Downregulation of Endothelin-1 by Farnesoid X Receptor in Vascular Endothelial Cells

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Abstract—The farnesoid X receptor (FXR) is a member of the nuclear receptor superfamily that is highly expressed in liver, kidney, adrenals, and intestine. FXR may play an important role in the pathogenesis of cardiovascular diseases via regulating the metabolism and transport of cholesterol. In this study, we report that FXR is also expressed in rat pulmonary artery endothelial cells (EC), a “nonclassical” bile acid target tissue. FXR is functional in EC, as demonstrated by induction of its target genes such as small heterodimer partner (SHP) after treatment with chenodeoxycholic acid, a FXR agonist. Interestingly, activation of FXR in EC led to downregulation of endothelin (ET)-1 expression. Reporter assays showed that activation of FXR inhibited transcriptional activation of the human ET-1 gene promoter and also repressed the activity of a heterologous promoter driven by activator protein (AP)-1 response elements. Electrophoretic mobility-shift and chromatin immunoprecipitation assays indicated that FXR reduced the binding activity of AP-1 transcriptional factors, suggesting that FXR may suppress ET-1 expression via negatively interfering with AP-1 signaling. These studies suggest that FXR may play a role in endothelial homeostasis and may serve as a novel molecular target for manipulating ET-1 expression in vascular EC. (Circ Res. 2006;98:192-199.)

Key Words: farnesoid X receptor ■ bile acids ■ endothelin-1 ■ endothelial cells ■ gene regulation

Endothelin (ET)-1, a peptide of 21 amino acid residues, is the most potent vasoconstrictive substance known. Originally isolated from porcine aortic EC, ET-1 is now known to be 1 of a family of 3 mammalian vasoactive peptides that also includes ET-2 and ET-3. ET is produced predominantly by endothelial cells (EC), but it is also produced by leukocytes, macrophages, smooth muscle cells (SMC), cardiomyocytes, and mesangial cells. ET-1 produced in the EC is predominately released abluminally toward the muscular media suggesting a paracrine/autocrine role.

All 3 ETs bind to 2 types of receptors named ETA and ETB: in the cardiovascular system, ETA receptors are found in SMC and cardiac myocytes, whereas ETB receptors are primarily localized on EC and certain vascular SMC. The binding of ET-1 to SMC ETA and ETB receptors leads to vasoconstriction. On the other hand, the activation of endothelial ETB receptors by luminal ET-1 stimulates the release of NO and prostacyclin and plays a role in endothelium-dependent vasodilatation. ETB receptors also mediate the pulmonary clearance of circulating ET-1 and the reuptake of ET-1 by EC.

The lungs represent a primary target for ET-1 effects and are a special site for ET-1 metabolic pathways. A large body of evidence suggests that ET-1 may play an important role in the development of both primary and secondary pulmonary hypertension. The endothelin system also plays an important role in the pathophysiology of a variety of other cardiovascular diseases including congestive heart failure, renal failure, and cerebrovascular disease. Recently, vascular ET-1 has received increasing attention as a therapeutic target for the management of a number of vascular diseases.

Recent studies with reporter gene have revealed important insight into regulation of the human ppET-1 promoter. Regions essential for high basal levels of ppET-1 promoter activity in EC include binding sites for the factors activator protein (AP)-1 and GATA-2. An element binding the vascular endothelial zinc finger-1 protein and mediating EC-specific gene expression has recently been described in the ppET-1 promoter. Recently nuclear factor (NF)-κB and signal transducer and activator of transcription (STAT)-1 signaling has also been shown to be involved in ET-1 regulation in cells that are stimulated with either lipopolysaccharide (LPS) or cytokines. Interestingly, ET-1 expression has been shown to be negatively regulated by several members of the nuclear receptor superfamily of ligand-acti-
vated transcription factors including estrogen receptor (ER), retinoic acid receptor (RAR), peroxisome proliferator-activated receptors (PPAR) α and γ.14–18

The farnesoid X receptor (FXR) (NR1H4) is a member of the nuclear receptor superfamily that is highly expressed in liver, kidney, adrenals, and intestine.19 FXR is activated by bile acids (BAs), such as primary BA chenodeoxycholic acid (CDCA).20 In addition to BAs, synthetic FXR agonists have also been identified.21 Activation of FXR causes both feedback inhibition of cholesterol 7α-hydroxylase (CYP7A1), the rate-limiting enzyme in BA biosynthesis from cholesterol, and activation of intestinal BA binding protein.22 Interestingly, a recent study has shown that FXR is expressed in vascular SMC.23 Expression of FXR in vascular EC has also been suggested in tissue immunohistochemical staining.23 Treatment of SMC with a range of FXR ligands led to apoptosis in a manner that correlates with the ability of the ligands to activate FXR.23 In this study, we demonstrate via various means that FXR is expressed in rat pulmonary EC. FXR is functional in EC as demonstrated by induction of 1 of its target genes, small heterodimer partner (SHP), after treatment with CDCA. Furthermore, activation of FXR in EC led to downregulation of ET-1 expression possibly through inhibition of AP-1 signaling. These studies suggest that FXR may play an important role in endothelial homeostasis and may serve as a novel molecular target for manipulating ET-1 expression in vascular EC.

Materials and Methods

Materials
CDCA and LPS were purchased from Sigma (St Louis, Mo). GW4064 was synthesized following a published protocol.21 All products for cell culture were purchased from Invitrogen (Carlsbad, Calif). pCMX, pCMX-FXR, and pCMV-βgal were described previously.24 pCMX-VP16 and pCMX-VP16γ (gifts from Drs Enrique Saez and Ronald Evans at the Salk Institute) were generated by fusing the VP16 activation domain from the herpes simplex virus to the N terminus of the FXR and PPAR-γ cDNA, respectively. pCMX-FXR-DN, a plasmid expressing a dominant negative rat FXR (an AF-2 domain deletion mutant), was constructed following a published protocol.25 pNF-kB-Luc and pAP1-Luc were purchased from Promega (Madison, Wis). Expression plasmids, RSV-c-Jun and RSV-c-Fos, were kindly provided by Dr Bassel E. Sawaya (Temple University, Philadelphia, Pa).

Cell Culture
Rat pulmonary artery endothelial cells (RPAEC) and rat pulmonary microvascular endothelial cells (RPMVEC) were prepared and characterized according to a published protocol26 and cells of passages 15 to 20 were cultured in phenol red-free DMEM supplemented with 20% (vol/vol) FBS, streptomycin (100 μg/mL), penicillin (100 U/mL), and 10 mmol/L HEPES (complete medium). When treated with CDCA or LPS, RPAEC were cultured in Opti-MEM I medium supplemented with 0.5% FBS and without antibiotics (conditioned medium). CV-1 cells were obtained from American Type Culture Collection (Manassas, Va) and were cultured in DMEM supplemented with 10% (vol/vol) FBS, 0.1 mmol/L nonessential amino acids, 1 mmol/L sodium pyruvate, streptomycin (100 μg/mL), and penicillin (100 U/mL). HeLa cells were purchased from American Type Culture Collection and cultured in DMEM supplemented with 10% (vol/vol) FBS, streptomycin (100 μg/mL), and penicillin (100 U/mL). When treated with CDCA, CV-1 and HeLa cells were cultured in the above-mentioned conditioned medium.

Plasmid Construction
Human ET-1 promoter containing fragment (~3604) was amplified by PCR with the oligonucleotides 5′-TAGGTACCATTTTCTGAAGGAGGTT-3′ (forward primer) and 5′-CAAGCTTCTCTGAAAAAGGGATCAAAAC-3′ (reverse primer) using a genomic bacterial artificial chromosome clone RP3–451B15 (BACPAC Resources, Calif) as template. The fragment was cloned into pGL3-basic vector (Promega) after digestion with KpnI and Nhel, and the resulting plasmid was named as pETI1-Luc.

RT-PCR and Real Time RT-PCR
Total RNA was extracted from cells with TRIzol reagent (Invitrogen) and the first-strand cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen). PCR amplification of FXR cDNA was then performed as described.23 Real-time RT-PCR assay of FXR, SHP, and ET-1 was performed as detailed in the online data supplement available at http://circres.ahajournals.org.

Western Blot Analysis for FXR
Protein extraction and Western blot analysis were performed as described.27 Rabbit anti-FXR antibody (sc-13063) was purchased from Santa Cruz Biotechnology. Horseradish peroxidase-labeled goat anti-rabbit IgG and the ECL chemiluminescence kit were purchased from Amersham Biosciences (Piscataway, NJ).

FXR Immunostaining of Rat Lung Tissues and Cultured Pulmonary EC
Immunodetection of FXR was performed following our published protocol28 as detailed in the online data supplement.

Endothelin-1 ELISA
RPAEC were plated in 6-well plates and allowed to grow overnight. Cells were then treated with CDCA or GW4064 in conditioned medium for 24 hours, and ET-1 in the supernatant was examined with Endothelin-1 Biotrak Assay kit (Amersham Biosciences). In a separate experiment, cells were treated with LPS for an additional 16 hours after CDCA treatment, and ET-1 was then similarly examined. The ET-1 ELISA kit has a 100% cross-reactivity with ET-1 (synthetic), a >100% cross-reactivity with ET-2 (synthetic), a <0.001% cross-reactivity with ET-3 (synthetic), and a <0.07% cross-reactivity with big ET-1 (human).

Transfection Assays
RPAEC were grown to 60% to 70% confluence in 48-well plates. Cells were transiently transfected using Lipofectamine2000 (Invitrogen) with luciferase reporters (pETI1-Luc, pNF-κB-Luc, or pAP1-Luc) in the presence or absence of pCMX-VP16 or pCMX-VP16γ, or pCMX-FXR. pCMX was added to ensure identical transfection efficiencies. Cell extracts were prepared after transfection, and the luciferase and β-galactosidase assays were performed as described,29 and luciferase activity was normalized against β-galactosidase activity. Transfection experiments were performed at least 3 times in triplicate. Data were represented as fold induction over reporter gene alone. Similar transfection assays were also performed in HeLa and CV-1 cells.

In Vitro Transcription Translation
Rabbit reticulocyte coupled transcription/translation system (TnT) (Promega) was used to generate in vitro translated FXR, c-Fos/c-Jun, or p65 according to the instructions of the manufacturer. Plasmid DNAs (1 μg/reaction) were used for the TnT reactions performed at 30°C for 90 minutes in a total volume of 50 μL. The products were directly used in subsequent studies.

Electrophoretic Mobility Shift Assay
An AP-1 or NF-κB double-stranded oligonucleotide (Promega) was end-labeled with [γ-32P]-ATP using T4 polynucleotide kinase. The
labeled probes were incubated with nuclear extracts from HeLa cells (Promega) or in vitro–translated c-Fos/c-Jun or p65, together with various amounts of in vitro–translated FXR for 20 minutes at room temperature in 20 μL of buffer containing 10 mmol/L Tris (pH 7.5), 50 mmol/L NaCl, 1 mmol/L DTT, 1 mmol/L EDTA, 5% glycerol, 0.3 μg of BSA, and 2 μg of poly(dI-dC). The reactions were analyzed by electrophoresis in a nondenaturing 5% polyacrylamide gel in 0.5 Tris-borate-EDTA. The gels were dried and exposed at −80°C for autoradiography.

Chromatin Immunoprecipitation Assays
Chromatin immunoprecipitation (ChIP) assays were performed using a ChIP assay kit (Upstate) according to the instructions of the manufacture. Soluble chromatin was prepared from RPAEC treated with 50 μmol/L CDCA for 24 hours followed by stimulation with LPS (100 ng/mL) for 1 hour. Chromatin was immunoprecipitated with antibodies (2 μg) directed against phospho-c-Jun (sc-822). Final DNA extractions were PCR amplified using primer pairs that cover an AP-1 consensus sequence in the ET-1 promoter as follows: forward, 5′-GCCTCTGAAGTAGCCGTGA-3′; antisense, 5′-GGGGTGAAACAGCACCGACTT-3′.

Statistical Analysis
All data are expressed as means±SEM unless otherwise stated. Comparisons between 2 groups were made with unpaired Student’s t test. Comparisons between 3 or more groups were made with ANOVA followed by Tukey–Kramer post hoc analysis. In all cases, P<0.05 was considered statistically significant.

Results
FXR Is Expressed in Pulmonary Vascular EC
RT-PCR analysis of RNA from RPAEC clearly displayed constitutive expression of transcript for FXR, although their levels were lower than that in rat liver (Figure 1A). Sequenc-
ing of the amplified fragment matches the sequence of rat FXR cDNA reported in GenBank (NM_021745). Interestingly, RPMVEC appear to express lower levels of FXR compared with RPAEC (Figure 1A). Similar results were obtained when FXR (55kD) expression was analyzed by Western blot (Figure 1B). The immunostaining of EC with FXR antibody is shown in Figure 1C (a and b). FXR had a predominantly nuclear localization with weak staining throughout the cytoplasm in both RPAEC and RPMVEC. Again, FXR appears to be expressed at a higher level in RPAEC than that in RPMVEC. The levels of positive staining in both types of EC were significantly reduced by the use of blocking peptide (data not shown). The FXR expression in rat lung sections is shown in Figure 1C (c and d). Both cytoplasmic and nuclear expression was noticed. FXR was clearly expressed in EC that are lining blood vessels (Figure 1C, c), as well as the EC in alveolar region (Figure 1C, d). Preliminary studies showed that FXR was also expressed in mouse and human pulmonary EC as determined by RT-PCR (data not shown).

**Induction of SHP Expression in EC After CDCA Treatment**

After demonstration of FXR expression in RPAEC, we then determined whether FXR was functional in EC by examining the expression of SHP after treatment with CDCA. SHP is 1 of the FXR target genes that is induced after FXR activation. As shown in Figure 2, treatment of RPAEC with CDCA led to increases in the mRNA levels of SHP in a concentration-dependent fashion, suggesting that FXR expressed in RPAEC is biologically active.

**FXR Autoregulation in EC**

Figure 3 shows that treatment with a FXR ligand, CDCA, upregulated the expression of FXR itself in RPAEC. CDCA treatment led to increase in the levels of FXR, as determined by both real-time RT-PCR (Figure 3A) and Western blot (Figure 3B), suggesting that FXR may act locally in EC in an autoregulatory fashion. The FXR autoregulation in EC is consistent with that reported in hepatocytes.30

**CDCA Treatment Downregulates ET-1 Expression in RPAEC**

The above studies clearly demonstrated that FXR is expressed in RPAEC and is functional. As an initial approach to investigate the biological function of FXR in EC, we then examined whether activation of FXR by CDCA would affect ET-1 expression. In EC, ET-1 is detectable under basal conditions and its expression is significantly upregulated by a number of stimuli such as LPS.31 Figure 4A shows that CDCA treatment resulted in decreased expression of ET-1 mRNA in a concentration-dependent manner. CDCA treatment also resulted in decreased amounts of secreted ET-1 in the supernatants. After demonstration of inhibition of the basal level of ET-1 expression in EC a similar experiment was performed in EC that were stimulated with LPS. EC were treated with CDCA or vehicle dimethyl sulfoxide (DMSO) for 24 hours before LPS exposure. In agreement with previous studies,12 treatment of RPAEC with LPS led to increases in ET-1 expression as determined by real-time RT-PCR (Figure 4B). Pretreatment of EC with CDCA resulted in a significant decrease in the LPS-stimulated ET-1 mRNA expression (Figure 4B) and decreased amounts of secreted ET-1 in the culture supernatants.

Figure 5 shows that ET-1 expression in EC was also inhibited by GW4064. GW4064 is a ligand that is highly specific for FXR and is often used as a “chemical tool” to
show that BA target genes are regulated in a FXR-specific manner. Thus, results from Figures 4 and 5 suggest that activation of FXR plays a role in CDCA- or GW4064-mediated inhibition of ET-1 expression in EC.

FXR Represses Transcriptional Activation of the Human ET-1 Gene Promoter

Downregulation of ET-1 mRNA expression by CDCA or GW4064 suggests that activation of FXR modulates ET-1 expression at transcriptional level. We then hypothesized that activation of FXR inhibits ET-1 expression via exerting its inhibitory activity on ET-1 promoter. To test this hypothesis, we constructed a luciferase reporter expression plasmid (pET1-Luc) that is driven by human ET-1 promoter. EC were transfected with pET1-Luc in the absence or presence of FXR expression vector. As shown in Figure 6A, CDCA alone had a slight inhibitory effect on ET-1 promoter activity, but the inhibitory effect was more obvious in the presence of LPS. The inhibitory effect may result from the activation of endogenous FXR. However, the inhibitory effect of CDCA on both the basal and LPS-stimulated ET-1 promoter activity was significantly increased when the cells were cotransfected with pCMX-FXR (Figure 6A). Cotransfection of FXR alone showed minimal effect on ET-1 promoter activity (Figure 6A). Figure 6B shows that the inhibitory effect of CDCA/FXR on LPS stimulated ET-1 promoter activity was substantially abolished when RPAEC were pretransfected with a FXR-DN expression plasmid, suggesting that CDCA inhibits ET-1 promoter activity largely via activation of FXR. The mechanism by which FXR-DN attenuates the effect of CDCA/FXR is not clearly understood at present. It remains to be determined whether FXR-DN (an AF-2 domain deletion mutant) competes with FXR for cofactors that are involved in inhibition of ET-1 promoter activity. To further elucidate an inhibitory role of FXR in regulating ET-1 promoter...
activity, we then cotransfected phET1-Luc with an expression plasmid encoding a constitutively activated FXR, vpFXR. VpFXR was generated by fusing the VP16 activation domain to the N terminus of FXR cDNA and its constitutive activity was demonstrated using a tk-EcRE-Luc, an established FXR reporter gene24 (Figure 6C). Figure 6D shows that coexpression of vpFXR in EC significantly inhibited both the basal and the LPS-induced ET-1 promoter activity, clearly demonstrating that a genetic activation of FXR inhibits ET-1 promoter activity. Similar results were obtained in HeLa cells (Figure 6E and 6F). However, no increase in ET-1 promoter activity was observed in the latter cells after LPS treatment (data not shown) possibly because of a lack of expression of Toll-like receptor 4, a transmembrane receptor that specifically recognizes LPS.

FXR Represses the Activity of a Heterologous Promoter Driven by AP-1 Response Elements
Inspection of ET-1 promoter failed to identify FXR binding sites. To delineate the molecular mechanism of FXR-mediated ET-1 suppression, we examined the effect of FXR on AP-1 transcriptional factors that have been shown to positively regulate ET-1 expression.13,31–34 We hypothesized that activation of FXR may inhibit ET-1 expression via interfering with AP-1 signaling. As shown in Figure 7, FXR inhibited the activity of a heterologous promoter driven by AP-1 response elements in a manner that was remarkably similar to its effect on human ET-1 promoter (Figure 6). Pharmacological (CDCA) or genetic (vpFXR) activation suppressed both the basal and LPS-stimulated AP-1 activity in RPAEC (Figure 7A and 7B). Similar to what was shown for ET-1 promoter (Figure 6A), a partial inhibition of LPS-stimulated AP-1 activity by CDCA alone (Figure 7A) was also likely attributable to activation of endogenous FXR in EC. The inhibition of the basal activity of AP-1 was also observed in HeLa cells (Figure 6C and 6D).

FXR Reduces the Binding Activity of AP-1 Transcriptional Factors
As an initial approach to understand the mechanism by which FXR inhibits AP-1 signaling, we performed electrophoretic mobility shift assay (EMSA) to examine whether FXR inhibited the binding of transcription factors to the AP-1 consensus site. FXR was generated by an in vitro transcription-translation system. As shown in Figure 8A, FXR inhibited the binding of transcription factors in HeLa nuclear extract to the AP-1 consensus site in a dose-dependent manner. FXR similarly inhibited the binding of in vitro–translated c-Fos/c-Jun to the AP-1 consensus site (Figure 8B).

After demonstration of FXR-mediated inhibition of AP-1 binding activity by EMSA, we then examined whether treatment with a FXR ligand, CDCA, will lead to decreased binding of AP-1 transcriptional factors to ET-1 promoter in
Furthermore, EC vary in their gene expression in different functional states. It is interesting to notice that FXR is expressed at a higher level in cultured EC isolated from pulmonary artery compared with cultured EC obtained from pulmonary microvasculature. It remains to be determined via a sensitive assay whether such difference in FXR expression also exists in pulmonary vasculature in intact animals. It also remains to be examined whether FXR expression varies in blood vessels from different organs/tissues.

The mechanism by which FXR regulates ET-1 expression is not fully understood. A GenBank database search did not reveal any FXR binding consensus sequence in either rat or human ET-1 promoter, suggesting that it is unlikely that FXR inhibits ET-1 expression via directly interacting with ET-1 promoter. Previous studies with PPAR-α or PPAR-γ suggest that they may inhibit ET-1 expression via interference of AP-1 signaling through inhibition of c-Jun or c-Fos binding to an AP-1 site. In addition, it has been suggested that PPARs interfere with AP-1, STAT, and NF-κB signaling pathways via competition for essential cofactors. FXR may similarly modulate ET-1 expression in EC via inhibition of AP-1 signaling as supported by the following observations: (1) pharmacological or genetic activation of FXR inhibited the activity of a heterologous promoter driven by AP-1 response elements (Figure 7) and (2) FXR inhibited the binding of transcription factors to the AP-1 consensus site as shown in EMSA (Figure 8A and 8B). A likely role of BA/FXR-mediated inhibition of AP-1 activity in suppressing ET-1 expression in EC was further supported by results from ChIP assays in which CDCA treatment led to decreased binding of c-Jun to an AP-1 binding site in ET-1 promoter in RPAEC (Figure 8C). We have also shown that activation of FXR resulted in inhibition of the activity of a heterologous promoter driven by AP-1 response elements (supplemental Figure I). Interestingly, FXR inhibited the binding of transcription factors in HeLa nuclear extract but not in vitro translated p65 to the NF-κB consensus site (supplemental Figure II), suggesting that a cofactor(s) that is absent in the in vitro transcription-translation system is required for the FXR-mediated inhibition of NF-κB binding activity. More studies are needed to better understand a role of interaction of FXR with NF-κB transcriptional factors in BA-mediated inhibition of ET-1 expression in EC.

The physiological or pathophysiological role of FXR in pulmonary vasculature is not clearly understood at present. However, it is interesting to notice that intrapulmonary vasodilatation is seen in certain patients with liver disease in which plasma levels of BAs are significantly increased. This phenomenon, known as hepatopulmonary syndrome (HPS), has also been reproduced in rats with common bile duct ligation. Current studies have been focused on understanding the role of pulmonary overproduction of NO in this pathological process. It is likely that activation of FXR also plays a role, considering the inhibitory effect of BA/FXR on pulmonary ET system. Studies are currently ongoing to investigate the biological consequences of targeted delivery of BA/FXR to pulmonary circulation in intact rats.

Results from this study may also have therapeutic implications. Recently, FXR ligands have been proposed as novel...
drugs to target cardiovascular diseases by affecting lipid metabolism in the liver. Our results suggest that such therapy may also benefit from its direct effect on vasculature, particularly its effect on ET-1 expression because ET-1 overexpression is implicated in the development of a number of vascular diseases including atherosclerosis. More studies are warranted regarding the molecular mechanism by which FXR regulates ET-1 expression in vasculature.

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METHODS:

*Real Time RT-PCR Assay of FXR, SHP, and ET-1*— Extraction of total RNA and the synthesis of the first strand cDNA were performed as described in *Materials and Methods*. Pre-designed Assays-on-Demand TaqMan probes and primer pairs for rat FXR, SHP, ET-1, and β-glucuronidase were obtained from Applied Biosystems Incorporated (ABI). Each amplification mixture (25 µl) contains 25 ng of cDNA, 1.25 µl of primers and FAM-labeled fluorogenic probe, and 12.5 µl of Universal PCR Master mix. Amplification is performed using the following conditions: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. Transcript abundance, normalized to β-glucuronidase expression, is expressed as a fold increase over a calibrator sample.

*FXR Immunostaining of Rat Lung Tissues and Cultured Pulmonary EC*— Staining of lung sections with FXR antibody was performed following our published protocol28. Briefly, the lungs were fixed in 2% paraformaldehyde in PBS and five micrometer lung cryosections were prepared. Following permeabilization with Triton X-100, lung sections were incubated in a 1:100 dilution of rabbit anti-rat FXR (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h, followed by labeling with Alexa 488-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR) for 1 h. To label EC, sections were incubated in a 1:100 dilution of mouse anti-rat PECAM (Pharmingen, San Diego, CA) for 1 h followed by incubation with rhodamine-labeled goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h. Sections were finally stained
with Hoescht dye 33,258 (Sigma) and mounted in Gelvatol (Monsanto, St. Louis, MO) and images were collected as described.¹

For staining of cultured EC, cells were grown overnight on fibronectin-treated glass coverslips. Staining of FXR was similarly performed as described above. Cellular skeleton (actin) was then stained with rhodamine-labeled phalloidin and cells were observed under a confocal microscope.

References

Supplemental Fig. 1. FXR represses the transcriptional activity of a heterologous promoter driven by NF-κB response elements.

RPAEC or HeLa cells were similarly transfected as described in legend to Fig. 6 except that phET1-Luc was replaced with pNF-κB-Luc. **A**, Transfection of pNF-κB-Luc in the presence or absence of pCMX-FXR in RPAEC. The treatment was the same as described in legend to Fig. 6A. **B**, Transfection of pNF-κB-Luc in the presence or absence of pCMX-vpFXR or pCMX-vpPPARα in RPAEC. The treatment was the same as described in the legend to Fig. 6C. Luciferase activity was measured and normalized against the activity of cotransfected β-galactosidase. **C** and **D**, Similar experiments were performed as described for panels **A** and **B** except HeLa cells were used. *P*<0.05; **P**<0.01 (vs the cells transfected with the corresponding reporter gene alone and treated with the corresponding reagents).

Supplemental Fig. 2. FXR reduces the binding activity of NF-κB transcription factors.

A NF-κB double-stranded oligonucleotide (Promega) was end-labeled with [α-32P]-ATP using T4 polynucleotide kinase. The labeled probes were incubated with HeLa nuclear extracts (A) or in vitro–translated p65 (B), together with various amounts of in vitro–translated FXR for 20 minutes. The reactions were analyzed by electrophoresis in a nondenaturing 5% polyacrylamide gel in 0.5×Tris-Borate-EDTA. The gels were dried and exposed at -80°C for autoradiography. Shown in the figure are the representative data from 4 experiments.
Supplemental Fig. 1(A – D)
Supplemental Fig. 2A & B