Chronic stimulation of farnesoid X receptor impairs nitric oxide sensitivity of vascular smooth muscle

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Kida T, Murata T, Hori M, Ozaki H. Chronic stimulation of farnesoid X receptor impairs nitric oxide sensitivity of vascular smooth muscle. Am J Physiol Heart Circ Physiol 296: H195–H201, 2009. —Farnesoid X receptor (FXR), a member of the nuclear receptor superfamily that is highly expressed in enterohepatic tissue, is implicated in bile acid, lipid, and glucose metabolisms. Although recent studies showed that FXR is also expressed in vascular endothelial cells and smooth muscle cells, its physiological and/or pathological roles in vasculature tissue remain unknown. The aim of this study is to examine the chronic effect of synthetic FXR agonist GW4064 on vascular contraction and endothelium-dependent relaxation using tissue culture procedure. In cultured rabbit mesenteric arteries, the treatment with 0.1–10 μM GW4064 for 7 days did not influence vascular contractility induced by high K⁺ (15–65 mM), norepinephrine (0.1–100 μM), and endothelin-1 (0.1–100 nM). However, the chronic treatment with GW4064 (1–10 μM for 7 days) dose dependently impaired endothelium-dependent relaxation induced by substance P (0.1–30 nM). In hematoxylin-eosin cross sectioning and en face immunostaining, GW4064 had no effects on the morphology of endothelial and smooth muscle cells. In endothelium-denuded arteries treated with GW4064 (1–10 μM) for 7 days, 3 mM-100 μM sodium nitroprusside-induced vasorelaxation, but not membrane-permeable cGMP analog 8-bromoguanosine-cGMP (8-Br-cGMP; 1–100 μM)-induced vasorelaxation, was significantly impaired. In these GW4064-treated arteries, 1 μM sodium nitroprusside-induced intracellular cGMP elevations were impaired. In RT-PCR, any changes were detected in mRNA expression level of α₁- and β₁-subunit of soluble guanylyl cyclase. These results suggest that chronic stimulation of FXR impairs endothelium-dependent relaxation, which is due to decreased sensitivity of smooth muscle cells to nitric oxide.

nuclear receptor; guanosine 3′,5′-cyclic monophosphate; endothelium-dependent relaxation; GW4064; rabbit mesenteric artery

FARNESOID X RECEPTOR (FXR) is a member of the nuclear receptor superfamily. Highly expressed in the liver, small intestine, and kidney, FXR regulates the expression of genes involved in cholesterol, triglyceride, and glucose homeostasis (1, 2). FXR regulates the transcription of target genes through the induction of the atypical nuclear receptor small heterodimer partner (SHP) (4, 10). Despite lacking a DNA-binding domain, SHP is reported to repress transcription by binding to transcription factors of target genes (22). An alternate mechanism of regulation by FXR occurs through the heterodimerization with the retinoic X receptor, which allows binding to specific response elements of target genes (21).

Endogenous ligands of FXR are bile acids, major products of cholesterol metabolism (11, 18). Bile acids are synthesized in the liver, stored in the gallbladder, and released into the small intestine, where they are crucial for the absorption of lipids and lipid-soluble nutrients (11). Although the serum bile acid level is much lower than that found in the portal circulation, vasculature is known to be constantly exposed to a certain amount of bile acids. In healthy humans, the concentration of serum bile acids is known to fluctuate with diet (3, 13). Furthermore, in liver diseases such as cirrhosis, the serum value of bile acids is dramatically raised, often reaching several hundred micromolars (13).

Recent studies reported that FXR is also expressed both in vascular endothelial cells (5) and smooth muscle cells (1), suggesting its physiological and/or pathological role in circulation. Indeed, several in vitro studies reported FXR agonism causes pathophysiological responses in vascular smooth muscle cells and endothelial cells, e.g., induction of apoptosis (1), upregulation of angiotensin type 2 receptor expression (24) in smooth muscle cells, suppression of endothelin-1 expression (5), and upregulation of endothelial nitric oxide (NO) synthase (eNOS) expression (8). However, chronic effects of FXR stimulation on vascular tissue remain unknown.

Therefore, in the present study, we sought to assess the chronic effect of the FXR agonist GW4064 (12) on vascular contractility and endothelium-dependent relaxation (EDR) using an organ culture procedure. This procedure allows us to treat vascular tissue with test agents at constant concentrations under conditions similar to chronic in vivo FXR stimulation and examine its effects both on vascular contractile response and tissue morphology (16, 23). Here we show that chronic stimulation of FXR impairs EDR, which is due to a decrease in the NO sensitivity of smooth muscle.

MATERIALS AND METHODS

Tissue preparation and organ culture procedure. Male Japanese White rabbits (2 kg) were euthanized by stunning and exsanguination. Animal experiments and care were approved by the local Ethical Review (approval code P07-138) and were performed in strict compliance with the guidelines outlined within the Guide to Animal Use and Care from the University of Tokyo, which conforms to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1996). The organ culture procedure was performed as described previously (16). In brief, main branches of the superior mesenteric arteries were isolated aseptically. After fat and adventitia in sterile HBSS were removed, each artery was cut into rings ~1.5 mm wide.
The arterial rings were then placed in 2 ml of DMEM in the presence or absence of GW4064 (0.1–10 μM) or 6-ethyl chenodeoxycholic acid (6-ECDCDA; 10–100 μM) supplemented with 1% penicillin-streptomycin. The rings were maintained at 37°C under an atmosphere of 95% air-5% CO₂ for 1 h to 7 days. The medium was changed every 2 days until the start of the experiments.

**Measurement of muscle tension.** Muscle tension was recorded isometrically as described previously (16), with a force-displacement transducer (Orientec) connected to a strain amplifier (Yokogawa) under a resting tension of 10 mN. To ensure the effect of GW4064 on the vascular smooth muscle, the endothelium was removed by gently rubbing the intimal surface with forceps after the organ culture procedure. Data are shown as percentages of the relaxation of steady-state precontraction.

**Cross-sectional hematoxylin-eosin staining.** After the incubation, arterial rings were fixed with neutral buffered 10% formaldehyde for 2 days and embedded in paraffin. The 4-μm-thick sections were stained with hematoxylin and eosin (HE). The slides were examined by light microscopy (Nikon Eclipse E800).

Whole mount immunostaining. Whole mount immunostaining was performed as described previously (15). After the incubation, the arterial rings were fixed with 4% paraformaldehyde. The fixed rings were placed in 0.3% Triton X-100 in PBS for 30 min and 3% bovine serum albumin in PBS for 1 h. The arterial rings were then probed with anti-CD31 monoclonal antibody (Dako) at a 1:50 dilution. The slides were captured using a confocal laser scanning microscope LSM510 imaging system (Carl Zeiss).

**Measurement of cGMP content.** After the incubation, the endothelium was removed by gently rubbing the intimal surface with a forceps. The arterial rings were placed in a normal physiological salt solution (PSS) and equilibrated for 2 h. The composition of PSS is the same as that used in muscle tension measurements. To facilitate forceps. The arterial rings were placed in 0.3% Triton X-100 in PBS for 30 min and 3% bovine serum albumin in PBS for 1 h. The arterial rings were then probed with anti-CD31 monoclonal antibody (Dako) at a 1:50 dilution. The images were captured using a confocal laser scanning microscope LSM510 imaging system (Carl Zeiss).

**Semiquantitative RT-PCR.** After the incubation, total RNA was extracted from the arterial rings, and RT-PCR was performed as described previously (17). The first strand of cDNA was synthesized using random 9 mer RT-primer and AMV Reverse Transcriptase XL described previously (17). The first strand of cDNA was synthesized (200 cGMP measurement, the arterial rings were pretreated with IBMX (1 mM) or 6-ethyl chenodeoxycholic acid (6-ECDCDA) for 3 h and equilibrated in 6% TCA, and centrifuged at 2,000 rpm for 30 min to prevent the rapid turnover of accumulated cGMP by phosphodiesterases (PDEs). Subsequent to treatment with a test agent, the arterial rings were immediately frozen in liquid nitrogen, homogenized in 6% TCA, and centrifuged at 2,000 rpm for 15 min at 4°C. Supernatants were analyzed by an Amersham cGMP enzyme immunoassay kit (Amersham HealthCare); the pellets were used to determine protein concentration by the Lowry method. cGMP concentration was expressed as femtomoles per microgram protein content.

**Fig. 1. mRNA expression level of farnesoid X receptor (FXR) and small heterodimer partner (SHP) in rabbit mesenteric arteries.** Total RNA was extracted from mesenteric arteries cultured for 1 h in the presence or absence of GW4064 (0.1–10 μM) or freshly isolated liver (positive control), and RT-PCR was then performed. Agarose-gel electrophoresis demonstrates RT-PCR products of expected size corresponding to mRNA encoding GAPDH (308 bp), FXR (245 bp), and SHP (197 bp). A: typical trace of agarose-gel electrophoresis of RT-PCR products. B: quantitative graph showing FXR and SHP mRNA expression levels ($n = 3$ to 4). *Significantly different from the results in control arteries at $P < 0.05$; **significantly different from the results in control arteries at $P < 0.01$.
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nitroprusside (Wako Pure Chemical), TRIZol reagent, ethidium bromide (Invitrogen), random 9 mer RT-primer (Takara Biomedical), AMV Reverse Transcriptase XL (Life Sciences), Ampli Taq Gold (Roche), and penicillin-streptomycin (Gibco). GW4064 was kindly donated by GlaxoSmithKline. 6-ECDCA was synthesized following a published protocol.

RESULTS

Induction of SHP mRNA expression induced by FXR stimulation. In freshly isolated mesenteric arteries, FXR was constitutively expressed at mRNA level (intensity ratio to GAPDH, 0.53 ± 0.03). The FXR mRNA level in the liver, which was higher than that in mesenteric arteries, was assessed as the positive control (intensity ratio to GAPDH, 1.36 ± 0.11). It is well known that a FXR target gene, SHP, is inducibly expressed at mRNA level (intensity ratio to GAPDH, 0.03). The FXR mRNA level in the liver, which was higher than that in mesenteric arteries, was assessed as the positive control (intensity ratio to GAPDH, 1.36 ± 0.11). It is well known that a FXR target gene, SHP, is inducibly expressed at mRNA level (intensity ratio to GAPDH, 0.03). The FXR mRNA level in the liver, which was higher than that in mesenteric arteries, was assessed as the positive control (intensity ratio to GAPDH, 1.36 ± 0.11). It is well known that a FXR target gene, SHP, is inducibly expressed at mRNA level (intensity ratio to GAPDH, 0.03). The FXR mRNA level in the liver, which was higher than that in mesenteric arteries, was assessed as the positive control (intensity ratio to GAPDH, 1.36 ± 0.11).

We next examined the chronic effect of FXR stimulation on vascular contractility using endothelium-denuded mesenteric arteries cultured for 7 days and found that chronic treatment with GW4064 (0.1–10 μM) did not influence smooth muscle contractions induced by high K⁺ (15–65 mM) or norepinephrine (0.1–100 μM). The absolute contractile force (millinewtons per milligrams wet weight) in the respective presence of 65 mM K⁺ and 100 μM norepinephrine was as follows for the following agents: control, 53.4 ± 6.7 and 67.2 ± 9.7; 0.1 μM GW4064, 43.9 ± 4.1 and 59.2 ± 5.9; 1 μM GW4064, 55.7 ± 8.1 and 67.2 ± 9.7; and 10 μM GW4064, 52.3 ± 4.2 and 50.9 ± 7.4 (n = 6 each).

We next examined the chronic effect of FXR stimulation on EDR. A high K⁺ (25 mM) was chosen to induce precontraction because it elicited stable contractions of similar amplitudes in all the experiments (n = 10 to 11). As shown in Fig. 2A, treatment with 1 μM GW4064 for 7 days significantly impaired substance P (0.1–30 nM)-induced maximal EDR. In the arteries treated with higher concentrations of GW4064, the substance P-induced maximal EDR was inhibited on days 4 and 7. Figure 2B shows the effect of treatment with GW4064 (0.1–10 μM) for 7 days on the dose-response curve for EDR induced by substance P. GW4064 (1–10 μM) attenuated substance P-induced EDR in a dose-dependent manner (maximum relaxation, control, 43.9 ± 4.1%; n = 7; 1 μM GW4064, 51.0 ± 4.4%; n = 4; and 10 μM GW4064, 6.5 ± 3.3%, n = 6; P < 0.01).

Figure 2C shows the effects of treatment with another FXR agonist, 6-ECDCA (10–100 μM), for 7 days on the relaxation induced by substance P. 6-ECDCA attenuated substance P-
induced EDR in a dose-dependent manner. This was demonstrated in the model using precontractions elicited by 25 mM K+ [maximum relaxation using EC50, control, 85.0 ± 5.0% and 0.56 (0.46–0.68) nM, n = 6; 10 μM 6-EDCDA, 64.4 ± 7.6% and 0.85 (0.60–1.20) nM, n = 10; 30 μM 6-EDCDA, 75.1 ± 6.0% and 1.16 (0.83–1.61) nM, n = 7; and 100 μM 6-EDCDA, 22.1 ± 9.0% and 1.17 (0.36–3.79) nM, n = 6].

In all of the arteries without endothelium, substance P was ineffective. The substance P-induced EDR in the cultured mesenteric arteries was eliminated by pretreatment with a NO synthase inhibitor, L-NAME, (300 μM; 20 min before the addition of 25 mM K+; data not shown), suggesting that the EDR of mesenteric arteries is attributable mainly to NO production.

As shown in Fig. 2D, 1 μM GW4064 (7 days) significantly attenuated and 10 μM GW4064 (7 days) completely abolished Ca2+ ionophore, ionomycin (0.1 nM–1 μM)-induced EDR in 25 mM K+--induced contraction (maximum relaxation, control, 80.5 ± 6.7%; 1 μM GW4064, 54.2 ± 11.6%; and 10 μM GW4064, −0.85 ± 16.3%, n = 5 each). In the 10 μM GW4064-treated arteries, 0.3–1 μM ionomycin caused contraction instead of relaxation. These phenomena may be due to a direct contractile effect of Ca2+ ionophore on smooth muscle.

**Chronic effects of GW4064 on the morphology of smooth muscle cells and endothelial cells.** To clarify the effects of GW4064 (0.1–10 μM) on the morphology of the arteries, we performed HE cross staining in the arteries cultured for 7 days. As shown in Fig. 3A, treatment with GW4064 (0.1–10 μM) for 7 days did not change the morphology of the arteries; the smooth muscle cells in the media layer were arranged in an ordinary manner and had typical spindle-shaped nuclei. Endothelial cells were attached tightly to the tunica interna. To further examine whether treatment with GW4064 (0.1–10 μM) for 7 days causes morphological changes, such as excoriation or apoptosis-like change in endothelial cells, we performed whole mount en face CD31-immunostaining. As shown in Fig. 3B, endothelial cells were arranged like cobblestones in all the arteries, and no conspicuous change was found between GW4064 (0.1–10 μM)-treated and control arteries.

Furthermore, we confirmed that chronic treatment with GW4064 (0.1–10 μM, for 7 days) did not cause apoptotic cell death both in smooth muscle and endothelial layer using terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay (n = 4 to 5; data not shown).

**Chronic effects of GW4064 on NO sensitivity and cGMP production.** In all the arteries without endothelium, sodium nitroprusside (SNP; 3 nM-100 μM) caused vasorelaxation of the contractions elicited by 25 mM K+ in a dose-dependent manner, as shown in Fig. 4A. However, GW4064 (1–10 μM) significantly decreased the NO sensitivity of smooth muscle in a dose-dependent manner [EC50, control, 0.037 (0.026–0.051) μM, n = 5; 1 μM GW4064, 0.172 (0.138–0.213) μM, n = 9; and 10 μM GW4064, 1.18 (0.96–1.46) μM, n = 7].

Figure 4B shows the effects of treatment with GW4064 (1–10 μM) for 7 days on the content of cGMP in endothelium-denuded arteries. GW4064 (1–10 μM) decreased the cGMP content under resting conditions in a dose-dependent manner (control, 129.4 ± 25.4 fmol/μg, n = 6; 1 μM GW4064, 81.9 ± 14.1 fmol/μg, n = 5; and 10 μM GW4064, 26.5 ± 4.7 fmol/μg, n = 5). The cGMP content after SNP (1 μM)
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Fig. 4. A: effects of treatment with GW4064 (0.1–10 μM) for 7 days on relaxation induced by sodium nitroprusside (SNP) in endothelium-denuded mesenteric arteries. SNP (3 nM–100 μM) was added cumulatively after 25 mM high K⁺-induced contraction reached steady-state level. B: effects of treatment with GW4064 (1–10 μM) for 7 days on cGMP content in the endothelium-denuded mesenteric arteries. The graph shows the cGMP content in the absence or presence of 1 or 100 μM SNP (5 min). C: effects of treatment with GW4064 (0.1–10 μM) for 7 days on relaxation induced by 8-bromoguanosine-cGMP (8-Br-cGMP) in the endothelium-denuded mesenteric arteries. 8-Br-cGMP (1–100 μM) was added cumulatively after 25 mM high K⁺-induced contraction reached steady-state level. D: effects of treatment with GW4064 (0.1–10 μM) for 7 days on relaxation induced by iloprost in the endothelium-denuded mesenteric arteries. Iloprost (0.1 nM–1 μM) was added cumulatively after 25 mM high K⁺-induced contraction reached steady-state level. *Significantly different from the results in control arteries at P < 0.05; **significantly different from the results in control arteries at P < 0.01.

In this study, we showed that chronic stimulation of FXR impaired EDR in rabbit mesenteric arteries, which is due mainly to the decreased sensitivity of smooth muscle to NO. To our knowledge, this is the first demonstration of the chronic effects of FXR stimulation on vascular function.

As described in the introduction, although FXR plays important roles in the expression of genes involved in cholesterol, triglyceride, and glucose homeostasis in the liver, recent reports suggest FXR is expressed in various organs and cell types inducing physiological and pathophysiological functions. In this study, we first confirmed that FXR mRNA was constitutively expressed in mesenteric arteries; however, its level was lower than that in the liver (Fig. 1). This observation is consistent with previous reports showing FXR mRNA is expressed at lower levels in human umbilical vein endothelial cells (20) and human saphenous vein smooth muscle cells (1) compared with HepG2 cells (a human hepatocellular carcinoma cell line). In addition, we detected the induction of SHP mRNA expression stimulated by the FXR specific ligand GW4064. Since the expression of SHP is known to be induced upon FXR stimulation (1, 5), it indicates GW4064 increases transcriptional regulatory activity of FXR under our experimental conditions. Another notable finding is that the expres-

treatment for 5 min was also decreased by GW4064 in a dose-dependent manner (control, 326.8 ± 34.1 fmol/μg, n = 4; 1 μM GW4064, 199.1 ± 33.6 fmol/μg, n = 5; and 10 μM GW4064, 16.0 ± 2.4 fmol/μg, n = 5). Stimulation with 100 μM SNP increased the cGMP content in 1 μM GW4064-treated arteries to 386.6 ± 105.1 fmol/μg (n = 5), which was comparable with that of control arteries (327.8 ± 106.2 fmol/μg, n = 4). In 10 μM GW4064-treated arteries, the 100 μM SNP-induced cGMP elevations were still lower, but not significantly different, compared with those of control arteries (155.4 ± 51.6 fmol/μg, n = 5).

Figure 4C shows the effects of treatment with GW4064 (0.1–10 μM) for 7 days on endothelium-denuded vascular relaxation induced by the cGMP analog 8-Br-cGMP (1–100 μM). GW4064 (0.1–10 μM) did not attenuate the 8-Br-cGMP-induced relaxation in muscle precontracted by 25 mM K⁺ (n = 5 each).

We next investigated prostacyclin/cAMP-dependent relaxation in the arteries treated with GW4064. Of interest, as shown in Fig. 4D, the treatments with GW4064 (1–10 μM) for 7 days accelerated iloprost-induced relaxation in a concentration-dependent manner (maximum relaxation, control, 29.5 ± 6.6%, n = 8; 1 μM GW4064, 61.0 ± 6.9%, n = 7; and 10 μM GW4064, 74.4 ± 5.8%, n = 7; P < 0.01).

Chronic effects of GW4064 on sGC mRNA expression. We next examined whether mRNA expression of sGC is altered by treatment with GW4064. sGC consists of two subunits, α- and β-subunit, and the most abundant isoform in vascular smooth muscle is the α₁/β₁-heterodimer. As shown in Fig. 5, the treatment with GW4064 (0.1–10 μM, for 7 days) did not influence the mRNA expression level of the two subunits of sGC in endothelium-denuded arteries (n = 7 to 8).

DISCUSSION

In this study, we showed that chronic stimulation of FXR impaired EDR in rabbit mesenteric arteries, which is due mainly to the decreased sensitivity of smooth muscle to NO. To our knowledge, this is the first demonstration of the chronic effects of FXR stimulation on vascular function.

As described in the introduction, although FXR plays important roles in the expression of genes involved in cholesterol, triglyceride, and glucose homeostasis in the liver, recent reports suggest FXR is expressed in various organs and cell types inducing physiological and pathophysiological functions. In this study, we first confirmed that FXR mRNA was constitutively expressed in mesenteric arteries; however, its level was lower than that in the liver (Fig. 1). This observation is consistent with previous reports showing FXR mRNA is expressed at lower levels in human umbilical vein endothelial cells (20) and human saphenous vein smooth muscle cells (1) compared with HepG2 cells (a human hepatocellular carcinoma cell line). In addition, we detected the induction of SHP mRNA expression stimulated by the FXR specific ligand GW4064. Since the expression of SHP is known to be induced upon FXR stimulation (1, 5), it indicates GW4064 increases transcriptional regulatory activity of FXR under our experimental conditions. Another notable finding is that the expres-
arteries precontracted with a high concentration of KCl in a time- and dose-dependent manner in organ cultured mesenteric arteries. To test, we found that GW4064 impaired substance P-induced NO and endothelin-1 in the endothelium-denuded arteries. In contrast, we indicated the chronic treatment with GW4064 did not influence calcium sensing assay (19). This difference in binding strength may be a potential reason for our current results. In mesenteric arteries with intact endothelium, GW4064 treatment impaired EDR, induced not only by receptor stimulation but also by the Ca\(^{2+}\) ionophore ionomycin (Fig. 2D). This observation let us rule out the possibility that GW4064 may influence the receptor-mediated Ca\(^{2+}\) signal cascade in endothelial cells.

In the next series of experiments, we examined mechanisms underlying FXR-mediated endothelial dysfunction. According to a previous report, bile acids (chenodeoxycholic and deoxycholic acids), as well as synthetic FXR ligands including GW4064, induced apoptosis in smooth muscle cells (1). Therefore, we attempted to evaluate the possibility of GW4064-mediated FXR activation resulting in morphological changes in mesenteric arteries, possibly contributing to vascular dysfunction. However, in HE staining (Fig. 3A), en face CD31 immunostaining (Fig. 3B), and TUNEL assay, we did not detect any morphological changes caused by GW4064 in smooth muscle or endothelial cells, suggesting GW4064-mediated FXR activation does not induce any apoptotic effect on the vascular wall under our experimental conditions.

We next found that the chronic stimulation of FXR by GW4064 reduced the sensitivity of vascular smooth muscle to NO (Fig. 4A). In smooth muscle cells, NO activates sGC, which in turn synthesizes cGMP. cGMP stimulates the cGMP-mediated relaxation. It has been reported that the strength of binding of FXR to a previous report, bile acids (chenodeoxycholic and deoxycholic acids), as well as synthetic FXR ligands including GW4064, induced apoptosis in smooth muscle cells (1). Therefore, we attempted to evaluate the possibility of GW4064-mediated FXR activation resulting in morphological changes in mesenteric arteries, possibly contributing to vascular dysfunction. However, in HE staining (Fig. 3A), en face CD31 immunostaining (Fig. 3B), and TUNEL assay, we did not detect any morphological changes caused by GW4064 in smooth muscle or endothelial cells, suggesting GW4064-mediated FXR activation does not induce any apoptotic effect on the vascular wall under our experimental conditions.

Because of FXR itself was also elevated by GW4064 in mesenteric arteries. This phenomenon was similar to that observed in in vitro studies on hepatocytes (7) and endothelial cells (5). Taken together, these results suggest that GW4064 could activate the FXR signaling pathway in organ-cultured rabbit mesenteric arteries.

In the present study, we focused on the chronic effects of FXR stimulation on vascular function by using a vascular organ culture procedure. As we reported previously, the rabbit mesenteric arteries did not exhibit any functional or morphological changes after 7 days in organ culture (16, 23), and thus this procedure makes it possible to assess the influence of chronic stimulation of FXR on vascular properties. The results indicated the chronic treatment with GW4064 did not influence vascular contractility stimulated by a high K\(^+\), norepinephrine, and endothelin-1 in the endothelium-denuded arteries. In contrast, we found that GW4064 impaired substance P-induced EDR in a time- and dose-dependent manner in organ cultured arteries precontracted with a high concentration of K\(^+\) (Fig. 2, A and B). Consistent impairments were also obtained by another FXR agonist, 6-ECDCA (Fig. 2C), suggesting chronic transcriptional activation of FXR could impair EDR in the vascular wall. When compared with GW4064, a relatively higher concentration of 6-ECDCA was required to hamper vasodilation. It has been reported that the strength of binding between 6-ECDCA and FXR is three times lower than that between GW4064 and FXR as assessed by a cell-free ligand-sensing assay (19). This difference in binding strength may be a potential reason for our current results. In mesenteric arteries with intact endothelium, GW4064 treatment impaired EDR, induced not only by receptor stimulation but also by the Ca\(^{2+}\) ionophore ionomycin (Fig. 2D). This observation let us rule out the possibility that GW4064 may influence the receptor-mediated Ca\(^{2+}\) signal cascade in endothelial cells.

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and β₁-subunit, induction of endogenous inhibitors, and reduced interaction of substrates or cofactors.

Although we showed that chronic FXR stimulation impaired NO sensitivity in vascular smooth muscle layer, we did not examine its direct effect on NO productivity in endothelial layer, which may affect the EDR. Li et al. (8) recently reported that treatment with both GW4064 and one of the bile acids, chenodeoxycholic acid, upregulated eNOS mRNA expression and thus increased NO production in isolated endothelial cells. The discrepancy is that although our results suggest that the stimulation of FXR leads to hypertensive phenotype, their results suggest that it leads to hypotensive phenotype, which may be due to some factors including difference in the duration of FXR stimulation and experimental model (cultured vascular tissue or in vitro cultured cell).

Although the importance of FXR in pathophysiology or pathophysiology in vasculature remains unclear, it is well known that many patients with severe cirrhosis develop portal hypertension, and some candidates of liver transplantation have pulmonary hypertension. There is a possibility that chronic exposure of vascular tissue to high level of bile acids, and subsequent FXR activation, is involved in the progression of these circulatory disorders observed in patients with cirrhosis.

In conclusion, we showed here that chronic stimulation of FXR by its synthetic agonist GW4064 leads to the impairment of EDR in rabbit mesenteric arteries, which is mainly due to decreased NO sensitivity of smooth muscle. Although the physiological or pathophysiological importance of FXR-mediated impairment of EDR is not clearly understood at present, the fact that serum bile acid concentrations increase dramatically in liver disease indicates FXR may be involved in the pathophysiological processes of these diseases and their complications.

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