Obiektive—Although initially seemingly paradoxical because of the lack of nucleus, platelets possess many transcription factors that regulate their function through DNA-independent mechanisms. These include the farnesoid X receptor (FXR), a member of the superfamily of ligand-activated transcription factors, that has been identified as a bile acid receptor. In this study, we show that FXR is present in human platelets and FXR ligands, GW4064 and 6α-ethyl-chenodeoxycholic acid, modulate platelet activation nongenomically.

Approach and Results—FXR ligands inhibited the activation of platelets in response to stimulation of collagen or thrombin receptors, resulting in diminished intracellular calcium mobilization, secretion, fibrinogen binding, and aggregation. Exposure to FXR ligands also reduced integrin αIIbβ3, outside-in signaling and thereby reduced the ability of platelets to spread and to stimulate clot retraction. FXR function in platelets was found to be associated with the modulation of cyclic guanosine monophosphate levels in platelets and associated downstream inhibitory signaling. Platelets from FXR-deficient mice were refractory to the actions of FXR agonists on platelet function and cyclic nucleotide signaling, firmly linking the nongenomic actions of these ligands to the FXR.

Conclusions—This study provides support for the ability of FXR ligands to modulate platelet activation. The atheroprotective effects of GW4064, with its novel antiplatelet effects, indicate FXR as a potential target for the prevention of atherothrombotic disease. (Arterioscler Thromb Vasc Biol. 2016;36:2324-2333. DOI: 10.1161/ATVBAHA.116.308093.)

Key Words: blood platelets • farnesoid X–activated receptor • fibrinogen • signal transduction • transcription factors

The farnesoid X receptor/bile acid receptor (FXR; NR1H4) is a member of the nuclear receptor superfamily of ligand-activated transcription factors, which binds and acts as heterodimer with retinoid X receptors that have also been found to be expressed in human platelets, and is highly expressed in liver, kidney, adrenal glands, intestine, and vascular tissues.1,2 FXR regulates the expression of genes involved in cholesterol and glucose homeostasis, liver regeneration, and gastrointestinal defense.3-4 FXR has also been shown to have anti-inflammatory and atheroprotective effects after ligand stimulation.5 Endogenous ligands of FXR are bile acids with ligands, including chenodeoxycholic acid and deoxycholic acid.6 Synthetic FXR ligands have also been identified, such as GW4064 and 6α-ethyl-chenodeoxycholic acid (6-ECDCA).7,8 FXR regulates the transcription of target genes through the induction of the atypical nuclear receptor small heterodimer partner, which mediates some of the inhibitory effects of FXR ligands on bile acid and lipid metabolism.9,10 Platelets are anucleate blood cells with a central role in hemostasis but are also involved in inflammation, immunity, tumor progression, and thrombosis.11 Although lacking genomic DNA, platelets contain a diverse transcriptome, which allows signal-dependent protein translation and microRNA processing.12-14 We and others have previously identified the presence of transcription factors in mammalian platelets, including peroxisome proliferator–activated receptors (PPARs),15,16 retinoid X receptor,17 glucocorticoid receptor,18 liver X receptor,19 and the nuclear factor-κB.20 Previous reports have demonstrated that FXR activation seems to protect against atherosclerotic plaque formation,21 but these

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effects are more pronounced than expected, based on its lipid-lowering actions alone.22 We, therefore, hypothesized that the atheroprotective effects of FXR ligands may be mediated, in part, through potential modulation of platelet function. In this report, we demonstrate that FXR is present in platelets and that FXR ligands inhibit a range of platelet functions and thrombus formation, suggesting a potential new target for the prevention of atherosclerosis and thrombosis based on acute DNA-independent actions of this receptor in platelets.

**Materials and Methods**

Materials and Methods are available in the online-only Data Supplement.

**Results**

**Presence of FXR in Platelets**

FXR protein expression was investigated in human and mouse platelets. Immunoblot analysis of cell lysates confirmed the presence of FXR in human and mouse platelets (Figure 1A). The localization of FXR in human platelets was analyzed by immunofluorescence microscopy. In resting platelets, FXR was found to be dispersed throughout the platelet cytoplasm in a punctate arrangement (Figure 1Bi), whereas in response to U46619, a thromboxane A2 receptor agonist, the localization of FXR seemed to partially translocate toward the plasma membrane (Figure 1Bii).

**FXR Ligands Inhibit Platelet Aggregation and Secretion**

The effect of FXR-selective ligands GW4064 and 6-ECDCA on the aggregation of human washed platelets in response to activators of platelet function was explored. Platelet aggregation in response to cross-linked collagen-related peptide (CRP-XL; 1 μg/mL), a glycoprotein VI (GPVI)-collagen receptor-selective ligand, was found to be inhibited in a concentration-dependent manner by GW4064 (Figure 2A and 2B). Inhibition of 22%, 40%, and 80% was observed with GW4064 (1, 10, and 20 μmol/L), which was more potent than 6-ECDCA in inhibiting platelet aggregation. An increase in light transmission was observed on treatment with 10 and 20 μmol/L GW4064 that may be associated with platelet swelling. Consistent with inhibition of GPVI-mediated responses, platelet aggregation in response to collagen (0.5 μg/mL) was also found to be inhibited in a concentration-dependent manner by GW4064. In contrast, high concentrations of the natural FXR ligand chenodeoxycholic acid were required to produce an inhibitory effect on collagen-stimulated platelets.

### Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Acronym</th>
<th>Definition</th>
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<tbody>
<tr>
<td>6-ECDCA</td>
<td>6α-ethyl-chenodeoxycholic acid</td>
<td></td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic guanosine monophosphate</td>
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<tr>
<td>CRP-XL</td>
<td>cross-linked collagen-related peptide</td>
<td></td>
</tr>
<tr>
<td>FXR</td>
<td>farnesoid X receptor</td>
<td></td>
</tr>
<tr>
<td>GPVI</td>
<td>glycoprotein VI</td>
<td></td>
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<tr>
<td>PPAR</td>
<td>peroxisome proliferator–activated receptor</td>
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<tr>
<td>VASP</td>
<td>vasodilator-stimulated phosphoprotein</td>
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(Figure 1 in the online-only Data Supplement). Differences in the potencies of GW4064, 6-ECDCA, and chenodeoxycholic acid in inhibiting platelet aggregation were in line with the differences in potency reported of these agonists in other cell
systems. To determine whether the target for FXR ligands is shared with other agonists, thrombin that activates platelets via G-protein–coupled receptors was tested and used at a concentration that was optimized to ensure a similar level of aggregation to that stimulated by CRP-XL. Lower levels of inhibition were noted with thrombin-induced platelet aggregation (0.1 U/mL) after treatment with GW4064 and 6-ECDCA, although overall the inhibition profiles were similar. Inhibition of 5%, 35%, and 55% was observed with GW4064 (1, 10, and 20 μmol/L; Figure 2C and 2D). Aggregation monitored during an extended period of 5 minutes’ duration confirmed this effect to be inhibition rather than delay in aggregation (data not shown).

Platelet aggregation is dependent on conformational changes of integrin αIIbβ3 through inside-out signaling that results in an increase in its affinity for fibrinogen. Platelet aggregation is dependent on conformational changes of integrin αIIbβ3 through inside-out signaling that results in an increase in its affinity for fibrinogen. Thus, flow cytometry was used to measure fibrinogen binding to platelets, as a marker for activation of the integrin αIIbβ3. CRP-XL–stimulated fibrinogen binding was reduced in the presence of GW4064 (Figure 2E), consistent with reduced aggregation and indicated the ability of the GW4064 to modulate inside-out signaling to integrin αIIbβ3, in platelets. The effects of 6-ECDCA were insufficiently potent to elicit a statistically significant reduction in fibrinogen binding. Thrombus generation is supported and enhanced by the release of many substances from platelet α- and dense granules, which are critical for the recruitment of additional platelets and for stabilization of the aggregate. To analyze the effects of FXR agonist treatment on platelet granule secretion, α- and dense granule secretion was assayed in the absence and presence of GW4064 or vehicle (containing dimethyl sulfoxide [0.1% (v/v)]). α-granule secretion was assessed by measuring the levels of P-selectin exposed on the surface of platelets stimulated with CRP-XL (1 μg/mL) and monitored simultaneously with aggregation in an optical lumi-aggregometer using a luciferase detection system (G). Calcium mobilization was assessed in Fura-2AM–loaded platelet-rich plasma preincubated with increasing concentration of GW4064 in the presence of EGTA to prevent influx of extracellular calcium and then stimulated with CRP-XL (1 μg/mL; H). Numeric data represent the percentage compared with control, mean±SD (n=4); *P≤0.05, **P≤0.01, ***P≤0.005, ****P≤0.001 (ANOVA with Bonferroni post-test).
only modest effects on P-selectin exposure, reaching statistical significance at a concentration of 20 μmol/L.

To investigate the role of FXR in dense granule secretion, ATP release was measured simultaneously with aggregation on washed platelet preparations using a luciferin-luciferase luminescence assay. GW4064 was found to reduce ATP secretion after CRP-XL stimulation (Figure 2G). Cytosolic mobilization of calcium plays a fundamental role in various aspects of platelet function, including reorganization of the actin cytoskeleton necessary for shape change, degranulation, and integrin α₃β₁ affinity modulation.26 We, therefore, examined the ability of FXR ligands to modulate intracellular mobilization of calcium. Fura-2-AM–loaded platelet-rich plasma was preincubated with GW4064 (1–10 μmol/L) or control (containing dimethyl sulfoxide [0.1% (v/v)]) for 5 minutes and then stimulated with CRP-XL (1 μg/mL). Treatment with GW4064 was associated with a modest (in comparison with ATP secretion and other assays of function using washed platelets) inhibition of CRP-XL–stimulated peak calcium concentration (Figure 2H).

**Actions of GW4064 on Platelets Are Mediated Through FXR**

To confirm whether FXR is required for the inhibitory effect of FXR ligands on platelet function, the ability of GW4064 to inhibit platelet function in mice deficient in FXR was explored. We confirmed that the levels of integrin α₃β₁, integrin αβ₃, GPVI, and GPlbα on the surface of FXR−/− platelets were similar to those from FXR+/- mice (Figure II in the online-only Data Supplement). GW4064 (1–20 μmol/L) treatment inhibited fibrinogen binding to FXR−/− platelets on stimulation with CRP-XL reaching ≅50% inhibition at 20 μmol/L. This dramatic inhibition was not observed in FXR+/- platelets, although higher concentrations of GW4064 did cause a modest reduction in fibrinogen binding (Figure 3). These data confirm that the principal mode of action of FXR agonists on platelet function is mediated through binding to FXR.

**FXR Ligand, GW4064, Affects Integrin α₃β₁-Mediated Outside-In Signaling**

After binding to fibrinogen, integrin α₃β₁ clustering transduces signals (outside-in signaling) into platelets to allow spreading and in the latter phase of its formation, clot retraction.27 The modulatory effects of GW4064 on outside-in integrin signaling through α₃β₁ were assessed by the measurement of clot retraction and platelet spreading under static conditions. Clot formation was initiated by adding thrombin to platelet-rich plasma in the absence of presence of GW4064 (1, 10 μmol/L), and the extent of clot retraction was monitored after 2 hours by measuring clot weight. Clot retraction was reduced in the presence of GW4064 (10 μmol/L) at 2 hours (indicated by increased clot weight) compared with vehicle-treated samples (Figure 4A and 4B). Consistent with this, GW4064-treated (10 μmol/L) platelets were unable to adhere and spread on fibrinogen to the same extent as control platelets at 45 minutes. Most GW4064-treated platelets failed to progress beyond filopodia formation with only a few cells progressing to lamellipodia formation and full spreading (Figure 4C and 4D). These data suggest that outside-in signaling through α₃β₁, which controls the coordinated process of clot retraction, is also modulated by GW4064.

**GW4064 Inhibits Thrombus Formation and Hemostasis**

The integrin α₃β₁ is critical for arterial thrombosis and hemostasis. After platelet activation, the α₃β₁ complex undergoes a conformational change that allows the adhesive protein fibrinogen to bind, forming a bridge between platelets that mediates platelet–platelet interactions and thrombus formation.28,29

Given the ability of FXR ligands to regulate platelet function, we sought to determine the potential implications of GW4064 on thrombus formation. Analysis of thrombus formation in vitro was performed by fluorescence microscopy using DiOC₆-labeled whole blood perfused under arterial flow conditions through Vena8 biochips coated with collagen, after preincubation with GW4064 (1,10 μmol/L) or vehicle (containing dimethyl sulfoxide [0.1% (v/v)]) for 5 minutes. Blood was perfused for 10 minutes, after which thrombus development was assessed by measurement of fluorescence intensity. In comparison with control samples (Figure 5A), 10 μmol/L GW4064 inhibited the thrombus fluorescence intensity by 65% (Figure 5C and 5D). These data suggest that GW4064 is able to modulate thrombus formation under arterial flow conditions in whole blood. To determine whether the effects of GW4064 on thrombus formation in vitro were because of inhibition of initial entrapment of platelets on collagen, or because of the inhibition of platelet aggregation and, therefore, thrombus growth, perfusion of blood was also performed in the presence and absence of the α₃β₁ antagonist Integrilin (4 μmol/L). In the presence of Integrilin, GW4064 (10 μmol/L) did not affect platelet adhesion to collagen, suggesting that the FXR agonist does not modulate GPlb-dependent adhesion under flow (Figure III in the online-only Data Supplement). These data indicate that the inhibition of thrombus formation by GW4064 is likely because of its ability to reduce platelet activation after adhesion.
To determine the potential impact of FXR on the acute regulation of platelet function in vivo, the effect of GW4064 on laser-induced thrombosis in mouse cremaster muscle arterioles was assessed. The effect of GW4064 infused intravenously before thrombus formation was explored and compared with thrombosis in vehicle-treated mice. Data analysis was performed for multiple thrombi formed in control and mice treated with GW4064. After laser injury, thrombus formation was monitored for 180 seconds. The initiation of thrombus formation was accelerated slightly, although the continued growth or stability of the thrombus was found to be reduced substantially in GW4064-treated mice compared with controls (Figure 5A and 5F). Inhibition of thrombus formation in the absence of endothelial cells (in vitro; Figure 5A through 5D) suggests that the inhibitory effects of GW4064 on thrombi formed in vivo are likely to be principally because of diminished platelet function, although indirect effects on platelet function mediated by other cells cannot be excluded. Taken together, these data establish a role for FXR ligands in the regulation of thrombosis.

To assess the importance of FXR ligands for hemostasis, tail-bleeding assays were performed. The bleeding time after dissection of 1 mm of tail tip was prolonged substantially in GW4064-treated mice (between 195 and 797 seconds) compared with controls (between 96 and 299 seconds; Figure 5G). These data are consistent with FXR agonists inhibiting platelet function and thereby suppressing hemostasis.

**GW4064 Modulates Platelet Cyclic Nucleotide Signaling**

Because of the inhibitory effects of FXR ligands on CRP-XL–induced aggregation, the phosphorylation levels of proteins involved early in the GPVI signaling pathway were assessed by immunoblot analysis. Platelet lysates were prepared after stimulation with CRP-XL (1 μg/mL) in the presence of GW4064 (1–10 μmol/L) or vehicle (containing dimethyl sulfoxide [0.1% (v/v)].) The levels of total platelet protein tyrosine phosphorylation were unaffected after GW4064 treatment as were the tyrosine phosphorylation levels of the spleen tyrosine kinase (Syk) and adapter protein linker for activation of T-cells (LAT; key early components in the GPVI signaling pathways; Figure IV in the online-only Data Supplement).

Because early signaling stimulated by GPVI was unaffected and FXR ligands were found to inhibit platelet responses to GPVI agonists and thrombin, we explored whether FXR mediates its actions on platelets through the modulation of cyclic nucleotide signaling, mechanisms that provide powerful inhibition of platelet functional responses to a wide range of platelet activators.

NO has both antithrombotic and vasodilatory effects. Under normal physiological conditions, the intact endothelium releases NO and prostacyclin to inhibit platelet adhesion and platelet aggregation by elevating the second messenger cyclic guanosine monophosphate (cGMP) and cAMP, respectively. The levels of cGMP and cAMP in GW4064-treated platelets were therefore measured after stimulation with CRP-XL. We first confirmed that we were able to measure cyclic nucleotide signaling in our experimental system. Consistent with expectations, the levels of cGMP (Figure 6A) were elevated on addition of the NO donor propylamine propylamine nonoate (10 μmol/L). In agreement with a recent report, treatment with the NO donor also resulted in an increase in cAMP levels (Figure 6B). The addition of GW4064 increased cGMP but not cAMP levels over the range of concentrations used in CRP-stimulated platelets (Figure 6A and 6B). Cyclic nucleotide levels are upregulated by synthesis through adenylyl cyclase and guanylyl cyclase and downregulated by degradation through phosphodiesterases.

To establish whether GW4064-increased cGMP levels were dependent on the modulation of phosphodiesterases, phosphodiesterase activity was measured using cGMP and cAMP as substrates. The phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine, as expected, was able to inhibit phosphodiesterase activity by 25% and 40% at concentrations of 10 and 40 μmol/L, respectively. GW4064 (1–20 μmol/L) did not show any inhibitory effects on phosphodiesterase activity with both cGMP and cAMP (Figure 6C and 6D) degradation unaffected by treatment with GW4064. Taken together, these data suggest that inhibition of platelet function by GW4064 is regulated by increased synthesis of cGMP and not through increased hydrolysis of this cyclic nucleotide.
Inhibitory Effect of FXR on Cyclic Nucleotide Signaling in Platelets

To analyze the potential requirement of FXR for the previously observed ability of GW4064 to elevate platelet cGMP levels in mice deficient in FXR was explored. The levels of cGMP were increased significantly on addition of the NO-donor propylamine propylamine nonoate (10 μmol/L) to both FXR−/− and FXR+/+ platelets. The addition of GW4064 (10 μmol/L) increased cGMP levels in CRP-stimulated FXR +/+, although in FXR −/− platelets intracellular cGMP levels were unchanged (Figure 7A).

The vasodilator-stimulated phosphoprotein (VASP) is a critical protein involved in cytoskeletal remodeling and regulating adhesive events that are involved in platelet activation.34 As an established marker of platelet inhibition and antiplatelet drug therapy,32 VASP is an effector that mediates cGMP-dependent inhibitory mechanisms in platelets. On elevation of cGMP, the cGMP-dependent protein kinase G phosphorylates VASP at position S239.

To further investigate whether GW4064 regulates cGMP-mediated inhibitory signaling in mice deficient in FXR, the phosphorylation of VASP at S239 was assessed by flow cytometry using phospho-specific antibodies.32 On addition of the NO-donor propylamine propylamine nonoate (10 μmol/L), VASP phosphorylation at S239 was increased in both FXR+/+ and FXR −/− mouse platelets (Figure 7B). Treatment with GW4064 (10 μmol/L) was found to increase phosphorylation of VASP at S239 in FXR+/+ platelets, where FXR −/− platelets were unresponsive to GW4064 treatment (Figure 7B). These data indicate that the inhibitory effects of FXR ligands mediated through the modulation of cGMP signaling in platelets may be attributed to their actions on FXR.

Discussion

Dyslipidemia is a major risk for cardiovascular disease and is associated with atherosclerosis and thrombotic complications.35,36 Inappropriate activation of platelets in the circulation is the major cause of atherothrombosis.35,36 Current approaches...
for suppression of platelet function to prevent thrombosis with aspirin and ADP receptor antagonists are associated with serious bleeding side effects. Thus, thrombotic disease remains a principal cause of mortality and morbidity worldwide, with increasing rates of incidence of these and underlying obesity-related metabolic disorders. Therefore, more efficacious and safer approaches are required. FXR is a key regulator of lipid and glucose metabolism, and FXR synthetic ligands have been shown to have atheroprotective effects, possibly through modulation of combined metabolic and vascular effects. Although platelets are anucleate cells, the presence of transcription factors in mammalian platelets has been reported, including PPARs, retinoid X receptor, liver X receptor, glucocorticoid receptor, and the nuclear factor-κB.

Figure 6. GW4064 modulates platelet cyclic nucleotide signaling. The levels of cGMP (A) and cAMP (B) were measured in platelets on stimulation with cross-linked collagen-related peptide (CRP-XL; 1 μg/mL) in the presence of GW4064 (1–10 μmol/L) that was found to selectively increase cGMP levels. The effects of GW4064 (1–10 μmol/L) on phosphodiesterase (PDE) activity were measured on the hydrolysis of cGMP (C) and cAMP (D) to establish whether this corresponded to greater cyclic nucleotide production or hydrolysis. Although the PDE inhibitor 3-isobutyl-1-methylxanthine (IBMX) inhibited cGMP and cAMP hydrolysis, GW4064 was without effect. The level of PDE activity obtained in the absence of GW4064 was taken as 100%. Data represent mean±SD (n=3), ***P≤0.005, ****P≤0.001 (ANOVA with Bonferroni post-test).

Figure 7. Platelet farnesoid X receptor (FXR) mediates cGMP signaling. Platelets derived from FXR+/+ and FXR−/− mice were treated with GW4064 or vehicle (containing dimethyl sulfoxide [0.1% (v/v)]) for 20 minutes before stimulation with cross-linked collagen-related peptide (CRP-XL; 1 μg/mL). A, cGMP levels were measured using the enzyme immunoassay Biotrak (EIA) system and (B) vasodilator-stimulated phosphoprotein (VASP) phosphorylation levels (S239) assessed by flow cytometry. Data represent FXR−/− mice platelets compared with FXR+/+ mice platelets (control), mean±SD (n=4), *P≤0.05, **P≤0.01, ***P≤0.005, ****P≤0.001 (ANOVA with Bonferroni post-test). R indicates untreated resting platelet samples.
PPARs are a family of ligand-activated nuclear receptors that, similar to FXR, bind regulatory elements in responsive genes after the formation of a heterodimeric complex with retinoid X receptor.38 We have shown previously that PPARγ interacts with Syk and LAT on the stimulation of platelets with collagen, and PPARγ ligands cause loss of these associations.39 This is important because these signaling proteins perform critical roles early in the signaling pathway that is stimulated by the platelet collagen receptor GPVI. More recently, we suggested that the ability of liver X receptor and PPARγ to interact might be based on bidirectional association either with other nuclear receptors or components of the GPVI signaling pathway in the presence or absence of their ligands.19 In the current study, we therefore evaluated the effects of FXR ligands on platelet function and thrombus formation and characterized potential mechanisms of action.

Our data demonstrate that FXR ligands are able to modulate multiple aspects of platelet function stimulated by adhesion receptors, GPCR agonists, and through integrin signaling. The FXR ligand, GW4064, did not cause marked inhibition of CRP-XL–induced tyrosine phosphorylation of Syk or LAT, suggesting that FXR does not serve to modulate the initiation of cell signaling mechanisms that are stimulated by GPVI and that more general downstream mechanisms with the potential to prevent activation through different platelet agonists must be involved.

FXR has been proposed to be a novel and promising therapeutic target for the treatment of atherosclerosis and heart diseases.40 The implications of FXR activation for vascular function is a subject of debate. A previous study suggested that chronic stimulation of FXR with GW4064 impaired endothelium-dependent relaxation because of decreased sensitivity of smooth muscle cells to NO.21 Because FXR ligands are able to inhibit the function of platelets, and they possess the ability to modulate NO signaling in vascular cells,23,41 we sought to determine whether the acute actions of GW4064 may modulate cyclic nucleotide signaling in platelets. Indeed, such signaling, in common with the observed effects of FXR agonists, is known to suppress platelet activation that is stimulated by a range of different platelet agonists, resulting in reduced intracellular calcium mobilization, secretion, fibrinogen binding, and aggregation.

Our study revealed that FXR ligands are able to modulate platelet function by increasing cGMP levels, which is not mediated through the inhibition of phosphodiesterases in platelets. GW4064 was found to increase the phosphorylation of VASP at position S239 in FXR+/+ platelets, which is regulated by cGMP-mediated signaling. Consistent with this, the FXR−/− platelets were unresponsive to the actions of GW4064 on cGMP accumulation and VASP phosphorylation at S239. These data indicate that the inhibitory effects of the FXR ligand GW4064, which are associated with increased intracellular accumulation of cGMP, may be attributed to its ability to activate FXR in these cells. In recent years, the presence of endothelial nitric oxide synthase in platelets has become a subject of debate, with some studies showing its presence and function42,43 and others its absence.44,45 We have no evidence for a direct role for FXR in stimulating NO generation, although whether through platelet endothelial nitric oxide synthase or NO generated by platelets through other means, this remains a potential axis through which the acute effects of FXR in platelets may be mediated.

Further work will be required to explore in detail the mechanism through which FXR modulates cyclic nucleotide signaling in platelets. It is important to note that FXR ligands modulate platelet function in an acute and clearly nongenomic manner. Whether the effects of FXR ligands on platelets are related to their shared abilities to regulate the effects of vascular NO (which have also been attributed to genomic actions of FXR) or other target molecules remains to be established.

Because of its roles in lipid and glucose metabolism, FXR has become a target for drug discovery. Bile acids are the major metabolites of cholesterol that exert genomic and nongenomic effects by activating the FXR.46 They are produced in the liver and are secreted into the small intestine where they facilitate the absorption of dietary and biliary lipids including cholesterol. Targeting bile acid metabolism and the enterohepatic circulation has, therefore, been considered an attractive mechanism for treating dyslipidemia.53 Normal levels of bile acids in the systemic circulation were reported to be ≥10 μmol/L in postprandial conditions.47,48 Bile acids have previously been associated with platelet dysfunction49; although to date, little is known about the interactions between bile acids and platelet signaling. A previous study reported that taurocolic acid inhibits platelet activation and promotes fibrinolysis; however, whether the effects of taurocholic acid on platelet function are related to their shared abilities to regulate bile acid metabolism or other target molecules remains to be established.50

Whether FXR agonists may be considered in the development of antithrombotic agents will require thorough evaluation of their potential to limit thrombosis, while balancing the need for effective hemostasis. Notably, this study demonstrates that 10 μmol/L GW4064 is able to inhibit platelet function moderately in vitro and in vivo, but it is also associated with enhanced bleeding in mice. Emerging evidence suggests that GW4064 is able to decrease plasma triglycerides and insulin resistance in genetic mouse models of obesity.35 Furthermore, several preclinical animal studies have demonstrated that synthetic FXR ligands protect against development of aortic plaque formation in models of atherogenesis.51 The analysis of FXR-deficient mice has demonstrated that despite a proatherosclerotic profile, these mice did not spontaneously develop plaques even with a high-fat diet. Together these lines of evidence combined with the outcomes of the present study suggest a complex mechanistic role for FXR in the pathogenesis of atherosclerosis that might arise from the combined metabolic and vascular effects.35,51,52

Our study provides evidence that FXR is present in platelets and that its ligands inhibit platelet function and thrombus formation, suggesting a potential new axis for the prevention of atherosclerosis and thrombosis based on acute nongenomic actions of this receptor in platelets.

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A.J.U., S.V., P.S., G.D.F., A.P.B., N.K., O.M.C., and D.D. performed experiments, analyzed results, and made figures; M.S.A. performed
experiments and analyzed results; T.S. and E.D. performed experiments; D.B.-B. and B.S. designed the research; L.A.M. and J.M.G. designed the research, performed experiments, analyzed results, made figures, and wrote the paper.

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Disclosures
None.

References
Moraes et al  FXR Modulates Platelet Function


**Highlights**

- The farnesoid X receptor acutely regulates platelet function in a nongenomic manner.
- Farnesoid X receptor ligands cause inhibition of platelet activation, secretion, aggregation, thrombus formation, hemostasis, and thrombosis.
- Farnesoid X receptor controls platelet function through the modulation of cyclic guanosine monophosphate signaling.
Farnesoid X Receptor and Its Ligands Inhibit the Function of Platelets

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Materials and methods

Reagents

Aggregation and cell stimulation assays were performed using collagen-related peptide (CRP-XL), a selective agonist for the platelet collagen receptor glycoprotein (GP) VI, from Professor Richard Farndale (University of Cambridge, Cambridge, United Kingdom), collagen (Horm collagen, Nycomed, Austria) and thrombin (Sigma Aldrich, UK). The FXR ligands GW4064 and 6α-ethyl-chenodeoxycholic acid (6-ECDCA) were purchased from Merck Millipore, UK. Chenodeoxycholic acid (CDCA) was purchased from Sigma, Aldrich, UK.

Mice

FXR systemic knockout and littermates were produced as described previously. All protocols involving the use of animals were approved by the University of Reading Local Ethical Review Panel and authorized by a Home Office License (UK).

Human washed platelet preparation, aggregation and dense granule secretion

Washed platelets were prepared from fresh blood obtained from healthy, aspirin-free human volunteers with informed consent. Platelets were prepared and re-suspended in modified Tyrodes-HEPES buffer to a final density of 4x10^8 cells mL^-1 for aggregation assays as described previously. Aggregation studies were performed at 37°C in an optical platelet aggregometer (Chronolog). Contaminating blood cells were counted by light microscopy and were mainly erythrocytes; leukocytes were rarely encountered with total cell contamination level was <1 per 13000 platelets. ATP secretion assays were performed as described previously.

Immunoblotting and cyclic nucleotide assays

SDS-PAGE and immunoblotting were performed using standard protocols as described previously. Rabbit anti-human 14-3-3ζ (Santa Cruz Biotechnology, USA) was used to detect 14-3-3ζ to ensure equivalent levels of protein loading in immunoblots. The anti-phosphotyrosine antibody (4G10) was obtained from Millipore, USA and phospho-specific antibodies against Syk and LAT were obtained from Epitomics, USA. Phospho-VASP antibody was obtained from Cell Signaling Technology, UK. The secondary antibodies for immunoblotting; Cy5® goat anti-rabbit IgG and Cy3® goat anti-mouse IgG antibodies were obtained from Invitrogen, UK. Phycoerythrin (PE)-Cy5 mouse antihuman CD62P and PE-Cy5 mouse IgG1 κ-isotype control used to measure P-selectin exposure were from BD Biosciences, UK. Luciferin-luciferase
luminescence substrate was obtained from (Chrono-log, Haverton, USA). PAPA nonoate, cGMP and cAMP ELISA detection and, cyclic nucleotide phosphodiesterase assays were obtained from Enzo lifesciences, UK and performed according to manufacture’s protocols. Total cellular cGMP ELISA detection for murine platelets (RPN 226) were obtained from Amersham Biosciences, (Buckinghamshire, UK) and performed according to manufacture’s protocols.

Flow cytometric analysis: α-granule secretion and fibrinogen binding to integrin α1β3

Platelets were analysed by flow cytometry using whole blood with increasing concentrations of GW4064 or vehicle (containing DMSO (0.1% (v/v)) prior stimulation with CRP-XL (1 µg mL⁻¹) at room temperature for 20 minutes. Platelets were labelled with FITC-labelled rabbit anti-human fibrinogen and PE/Cy5 anti-human-CD62P (P-selectin) as described previously. The cells were then fixed in 0.2% (v/v) formyl saline and analysed by counting 5000 events within the gated population using flow cytometry. Flow cytometric acquisition was performed using Accuri C6 flow cytometry (BD Accuri flow cytometers, USA). Data were analysed by calculating median fluorescence intensity (MFI).

Measurement of [Ca²⁺]i by spectrofluorimetry

Intracellular calcium mobilisation in platelets was measured in PRP pre-loaded with the fluorescent dye FURA-2AM as described previously and it the presence of EGTA (1mM) to prevent calcium influx. Briefly, PRP were incubated with FXR agonist or vehicle (containing DMSO 0.1% (v/v) for 5 min and then stimulated with CRP-XL (1 µg mL⁻¹) in a luminescence spectrophotometer (LS-50B; Perkin Elmer, Beaconsfield, UK). The ratio of emission values (excitation: 340/380 nm) was calculated and converted to calcium concentration using FLWinLab software (Perkin Elmer).

Clot retraction

Human PRP (200 µl) was mixed with 5μl of red blood cells and vehicle or GW4064, and the final volume raised to 1mL with modified Tyrodes-HEPES buffer as described. Fibrin clot formation was initiated by adding thrombin (1U/mL). Clot retraction around a glass capillary added prior to clot formation was observed over a period of 2 hours at room temperature. Clot weight was measured as a marker of clot retraction.

Platelet spreading
Washed mouse platelets were prepared at a density of $2 \times 10^7$ cells/ml and 200 µl of suspension allowed to spread on fibrinogen (100 µg/ml) coated cover-glass for 45 minutes. Unbound platelets were washed away using modified Tyrodes-HEPES buffer and adhered cells fixed using 2% formaldehyde in modified Tyrodes-HEPES buffer. Images were obtained using a Nikon A1-R Confocal microscope using 100X objective. The number of platelets in different stages of spreading such as initial adhesion, formation of filapodia, formation of lamellapodia and fully spread were analysed using ImageJ (NIH, USA).

**Thrombus formation in vitro**

Whole citrated blood labelled with the lipophilic dye 3,3'$\text{-}$dihexyloxacarbocyanine iodide (DIOC$_6$) was pre-incubated with vehicle (containing DMSO (0.1% (v/v)) or GW4064 and perfused over a collagen coated (coated using 400 µg/mL) Vena8 BioChips (Cellix Ltd, Ireland) at a shear rate of 20 dynes/cm$^2$ as reported previously.$^{4-5}$ Z-stack images of thrombi were obtained using a Nikon eclipse (TE2000-U) microscope or by confocal analysis using an A1R microscope (Nikon Instruments, UK). Fluorescence intensity was calculated by analysing the data using Slidebook5 software (Intelligent Imaging Innovations, USA).

**Analysis of Thrombosis**

Thrombus formation in mice and data analysis were performed as described previously.$^{3-5}$ Briefly, C57BL/6 mice were anesthetized by intraperitoneal injection of ketamine (125 mg/kg), xylazine (12.5 mg/kg) and atropine (0.25 mg/kg). Anaesthesia was maintained with 5 mg/kg pentobarbital as required and the mouse circulation was accessed via a cannulus placed in the jugular vein, and the platelets labelled with Alexa-488-conjugated anti-mouse-GPIb antibody. The cremaster muscle was exteriorized, connective tissue removed, and an incision was made to allow the muscle to be affixed as a single sheet over a glass slide. Injury to cremaster arterioles was induced with a Micropoint Ablation Laser Unit (Andor technology plc, Belfast, UK). Thrombi were observed using an upright Olympus BX microscope. Images were captured prior to and after the injury by a Hamamatsu charged-coupled device digital camera C9300 in 640 x 480 format (Hamamatsu Photonics UK Ltd, Welwyn garden City, UK) and analysed using slidebook5 software (Intelligent Imaging Innovations, Denver, CO).

**Analysis of transgenic mouse platelets**

Blood was obtained from FXR$^{+/+}$ and FXR$^{-/-}$ mouse platelets via cardiac puncture after termination. Blood (1 mL) was drawn into a syringe containing acidic citrate dextrose as
anticoagulant. Flow cytometry analysis was performed using whole blood with increasing concentrations of GW4064 prior stimulation with CRP-XL (1 µg mL$^{-1}$) at room temperature for 20 minutes with FITC-labelled rabbit anti-human fibrinogen as described previously. The cells were then fixed in 0.2% (v/v) formyl saline and analysed by counting 5000 events within the gated population using flow cytometry. For analysis of VASP phosphorylation on serine 239, platelets were permeabilised using 0.1% Triton and stained using a phosphospecific antibody (Cell Signaling technology, Watford, UK) following the methodology of Spurgeon et al. Flow cytometric acquisition was performed using Accuri C6 flow cytometry (BD Accuri flow cytometers, USA). Data were analysed by calculating median fluorescence intensity (MFI).

**Assessment of haemostasis**

C57BL/6 mice ( ~16 weeks old) (The Jackson Laboratory, USA) were anesthetized using ketamine (125 mg/kg), xylazine (12.5 mg/kg) and atropine (0.25 mg/kg) administered via the intraperitoneal route 20 minutes prior to the experiment and placed on a heated mat. FXR agonist, GW4064 (10 µM) or vehicle control (DMSO) was injected via femoral vein 10 minutes before 1mm of tail tip was removed using a scalpel blade and the tail tip was placed in sterile saline at 37°C. The time to cessation of bleeding was measured up to 20 minutes. Data were analysed by comparing the bleeding time obtained with vehicle (containing DMSO (0.1% (v/v))) or GW4064 treated mice.

**Statistical analysis**

Median fluorescence intensity values obtained in fibrinogen binding and granule secretion assays were converted into percentage for comparison. Data were analysed using ANOVA with Bonferroni post test as indicated, or where appropriate by t-test. Tail bleeding assay data were analyzed using the Mann-Whitney test.

**References**


improves glucose homeostasis in mouse models of obesity. Diabetes. 2011;60:1861-1871.


Supplemental Figures

Figure I – FXR ligands inhibit platelet aggregation induced by collagen. Washed platelets were incubated with increasing concentrations of CDCA (100 - 300 µM) or vehicle (containing DMSO (0.1% (v/v)) prior to stimulation for 180 s with Collagen (0.5 µg mL⁻¹) and aggregation measured at 37°C under constant stirring conditions. Numerical data represent the percentage compared with control, mean ± SD (n=4). t-test *p ≤ 0.05, ** p ≤ 0.01.

Figure II – Characterization of FXR-deficient platelets. The expression levels of αIIbβ3, α2β1,
GPVI and GPIb were analyzed on FXR\(^{-/-}\) and FXR\(^{+/+}\) platelets by flow cytometry (A). Data represent mean (of median fluorescence intensity) ± SD (n=4). t-test \(P > 0.05\) (non-significant - NS).

Figure III –GW4064 does not inhibit the adhesion of platelets to collagen under arterial flow conditions. Human whole blood was loaded with DiOC6 and incubated with vehicle (containing DMSO (0.1% (v/v)) (black) or 10 \(\mu\)M GW4064 (dashed line) in the presence of integrilin (10 \(\mu\)M), to prevent platelet aggregation, for 10 minutes before perfusion through collagen coated (100 \(\mu\)g/mL) Vena8Biochips at a shear rate of 20 dyn/cm\(^2\) for 2.5 minutes. Following confocal microscopy using an AIR system (Nikon Instruments, UK) platelet adhesion was determined by comparing fluorescence intensity in the vehicle and treated samples. Data represent mean ± SD (n=3). 2-way ANOVA with Bonferroni post test \(P > 0.05\) (non-significant).
Figure IV – FXR agonists do not alter platelet protein tyrosine phosphorylation levels. (A) Washed human platelets were stimulated with CRP-XL (1µg/mL) in the absence or presence of GW4064. Whole-cell protein tyrosine phosphorylation levels were assessed by immunoblot analysis. Data are representative of 4 experiments. R represents untreated resting platelets. (B) Platelet lysates were subjected to immunoblot analysis using phospho-specific antibodies for the tyrosine kinase Syk (Y323) and the adapter protein LAT (Y200). Fluorescence images were visualised using a fluorimager and analysed using Imagequant software. P>0.05, n=4, T-test (non-significant).
FXR ligands (bile acids etc) → FXR → ↑cGMP → ↑Ca^{2+} → Secretion → Aggregation → Thrombus formation