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Farnesoid X Receptor Activation Modulates Calcium Homeostasis in Rat Aortic Vascular Smooth Muscle Cells

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Abstract

The farnesoid X receptor (FXR) is a member of the nuclear receptor superfamily that is expressed in the vasculature; our previous work showed that FXR regulated vascular reactivity through NO mechanism. The underlying mechanism for the regulation of vascular tension by FXR remains unclear. The present work was designed to investigate whether FXR regulates calcium homeostasis in aortic vascular smooth muscle cells (VSMCs). Protein abundances of angiotensin II type 1 and 2 receptors (AT_1R, AT_2R), bradykinin type 1 and 2 receptors (B_1R, B_2R), and the inositol 1,4,5-trisphosphate receptor (IP_3R) were analyzed by Western blotting. Kallikrein activity and bradykinin content were assayed by using spectrophotometry and a bradykinin assay kit, respectively. Aortic contraction, intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$), sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) activities, and IP_3-evoked Ca^{2+} release were investigated, following FXR activation in the presence or absence of AT_2R and B_2R blockade. We found that the FXR agonists GW4064 and INT-747 increased the protein abundance of AT_2R and B_2R in rat aortic VSMCs. AT_2R blockade with PD123319 reversed the effects of FXR agonists on kallikrein activity, B_2R, and bradykinin levels. Moreover, we found that GW4064 and INT-747 decreased $[Ca^{2+}]_i$, increased SERCA activity, downregulated IP_3R_1 expression, and attenuated IP_3-induced Ca^{2+} release. These effects were partially reversed by AT_2R and B_2R blockade with PD123319 and HOE140, respectively. Our data suggest that FXR regulates vascular tension by modulating extracellular Ca^{2+} influx and intracellular Ca^{2+} release from the sarcoplasmic reticulum via activation of an AT_2R-B_2R pathway in rat aortic VSMCs.

Key Words: **angiotensin II type 2 receptor, bradykinin type 2 receptor, calcium homeostasis, farnesoid X receptor, inositol 1,4,5-trisphosphate receptor, sarco/endoplasmic reticulum Ca^{2+} ATPase**

Introduction

The farnesoid X receptor (FXR), a bile acid-activated nuclear receptor, plays crucial roles in cholesterol and triglyceride metabolism (3). Since its expression was discovered in the vasculature

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(2), FXR has received much attention as a potential therapeutic target for atherosclerosis (5, 12, 15).

Though controversies still exist, recent studies suggest potential roles for FXR in regulating vascular tone and reactivity. We previously reported that chenodeoxycholic acid (CDCA), a natural FXR ligand, inhibited vasoconstriction and induced relaxation in rat thoracic aorta rings through a nitric oxide (NO)-dependent mechanism (24). Our data were consistent with another study from rat pulmonary microvasculature endothelial cells, where treatment with CDCA or the FXR agonist, GW4064, resulted in an increased abundance of endothelial nitric oxide synthase (eNOS) protein, as well as production of nitrite and nitrate (11). A recent study showed that CDCA lowered blood pressure by decreasing endothelin 1 (ET-1) levels, raising eNOS expression, and ameliorating inflammation in spontaneously hypertensive rats (10). However, impaired NO sensitivity of vascular smooth muscle was observed in cultured rabbit mesenteric arteries after chronic treatment with GW4064 (8). Though FXR activation in rat pulmonary artery endothelial cells decreased transcription of endothelin-1 (6) and increased decorin in human coronary artery smooth muscle cells (7), the mechanisms of FXR effects on the vasculature remain unclear.

Treatment of aortic vascular smooth muscle cells (VSMCs) with CDCA or GW4064 led to transcriptional induction of the angiotensin II type 2 receptor (AT_2R) (22). AT_2R overexpression was reported to activate the vascular kallikrein-kinin system (KKS) and contribute to vasodilation through NOS/NO-mediated pathways in spontaneously hypertensive rats (20). In a previous study (23), we investigated the effects of FXR activation on KKS in vascular endothelial cells. We found that FXR stimulation was followed by AT_2R-bradykinin type 2 receptor (B_2R) upregulation in aortic vascular endothelial cells, consistent with Zhang's work (22). In terms of a mechanism, GW4064 and INT-747 upregulated expression of eNOS, but not inducible NOS; this effect was inhibited by AT_2R-B_2R blockade. These data indicated that FXR activation could regulate vascular tension by modulating vascular endothelial cell function, mainly through NOS/NO-mediated pathways. An increased intracellular calcium (Ca^{2+}) concentration ($[Ca^{2+}]_i$) in VSMCs is critical for vasoconstriction. In VSMCs, calcium homeostasis depends on extracellular Ca^{2+} entry and intracellular Ca^{2+} release from such Ca^{2+} storage sites as the sarcoplasmic reticulum and mitochondria (1). Plasma membrane calcium channels, sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA), and the inositol 1,4,5-trisphosphate receptor (IP_3R) regulate $[Ca^{2+}]_i$ homeostasis (1, 21). AT_2R-mediated extracellular Ca^{2+} modulation (17) and B_2R-mediated activation of calcium-dependent K^+ channels (18) are reportedly involved in vascular calcium homeostasis and regulation of vasoreactivity. However, it remains unknown whether FXR activation can regulate Ca^{2+} homeostasis in VSMCs through AT_2R-B_2R pathways.

This study was designed to investigate whether FXR stimulation induced AT_2R-B_2R activation in aortic VSMCs and, if so, whether this activation could regulate extracellular Ca^{2+} influx or intracellular Ca^{2+} release from the sarcoplasmic reticulum.

Materials and Methods

Animal handling and experimentation were conducted in accordance with the Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences, and followed the Chinese guidelines for research with experimental animals. The use and care of experimental rats was supervised and approved by the Animal Ethics Committee of Chinese PLA General Hospital.

Aortic VSMCs Isolation and Culture

Each Sprague-Dawley (SD) rat was anesthetized with pentobarbital sodium (40 mg kg^{-1}, intraperitoneally) and sacrificed by exsanguination *via* the abdominal aorta. The thoracic aorta was rapidly removed and placed in cold Krebs buffer solution, pH 7.40, consisting of (in mmol L^{-1}) NaCl, 118; KCl, 4.7; $NaHCO_3$, 25; $CaCl_2$, 2.4; $MgSO_4$, 1.2; glucose, 11; and KH_2PO_4 1.2.

The VSMCs were dissected from the medial layer of the rat thoracic aorta and cultured in Dulbecco's modified Eagle's medium (DMEM) (containing 5% fetal bovine serum (FBS), 100 units mL^{-1} penicillin, 100 units mL^{-1} streptomycin, and 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.40) at 37°C in a humidified atmosphere equilibrated with 95% air and 5% CO_2. The cells were grown on six-well plates or 35-mm culture dishes to near confluence. Primary aortic VSMCs (grown for <4 passages) were used for this study. The cells were incubated with FXR agonists (GW4064 or INT-747, as indicated) for 24 h, with or without antagonists (PD123319 or HOE140, as indicated).

Preparation of Microsomal Vesicles

Microsomal vesicles of aortic VSMCs were prepared following treatment with GW4064 or INT-747, with or without the antagonists PD123319 or HOE140, as indicated in the figures. Cells were detached by trypsinization, then washed with PBS; the cell sus-

pension was homogenized by 30 strokes in a tightly fitting Dounce homogenizer. Homogenates were centrifuged at 650 × *g* for 5 min to remove debris; supernatants were then centrifuged at 115,000 × *g* for 45 min. The resulting pellets were each resuspended in a solution of 250 mM sucrose, 120 mM KCl, 3 mM β-mercaptoethanol, and 20 mM Tris/HEPES, pH 7.40.

Western Blotting Analysis

Aortic VSMC suspensions were homogenized in cell lysis buffer. Protein concentrations were determined by using the Coomassie Protein Assay (Pierce, Rockford, IL, USA). Homogenate samples containing 20 μg protein were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Electrophoretically separated proteins were transferred onto nitrocellulose membranes (Hybond-P, Amersham Biosciences, Piscataway, NJ, USA) that were then incubated overnight at 4°C with the following antibodies: goat anti-AT_1R (1:800 dilution; Abcam, Cambridge, UK); rabbit anti-AT_2R (1:5000 dilution; Abcam); rabbit anti-B_1R (1:500 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA); goat anti-B_2R (1:800 dilution; Santa Cruz Biotechnology); rabbit anti-IP_3R_1 (1:1000 dilution; Abcam); or goat anti-β-actin (1:1000 dilution; Santa Cruz Biotechnology). Membranes were then washed and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 2 h at room temperature. Chemiluminescence detection reagents (Amersham, Cleveland, OH, USA) were added after washing and membranes were exposed to Hyperfilm (Amersham, Cleveland, OH, USA).

Kallikrein Activity Assay

Kallikrein activity was detected by the cleavage of D-Val-cyclohexyl-Ala-Arg-4-nitroaniline. Lysates of aortic VSMCs (50 μg mL^{-1}) were prepared and diluted to 150 μL with 50 mM Tris-HCl buffer, pH 8.0. Samples were transferred to 96-well plates; then, 150 μL 0.5 mM kallikrein substrate D-Val-cyclohexyl-Ala-Arg-4-nitroniline (Chromozym GK, Boehringer, Germany) was added and plates were mixed to start the enzyme reaction. The optical density (OD) value at 405 nm was read after 30 min of rocking at 37°C to measure 4-nitroaniline. Background OD values without kallikrein were subtracted from all readings.

Bradykinin Assay

Bradykinin secreted into cell supernatants was measured by using a commercially available bradykinin RIA kit (Phoenix Pharmaceuticals Inc., CA, USA), following the manufacturer's instructions. Briefly, cell supernatants were collected and lyophilized. After adding primary antibody, test tubes were vortexed and incubated for 18 h at 4°C. ^{125}I-peptide was added; then, tubes were incubated at 4°C for 18 h. Goat anti-rabbit IgG and normal rabbit serum were added; then, tubes were vortexed and incubated for 60 min at room temperature. Samples were then centrifuged at 1500 × *g* for 20 min after adding RIA buffer and gently vortexing. Radioactivity levels were evaluated in a Packard Cobra Gamma Counter (Downers Grove, IL, USA). A standard curve was generated, following the manufacturer's instructions, and used to determine bradykinin levels (expressed as pg mL^{-1} supernatant).

Extracellular Ca^{2+}-Induced Contractions of Aortic Arteries

Thoracic aortic arteries were cut into 2.5-mm long sections and endothelium was removed from the aortic rings with a 25-gauge needle. The aortic rings were then suspended on Grass isometric transducers (FT-03, Grass Technologies, Warwick, RI, USA) in a bath of 20 mL Ca^{2+}-free Krebs (pH 7.3-7.4) at 37°C, continuously aerated with 95% O_2 and 5% CO_2. Before conducting measurements, the NOS inhibitor Nω-nitro-L-arginine methyl ester (L-NAME, 10^{-5} M) was added to the Krebs solution to prevent NO release by any residual endothelium.

We obtained cumulative extracellular Ca^{2+} dose-response curves (doses from 10^{-5}–10^{-2} M) in aortas without endothelium, pretreated with or without agonists (GW4064 and INT-747) and antagonists (PD123319 and HOE140). Agonists and antagonists, where indicated, were added to the Krebs bath 30 min prior to the addition of Ca^{2+}. Increasing concentrations of Ca^{2+} were added to the bath, thus establishing a Ca^{2+} concentration that caused a stable and sustained contraction for 5 min. The magnitude of contraction induced by 60 mM KCl was measured for all aortic rings and used as the reference value (100%) for expressing contractile responses induced by extracellular Ca^{2+}. After washing the KCl from the bath with fresh Krebs solution and recovering the baseline resting tension, Ca^{2+}-induced dose-response curves were obtained.

Monitoring $[Ca^{2+}]_i$ by Fluo-3/AM Staining

The fluorescent Ca^{2+} indicator, Fluo-3 acetoxymethyl ester (Fluo-3 AM, Biotium Inc., Temecula, CA, USA), was used to monitor $[Ca^{2+}]_i$ in rat aortic VSMCs. Briefly, cells were treated with GW4064 and INT-747 (with or without PD123319 and HOE140) for 24 h; then, cells were loaded, in a darkroom for

60 min at room temperature, with Fluo-3 AM (10 μM) in Krebs-Ringer solution (10 mM HEPES, 145 mM NaCl, 2.5 mM KH_2PO_4, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 10 mM glucose, and 0.1% BSA, pH 7.4) containing 0.05% pluronic acid. After loading with the dye, the cells were washed with a physiological saline solution at room temperature for 20 min to remove unhydrolyzed Fluo-3 AM. Fluorescence in cells was detected by using a confocal microscope (excitation/emission: 503/530 nm) with a PerkinElmer LS50 fluorimeter. Fluorescence signals (F) were calibrated by first adding 0.5 mM Ca^{2+} (F_{max}) and then 0.5 mM EGTA (F_{min}). Free $[Ca^{2+}]_i$ was calculated by the equation: $[Ca^{2+}] = Kd(F-F_{min})(F_{max} - F)^{-1}$, with a dissociation constant of 864 nM (K_d), as determined in a cytosol-like medium at 37°C.

SERCA Activity Assay

SERCA activity was measured at 37°C in 400 μL reaction mixture containing 100 mM imidazole-HCl pH 7.40, 100 mM KCl, 5 mM $ATPNa_2$, 0.1 mM $MgCl_2$, and 5 mM $CaCl_2$. Total ATPase activity was determined by adding microsomal vesicles (20 μg protein) from aortic VSMCs to start the reaction and measuring phosphate (Pi) to assess the amount of degradation. After adding 5 mM ethylene glycol tetraacetic acid (EGTA) and allowing it to chelate Ca^{2+} for 5 min, the amount of Pi was measured again. To calculate SERCA activity, the difference between ATPase activities in the presence and absence of EGTA was measured. Before recording SERCA activity, ethanol was added to the thermostatically controlled cuvette and the reaction was incubated for 10 min. SERCA activity was expressed as μmol Pi produced per mg protein in 1 h.

IP_3-Induced Ca^{2+} Release

Microsomal vesicles from rat aortic VSMCs were incubated for 30 min at 37°C in a buffer containing 120 mM KCl, 20 mM Tris/HEPES, pH 7.40, 0.3 mM $MgCl_2$, 0.5 mM EGTA, 3 mM β-mercaptoethanol, 1 mM Mg-ATP, 10 mM phosphocreatine, 2 mM potassium oxalate, 10 U/mL creatine kinase, 5.0 μCi/mL $^{45}Ca^{2+}$, 2 μM ruthenium red, and 1 μM IP_3. Potassium oxalate acts as an intravascular Ca^{2+} sink, enabling an almost 50-fold increase in $^{45}Ca^{2+}$ uptake. Ruthenium red was added to block the RyR-mediated Ca^{2+} channel. After incubation, microsomal vesicles were vacuum-filtered over a 0.3-μm filter (Millipore Corp., Billerica, MA, USA) and washed with 150 mM KCl. Filters were counted in Budget Solve complete counting mixture (Research Products International Corp., Mt. Prospect, IL, USA). INT-747- or GW4064-induced Ca^{2+} release was recorded at 0, 20, 40, and 60 min. The sarcoplasmic reticulum-derived Ca^{2+} release evoked by IP_3 was expressed as the ATP-dependent Ca^{2+} uptake of microsomal vesicles.

Statistical Analysis

Results are expressed as means ± standard error of the mean (SEM). GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA) was used to perform statistical analyses and to draw figures. Statistical evaluation was performed by one-way analysis of variance (ANOVA); concentration-response curves were analyzed by repeated-measures two-way ANOVA. A P value < 0.05 was considered statistically significant (*P < 0.05; **P < 0.01; ***P < 0.001).

Results

Effects of FXR Activation on the AT_2R-B_2R Pathway in Aortic VSMCs

We first investigated whether the AT_2R-B_2R pathway was activated following FXR stimulation, as well as the effects of FXR agonism on the KKS system in rat aortic VSMCs. Compared with control, both GW4064 and INT-747 increased protein abundance of AT_2R in a dose-dependent manner (Fig. 1). We found that GW4064 and INT-747 stimulated kallikrein activity (P < 0.001) and increased bradykinin and B_2R levels (P < 0.001) in a dose-dependent manner (Fig. 2). We next used the AT_2R antagonist PD123319 to determine whether FXR stimulation of the KKS was dependent on the AT_2R signaling pathway. We found that treatment of aortic VSMCs with PD123319 blocked the effects of GW4064 or INT-747 on kallikrein activity and on levels of B_2R and bradykinin (Fig. 3).

Effects of FXR- AT_2R-B_2R Activation on Extracellular Ca^{2+} Influx

To determine whether FXR activation regulates vascular tension by modulating Ca^{2+} homeostasis, we measured $[Ca^{2+}]_i$ in aortic VSMCs. We found that $[Ca^{2+}]_i$ in aortic VSMCs was significantly reduced and aortic contractions in response to extracellular Ca^{2+} were inhibited by both GW4064 and INT-747. This inhibition was attenuated by AT_2R-B_2R blockade with PD123319 and HOE140 (Figs. 4 and 5).

Effects of FXR-Induced Activation of AT_2R-B_2R on Sarcoplasmic Reticulum Ca^{2+}

To investigate whether FXR activation modulates $[Ca^{2+}]_i$ through control of Ca^{2+} uptake/release by sarcoplasmic reticulum in aortic VSMCs, we measured

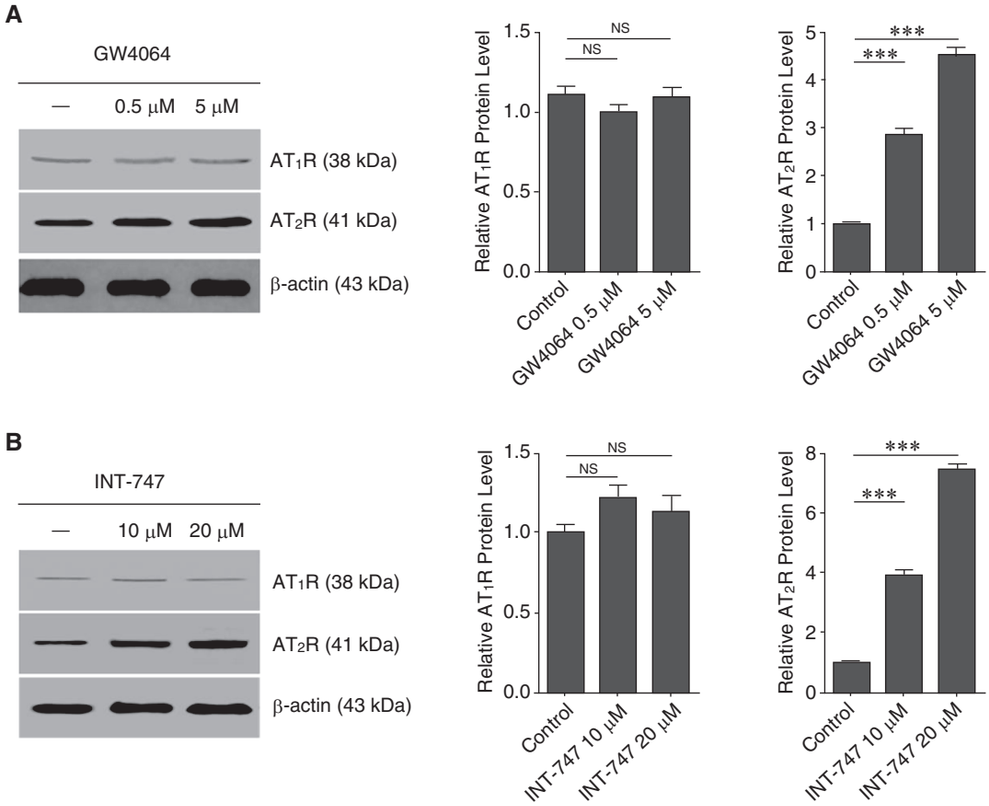

Fig. 1. Protein abundance of AT_1R and AT_2R following treatment of rat aortic VSMCs with the FXR agonists GW4064 (A) and INT-747 (B). Values are means ± SEM. n = 5 animals per group, ***$P < 0.001$. Left panel: representative protein bands, Right panel: relative protein quantification.

SERCA activity and expression of IP_3R_1. Compared with controls, both GW4064 and INT-747 enhanced SERCA activity; this effect was inhibited by PD123319 and HOE140 (Fig. 6). In aortic VSMCs, levels of IP_3R_1 protein decreased significantly (Fig. 7). Ca^{2+} uptake by microsomal vesicles at 0, 20, 40, and 60 min was augmented by GW4064 and INT-747 (Fig. 8A), and IP_3-evoked Ca^{2+} release was attenuated by GW4064 and INT-747. These effects were reversed by AT_2R-B_2R blockade with PD123319 and HOE140 (Figs. 8A, B).

Discussion

To our knowledge, this is the first report that FXR stimulation induces AT_2R-B_2R activation, modulating Ca^{2+} in rat aortic VSMCs by regulating extracellular Ca^{2+} influx and release of Ca^{2+} from the sarcoplasmic reticulum.

The FXR is a member of the ligand-activated nuclear receptor superfamily of transcription factors expressed in liver, kidney, adipose tissue, and the gastrointestinal tract. It has emerged as a therapeutic target for liver and metabolic disorders because it regulates cholesterol and bile acid homeostasis (3). Since FXR was discovered in the vasculature (2), its anti-atherosclerotic properties, in addition to its regulation of lipid metabolism, have received much attention (12, 14). In recent studies, FXR ligands have been shown to regulate vascular tension. For example, chronic FXR stimulation with GW4064 impaired endothelium-dependent relaxation by decreasing sensitivity of VSMCs to NO in rabbit mesenteric arteries (8). In contrast, we found that the FXR ligand, CDCA, attenuated vasoconstriction and induced vasodilation of aortic rings through an NO-dependent mechanism (24). Our observation is consistent with a report that FXR increased eNOS expression at the transcriptional level (11). Our findings are also consistent with a recent study showing that CDCA lowered blood pressure and regulated vascular tone in spontaneously hypertensive rats (10). The underlying mechanisms of these vascular effects of FXR receptor activation include reduced expression

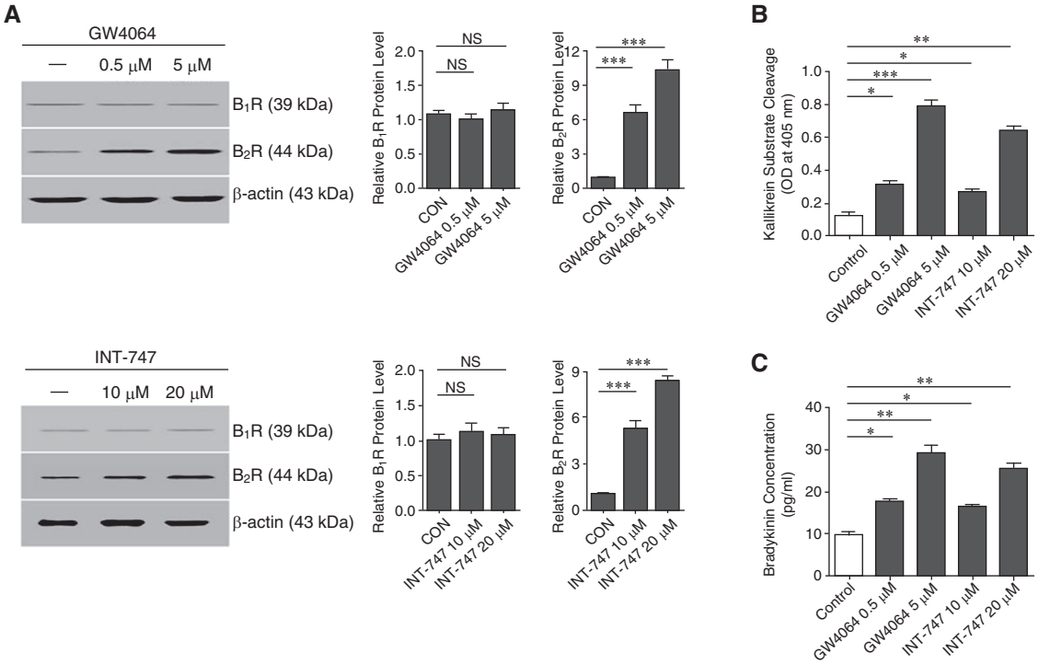

Fig. 2. Protein abundance of B_1R and B_2R (A), kallikrein activity (B) and bradykinin levels (C) following treatment of rat aortic VSMCs with the FXR agonists GW4064 and INT-747. Values are means ± SEM. n = 5 animals per group, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

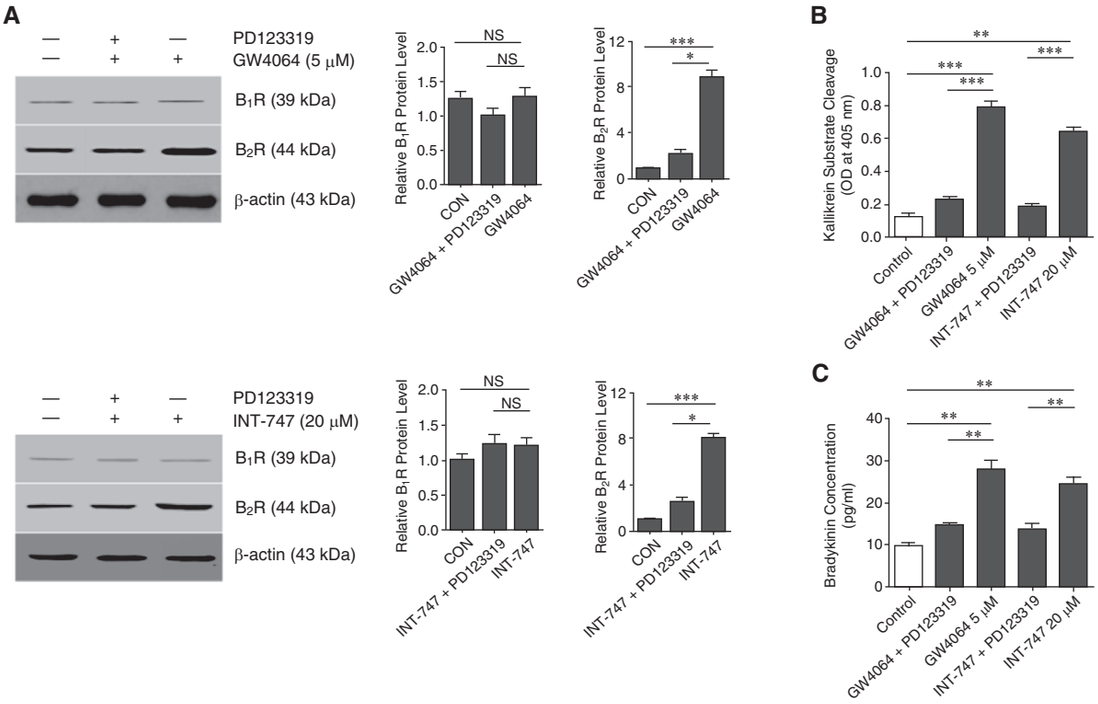

Fig. 3. Effects of AT_2R blockade on protein abundance of B_1R and B_2R (A), kallikrein activity (B) and bradykinin levels (C) following FXR activation with agonists GW4064 (A) or INT-747 (B) in rat aortic VSMCs. Values are means ± SEM. n = 5 animals per group, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. (A) Left panel: representative protein bands, Right panel: relative protein quantification.

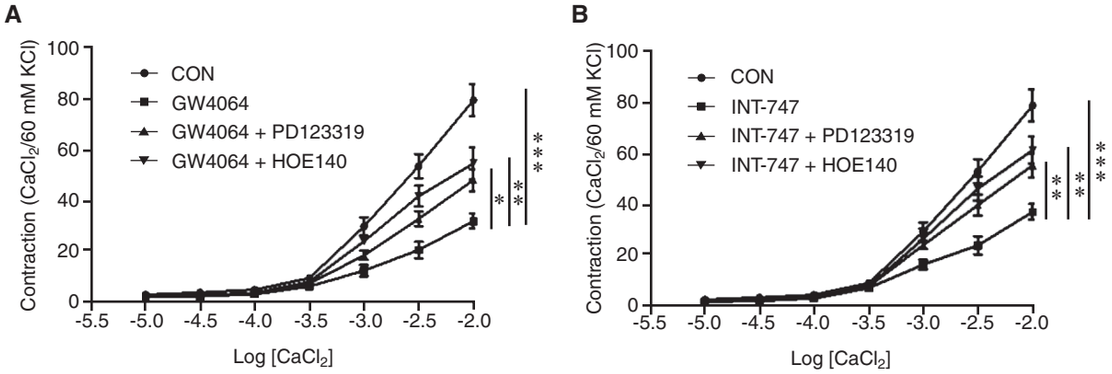

Fig. 4. Extracellular Ca^{2+}-induced contractions of aortic arteries following treatment with FXR ligands GW4064 (A) and INT-747 (B) with or without PD123319 or HOE140. Values are means ± SEM. n = 5 animals per group, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

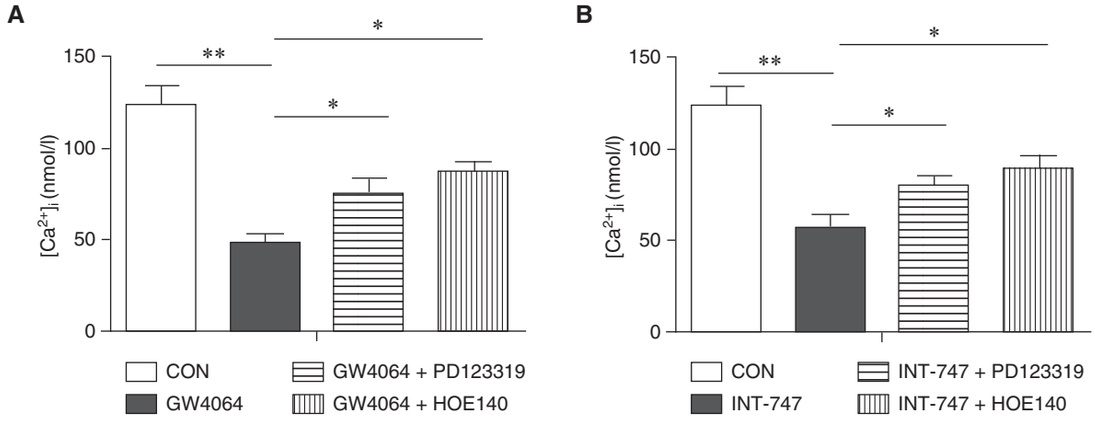

Fig. 5. $[Ca^{2+}]_i$ of aortic smooth muscle cells following treatment with FXR agonists GW4064 (A) and INT-747 (B) with or without PD123319 or HOE140. Values are means ± SEM. n = 5 animals per group, $*P < 0.05$, $**P < 0.01$.

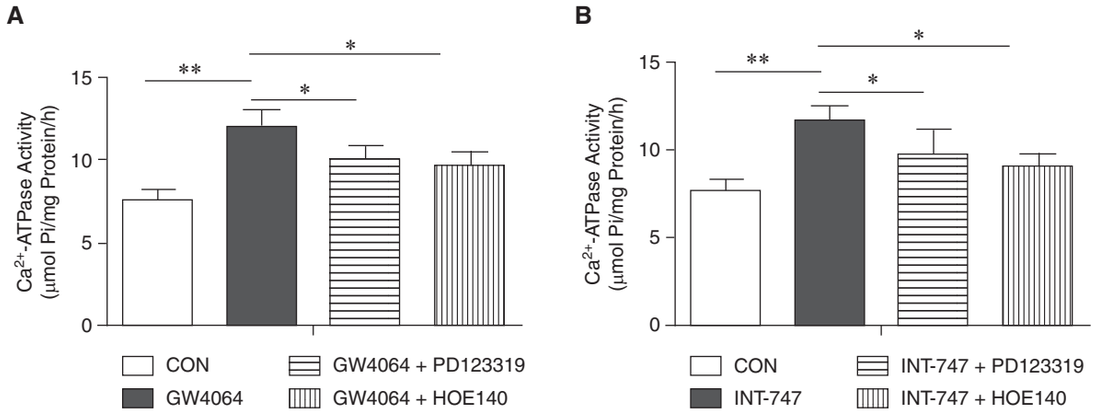

Fig. 6. SERCA activity in aortic smooth muscle cells following treatment with FXR agonists GW4064 (A) and INT-747 (B) with or without PD123319 or HOE140. Values are means ± SEM. n = 5 animals per group, $*P < 0.05$, $**P < 0.01$.

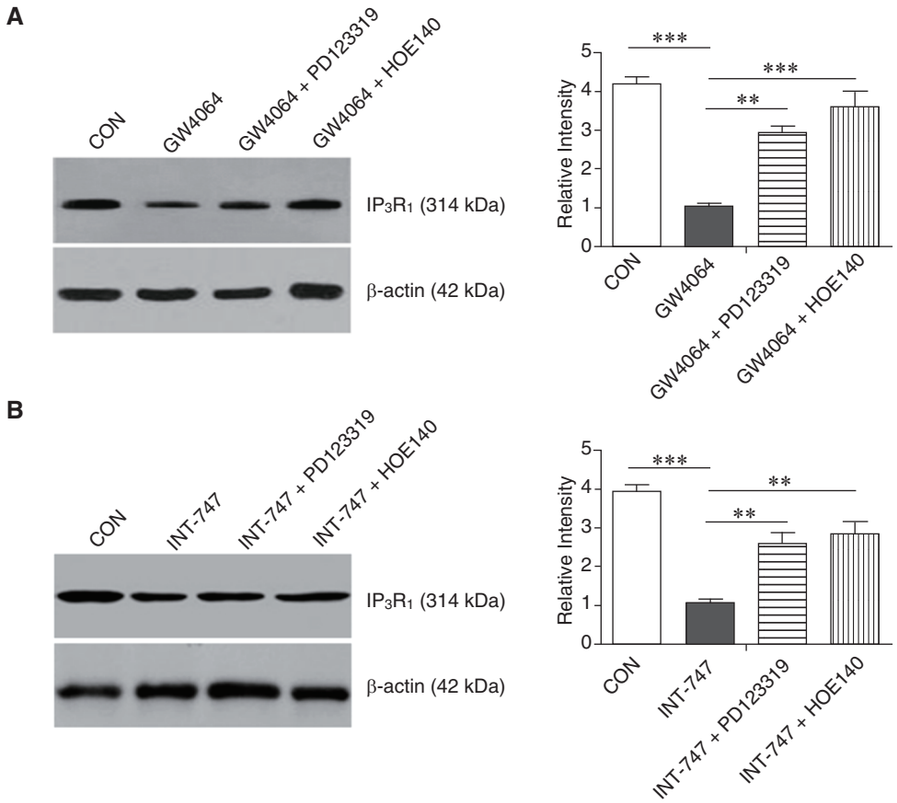

Fig. 7. Protein abundance of IP_3R_1 following treatment of rat aortic smooth muscle cells with the FXR agonists GW4064 (A) and INT-747 (B) with or without PD123319 or HOE140. Values are means ± SEM. n = 5 animals per group, $^{**}P < 0.01$, $^{***}P < 0.001$. Left panel: representative protein bands; Right panel: relative protein quantification.

of ET-1 (6, 10), activation of AT_2R (22), upregulation of dimethylarginine dimethylaminohydrolase expression (4), and regulation of large-conductance calcium-activated potassium channels (16) and eNOS expression (11). In another study, we found that the FXR ligands, GW4064 and INT-747, activated the AT_2R-B_2R pathway in rat aortic endothelial cells and regulated vascular tension by controlling the eNOS-NO system (23). Similarly, our present study showed that the AT_2R-B_2R pathway was activated following FXR stimulation in aortic VSMCs. Therefore, AT_2R-B_2R activation maybe a critical mechanism whereby FXR ligands regulate vascular structure and function.

Vascular tension depends upon functional endothelium and VSMCs. The $[Ca^{2+}]_i$ in resting VSMCs is very low and vasoconstriction requires $[Ca^{2+}]_i > 1$ μM. This is achieved *via* extracellular Ca^{2+} influx through L-type voltage-gated Ca^{2+} channels, store-operated Ca^{2+} channels, and receptor-operated Ca^{2+} channels, as well as Ca^{2+} release from the sarcoplasmic reticulum and mitochondria (1). FXR ligands were reported to regulate vascular osteogenic differentiation, thereby preventing vascular calcification in $ApoE^{-/-}$ mice (15). However, whether FXR activation modulates Ca^{2+} homeostasis in VSMCs has remained unclear. To study the physiological mechanisms of FXR activation in regulating vasoreactivity, we investigated the effects of FXR ligands on Ca^{2+} homeostasis in aortic VSMCs. We proposed that, in aortic VSMCs, FXR-induced AT_2R-B_2R activation would decrease extracellular Ca^{2+} influx and enhance Ca^{2+} uptake by the sarcoplasmic reticulum. In our present study, we found that FXR ligands inhibited vasoconstriction by decreasing Ca^{2+} influx; these effects were partially reversed by AT_2R-B_2R blockade. The sarcoplasmic reticulum is involved in the storage of intracellular Ca^{2+} in VSMCs. SERCA is responsible for Ca^{2+} uptake into the sarcoplasmic reticulum; the level of Ca^{2+} in the sarcoplasmic reticulum regulates extracellular Ca^{2+} influx into the cytosol *via* store-operated Ca^{2+} entry (19). Both GW4064 and INT-747 increased SERCA activity in rat aortic VSMCs, suggesting that

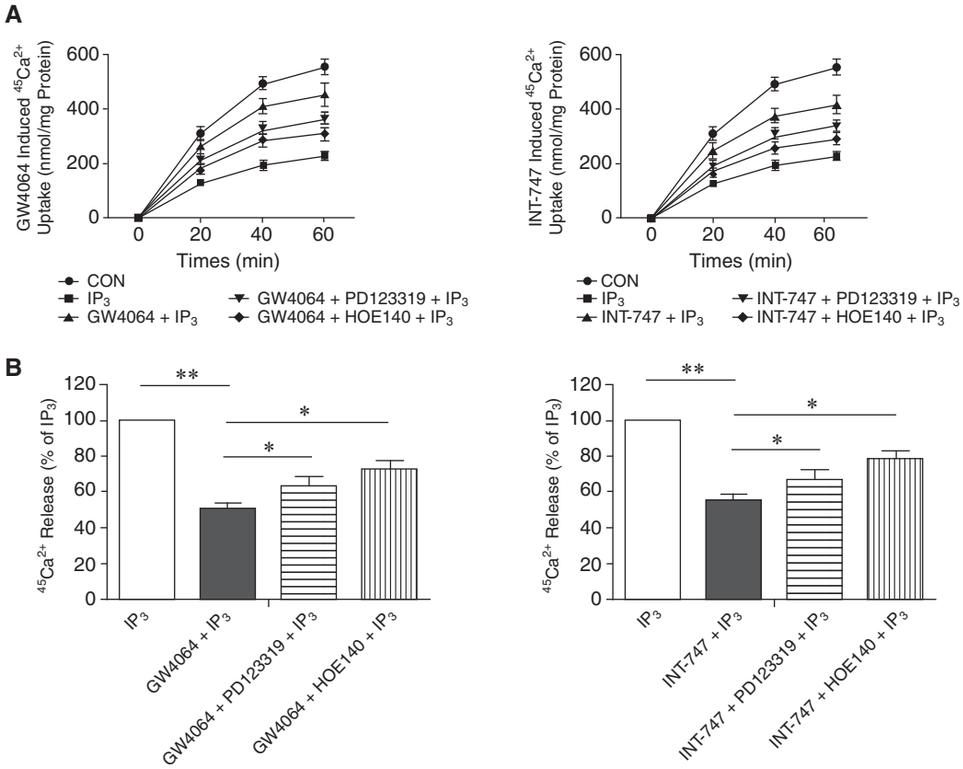

Fig. 8. Effects of FXR stimulation with GW4064 and INT-747 on $^{45}Ca^{2+}$ uptake (A) and IP_3-evoked Ca^{2+} release (B) in rat aortic smooth muscle cells with or without PD123319 or HOE140. Values are means ± SEM. n = 5 animals per group, $*P < 0.05$, $**P < 0.01$.

FXR activation results in increased Ca^{2+} uptake by the sarcoplasmic reticulum. In addition to SERCA, VSMCs express several intracellular Ca^{2+}-releasing channels in the sarcoplasmic reticulum, including IP_3Rs and ryanodine receptors (13). Upon binding IP_3, the IP_3R, an intracellular ligand-gated Ca^{2+} release channel, provides a pathway for Ca^{2+} to diffuse from the sarcoplasmic reticular lumen to the cytoplasm; the majority of this Ca^{2+} release is mediated by IP_3R_1 (9). By western blotting, we showed that IP_3R_1 protein abundance in rat aortic VSMCs was reduced by GW4064 and INT-747 and restored by the addition of PD123319 and HOE140. Consistent with these findings, IP_3-induced Ca^{2+} release from the sarcoplasmic reticulum was also reduced following FXR-AT_2R-B_2R activation by GW4064 and INT-747. Therefore, in aortic VSMCs, FXR activation inhibits both extracellular Ca^{2+} influx and intracellular Ca^{2+} release from the sarcoplasmic reticulum *via* the AT_2R-B_2R pathway. Ca^{2+} homeostasis in VSMCs is a complex phenomenon and, despite our findings, limitations still exist. First, isoform expression and the function of membrane Ca^{2+} channels should be clarified in future research to better understand extracellular Ca^{2+} influx following FXR stimulation. IP_3R_1 activation was reported to elevate plasma membrane large-conductance Ca^{2+}-activated K^+ (BKCa) channels, affecting Ca^{2+} sensitivity in VSMCs (25); the functional impacts of FXR-induced IP_3R_1 suppression on BKCa and other ion channels remain unknown. Second, Ca^{2+} transport by the sarcoplasmic reticulum of VSMCs is very complicated; thus, Ca^{2+} release channels other than SERCA and IP_3R_1 in that organelle require further investigation.

In summary, we identified the FXR-AT_2R-B_2R pathway in aortic VSMCs and demonstrated that reduced extracellular Ca^{2+} influx and Ca^{2+} uptake by sarcoplasmic reticulum are the underlying mechanisms involved in regulating vascular tension by activation of the FXR-AT_2R-B_2R pathway (Fig. 9). Overall, data presented here and in our previous study demonstrate that FXR activation contributes to vasodilation by functionally regulating endothelium and VSMCs *via* the AT_2R-B_2R pathway. Our findings may provide a physiological and pathological basis for targeting FXR in development of therapies for cardiovascular diseases, especially atherosclerosis and hypertension.

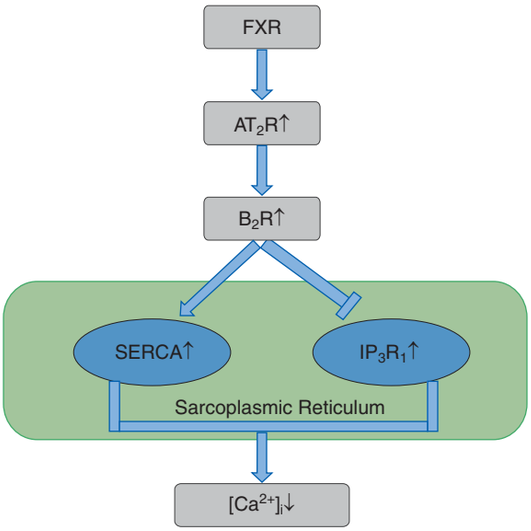

Fig. 9. Mechanism of calcium regulation by FXR in VSMCs. FXR, farnesoid X receptor; AT_2R, angiotensin II type 2 receptor; B_2R, bradykinin type II receptor; SERCA, sarco/endoplasmic reticulum Ca^{2+} ATPase; IP_3R_1, inositol 1,4,5-trisphosphate receptor type 1; $[Ca^{2+}]_i$, intracellular calcium concentration.

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Conflict of Interest

The authors declare that they have no conflicts of interest.

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