) analysis showing 6ECDCA (30 μmol/L) inhibits IL-1β–induced (10 ng/mL; 24 hours) COX-2 in HASMCs. e, HASMC migration induced by PDGF-BB (25 ng/mL) in the presence or absence of the FXR ligand 6ECDCA (6E; 30 μmol/L) at 72 hours. Data are mean±SEM of results from 4 separate cultures. *P<0.05 between FXR ligand treatment and respective control, †P<0.05 between control and PDGF-BB.
sclerosis and plaque stability is clearly in need of further investigation.

In conclusion, FXR is expressed and induces its target gene SHP in VSMCs, and activation of FXR and SHP lead to downregulation of important contributors to vascular inflammation and migration, notably COX-2 and iNOS. FXR may therefore be a novel therapeutic target for the treatment of vascular inflammation, remodeling, and atherosclerotic plaque stability.

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Disclosures

None.

References


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Supplemental Figures

**Figure I.** Physiological FXR ligands inhibit IL-1β-induced iNOS activity and cell death. Like the synthetic FXR ligands, “physiological” FXR ligands, (a) CDCA and DCA inhibit IL-1β-induced nitrite release, (b) CDCA reduces IL-1β-induced iNOS protein expression. As previously reported, CDCA and DCA weakly induce RASMC death (c). CDCA and DCA inhibited nitrite release with greater potencies than they induced cell death. Data represents the mean ± S.E.M. of 9 determinations from 3 separate experiments for graphs, and the Western blot is representative of 3 separate experiments.
Supplemental figure I; Li et al.

(a) Nitrite induction (% of control) vs [FXR ligand] (μM).

(b) Western blot images for iNOS and β-actin with IL-1β and CDCA treatments.

(c) MTT (% of control) vs [FXR ligand] (μM) for CDCA and DCA.