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FARNESOID X RECEPTOR REGULATES VASCULAR REACTIVITY THROUGH NITRIC OXIDE MECHANISM

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Farnesoid X receptor (FXR), a ligand-activated transcription factor and a member of nuclear receptor family, is not only highly expressed in the adrenal cortex, intestine, kidney and liver (1). However, the evidence on the roles of FXR in the vasculature is limited and whether FXR regulates vascular reactivity is poorly understood. In present study, we investigated the expression of FXR protein in rat vasculature by immunohistochemical method and tested the effects of FXR activation by chenodeoxycholic acid (CDCA) on thoracic aortic contraction and dilation. We also detected the level of nitrite/nitrate (NOx) and superoxide in the thoracic aortic segments. We found that FXR was expressed in rat carotid arteries, thoracic aorta, abdominal aorta and femoral arteries. FXR activation by CDCA significantly (P<0.01) inhibited the contractile responses of rat thoracic aorta rings to KCl and phenylephrine. The cumulative concentrations of CDCA caused a concentration-dependent relaxation, which could be partly impaired by L-NAME, an inhibitor of nitric oxide (NO) synthase. The NOx content in thoracic aorta significantly (P<0.01) increased when treated with CDCA. Meanwhile, the vascular redox status was not altered by high concentration of CDCA. The present study suggested that FXR regulated vascular reactivity through NO mechanism, which merits further attention.

Key words: farnesoid X receptor, nitric oxide, superoxide, vascular reactivity, chenodeoxycholic acid

INTRODUCTION

The farnesoid X receptor (FXR) is a ligand-activated transcription factor and a member of nuclear receptor family and is highly expressed in the adrenal cortex, intestine, kidney and liver (1). It serves as a receptor and physiological sensor for bile acids including chenodeoxycholic acid (CDCA) and deoxycholic acid (DCA) (2). There are four distinct FXR isoforms that differentially regulate gene expression in numerous tissues, such as small heterodimer partner (SHP), bile salt export pump, bile acid binding protein and phospholipids transfer protein (3). FXR activation down-regulates the transcription of cholesterol 7α-hydroxylase through induction of SHP protein, which causes feedback inhibition of cholesterol synthesis (4). Thus, FXR can be a potential target to treat hypercholesterolemia and related disorders (5, 6).

FXR has been found in vascular smooth muscle cells (7) and endothelial cells (8). However, the evidence on the physiological and pathophysiological roles of FXR in the vasculature is limited. FXR might provide a direct target for the treatment of proliferative diseases because vascular smooth muscle cells underwent apoptosis when treated with FXR ligands (7, 9). FXR ligands down-regulated interleukin-1beta (IL-1β)-induced inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 expression in rat aortic smooth muscle cells, and thereby inhibited vascular inflammation and suppressed smooth muscle cell migration (9). Bile acids also induced the expression of VCAM-1 and ICAM-1 by stimulation of NF-kB and p38 MAPK signaling pathways through the elevation in reactive oxygen species (ROS) in vascular endothelial cells (10). FXR activation in aorta attenuated IL-1β, IL-6, and tumor necrosis factor-α gene induction in response to Toll-like receptor 4 activation by lipopolysaccharide (11). Due to the anti-inflammatory properties of FXR in atherosclerosis and lipid homeostasis by regulating bile salt metabolism, FXR signaling pathways represent attractive therapeutic targets for the treatment of atherosclerosis (12). Chronic stimulation of FXR with GW4064, a FXR agonist, impaired endothelium-dependent relaxation because of decreased sensitivity of smooth muscle cells to nitric oxide (NO) (13). However, treatment of vascular endothelial cells with FXR ligands resulted in up-regulated expression of eNOS mRNA and protein and an increased production of NO (14), which indicated that FXR activation might contribute to vascular dilation. Therefore, further evidence is needed to clarify the effects of FXR activation on the vascular function due to the existing contrary evidence.

In the present study, we investigated the expression of FXR protein in rat arteries from different anatomic regions, and tried to clarify the effects of FXR activation by CDCA, a FXR agonist, on vascular contraction and dilation in the presence or absence of NOS inhibitors. We also detected the level of nitrite/nitrate (NOx) and superoxide in aortic arteries treated with CDCA. Here we demonstrated that FXR might regulate vascular reactivity through NO mechanism.
MATERIALS AND METHODS

The procedures used in this study were in accordance with Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences.

Animals and arterial tissue preparation

Male Sprague Dawley rats (250–300 g) were anesthetized with pentobarbital sodium (35 mg/kg, i.p.) and dissected. Common carotid arteries, thoracic aorta, abdominal aorta and femoral arteries were rapidly removed and placed in cold Krebs buffer solution consisting of (in mmol/L) NaCl, 118.3; KCl, 14.7; KH2PO4, 1.2; MgSO4 7H2O, 1.2; CaCl2, 2H2O, 2.5; NaHCO3, 25; dextrose, 11.1; and EDTA, 0.026; pH 7.40. The arteries were cleaned of fat and connective tissues, left with an intact endothelium. CDCA was diluted in dimethyl sulfoxide (DMSO). DMSO was used as a negative control at 0.05% volume/volume.

Drugs and reagents

All drugs and chemicals were obtained from Sigma Chemical (St. Louis, MO). Rabbit anti-FXR antibody was purchased from Santa Cruz Biotechnology. The BCA assay kit was the product of Pierce. Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG and DHE were the products of Invitrogen and Molecular Probes respectively.

Immunohistochemical localization

Serial sections (5 µmol/L) from paraffin-embedded rat common carotid arteries, thoracic aorta, abdominal aorta and femoral arteries were used for immunohistochemical localization of FXR. Briefly, sections were deparaffinized and rehydrated. Three independent sections per artery were examined. After preincubation with 20% normal bovine serum for 30 min at room temperature to block the nonspecific antigens, the sections were incubated with rabbit anti-FXR (H-130) antibody (diluted in 20% normal bovine serum 1:50; sc-13063, Santa Cruz Biotechnology Inc.) or 20% normal bovine serum only at 4°C overnight in a moist chamber. After washing all the sections with phosphate buffer solution (PBS), the sections were incubated with a biotinylated secondary antibody (1:400) (Vector Universal Elite Kit; Vector Laboratories, Inc., Burlingame, CA) for 2 hours at room temperature. The sections were rinsed, dehydrated in ethanol, cleared in xylene, and mounted.

Measurement of vascular contraction and dilation

Thoracic aorta rings (3 mm in length) were mounted on the stainless steel hooks, and placed on the stainless steel holders in tissue baths (15 mL) for vascular reactivity recordings with PowerLab (ADInstruments). The tissue baths were filled with tissue baths (15 mL) for vascular reactivity recordings with PowerLab (ADInstruments). The arteries were precontracted by KCl (60 mmol/L) or PE (10–5 mmol/L), and then concentration-dependent response curves to cumulative concentration of CDCA ranged from 12.5–400 µmol/L were generated. In another group, 100 µmol/L L-NAME (L-NAME) (0.1 mmol/L), a NOS inhibitor, was added to the tissue baths and then got the concentration-dependent response curves to cumulative concentration of CDCA ranged from 12.5–400 µmol/L. Vascular dilation is represented by percentages of maximal constriction to KCl at 60 mmol/L or PE at 10–5 mol/L.

Measurement of vascular nitrite/nitrate (NOx) content

Thoracic aorta arteries treated with CDCA (100, 200 and 400 µmol/L) were homogenized in 0.3 mmol/L perchloric acid buffer. The homogenates were centrifuged at 12,000 g for 5 min at 4°C and the supernatant was used for determination of NOx content by the chemiluminescence method. Commercialized NOx detection kit (Boehringer Mannheim, German) was used to determine NOx content. The standard curves were constructed by using various concentrations of NO3– and relating the optical density value produced to the given concentrations. The NOx content in each sample was determined by interpolation on the standard curve. The total protein concentration of sample was determined by the bicinchoninic acid (BCA) assay kit (Pierce, Rockford, USA).

Detection of vascular superoxide production

Dihydroethidium (DHE) is the most popular probe used to detect O2•– levels in vascular tissues. Briefly, thoracic aorta treated with CDCA (400 µmol/L) were immediately frozen in Tissue-Tek O.C.T. embedding medium. 30 µm thick frozen sections were prepared, and then were stained with 10 µmol/L DHE (Molecular Probes). Laser scanning confocal microscopic images were obtained after incubation in a light-protected humidified chamber at 37°C for 30 minutes.

Statistical analysis

The results were expressed as mean ±S.E.M. n equaled the number of animals studied. One-way ANOVA was used to analyze the effects of various concentration of CDCA on vascular contractile inhibition. For isometric ring studies, repeated measures analysis (two-way ANOVA) was used to analyze the concentration response curves. The values of P<0.05 were considered significant.

RESULTS

Immunohistochemical localization of farnesoid X receptor in rat vasculature

As shown in Fig. 1, farnesoid X receptor (FXR) protein (brown immunoperoxidase shown in the Figs. B, D, F, H) was detected in endothelial cells and smooth muscle cells of carotid arteries, thoracic aorta, abdominal aorta and femoral arteries.

Effects chenodeoxycholic acid on vascular contraction

As shown in Fig. 2, preincubation with increasing concentrations of chenodeoxycholic acid (CDCA) (12.5–100 mmol/L) caused concentration-dependent attenuation of vascular responses to KCl (Fig. 2A) and PE (Fig. 2B). At the 100 µmol/L CDCA concentration, the greatest inhibition of the
contractile responses to KCl and PE were 62.5±5.27% and 42.83±7.24% respectively. Treatment the aorta rings with DMSO showed no significant effects on aortic contraction.

Effects of chenodeoxycholic acid on vascular dilation

As shown in Fig. 3, in thoracic aortic rings precontracted with 60 mmol/L KCl or 10⁻⁵ mmol/L PE, the increasing concentrations of CDCA (12.5–400 µmol/L) caused a concentration-dependent relaxation and at the 400 µmol/L, the maximal aortic dilation was 101.67±2.55% and 100.2±1.25% respectively. However, when the arteries were pretreated with L-NAME, CDCA-induced aortic dilation was significantly attenuated compared with the absence of L-NAME (P<0.01). In addition, treatment the aorta rings with DMSO showed no significant effects on vascular dilation (data not shown).

Fig. 1. Representative photograph of immunohistochemical examination for FXR in common carotid artery (A, B), thoracic aorta (C, D), abdominal aorta (E, F) and femoral artery (G, H). Figs. A, C, E, G: arteries treated with 20% normal bovine serum. Figs. B, D, F, H: arteries treated with FXR antibody diluted in 20% normal bovine serum. Positive staining of FXR protein (shown as brown immunoperoxidase in the Figs. B, D, F, H) could be seen in the vascular endothelial cells and smooth muscle cells. n=5 in each group. Original magnification: ×200.
Measurement of vascular nitrite/nitrate (NOx) content

As shown in Fig. 4, treatment of CDCA (100, 200, 400 µmol/L) significantly increased \( P<0.01 \) NOx content in thoracic aorta. Treatment with DMSO did not affect the arterial level compared with control \( P>0.05 \).

Effects of treatment with chenodeoxycholic acid on vascular superoxide production

To clarify whether the redox status in the thoracic aorta was altered when treated with chenodeoxycholic acid (CDCA), we detected the level of \( \text{O}_2^{-} \) in thoracic aorta segments. As shown in Fig. 5, the level of \( \text{O}_2^{-} \) in thoracic aorta was not significantly altered at the 400 µmol/L by CDCA (Fig. 5C) or DMSO (Fig. 5B) compared with the absence of the CDCA or DMSO (Fig. 5A).

DISCUSSION

There were three major findings in present study: [1] FXR was expressed in normal rat carotid arteries, thoracic aorta, abdominal aorta and femoral arteries. [2] FXR activation by CDCA attenuated vascular response to vasoconstrictors and induced concentration-dependent relaxation through NO mechanism. [3] CDCA did not alter redox status in thoracic aorta even at high concentration.

FXR plays important roles in regulating lipid and glucose homeostasis (2, 4, 5, 15). Ever since Bishop-Bailey demonstrated the expression of FXR in the vasculature (7), FXR-related signaling pathways have been demonstrated to be involved in vascular smooth muscle cell apoptosis (7), vascular calcification (16) and vascular response regulation (13, 14). Therefore, vascular FXR has been considered as a novel and promising therapeutic target for the treatment of atherosclerosis and coronary heart diseases (17).

Hypotension and attenuated vascular tone in patients with severe cirrhosis is common. The peripheral vascular responses to either sympathetic or nonsympathetic agonists were impaired in severe cirrhosis, and the control of vascular tone was disturbed even in well compensated cirrhosis (18, 19). Angiotensin II, an important vasoactive peptide, contributed to basal vascular tone in patients with cirrhosis (20); however, the pressor response to angiotensin II was significantly lower in cirrhotic animals than in control ones (21). Although the evidence is accumulating, the mechanism of the hemodynamic changes described above is unclear. The level of serum bile acids increases significantly in hepatobiliary diseases. FXR is expressed mainly in tissues exposed to high level of bile acids, including liver, intestine,

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**Fig. 2.** The effects of various concentration of CDCA on vascular contraction induced by KCl (60 mmol/L) (A) and phenyllphrine (PE, \( 10^{-5} \) mmol/L) (B). The inhibition of CDCA on vascular contraction was expressed as percentage of KCl (60 mmol/L) and PE (\( 10^{-5} \) mmol/L)-induced tension in the absence of CDCA. Values are mean ±S.E.M. \( n=6 \) in each group. * \( P<0.05 \), ** \( P<0.01 \) vs. DMSO.

**Fig. 3.** Concentration-response curves for cumulative concentration of CDCA ranged from 12.5 to 400 µmol/L. CDCA induced aortic dilation precontracted by KCl (60 mmol/L) (A) and phenylephrine (PE, \( 10^{-5} \) mmol/L) (B) in the presence or absence of L-NAME, an inhibitor of nitric oxide synthase (NOS). (Values are mean ±S.E.M. \( n=6 \) in each group. * \( P<0.05 \), ** \( P<0.01 \) vs. the absence of L-NAME).
kidney and vasculature. FXR functions as the chief sensor of intracellular levels of bile acids and the main executor of bile acid-induced transcriptional programmers (22). CDCA, one of the most potent natural FXR agonist, directly interacts with the ligand-binding domain of FXR and enhances the transactivation function of FXR. Whether FXR activation by bile acids regulates vascular response to constrictors is still controversial. In present study, we found that preincubation the aortic rings with CDCA attenuated receptor and non-receptor induced vascular contraction. The fact that FXR activation by CDCA-induced aortic dilation in a concentration-dependent manner is contrary to Kida’s study (13) that chronic activation of FXR by GW4064 impaired NO sensitivity of vascular smooth muscle cells. Interestingly, L-NAME could not completely abolish CDCA-induced vascular dilation. Perhaps the dosage of 0.1 mmol/L was not enough to inhibit NOS or the other mechanisms might be involved in the process. Treatment of vascular endothelial cells with FXR ligands such as CDCA or GW4064 resulted in up-regulated expression of eNOS mRNA and protein expression at transcriptional level (14). Meanwhile, asymmetric dimethylarginine (ADMA), a major endogenous NOS inhibitor, received much attention in the past years. Elevated ADMA levels are associated with reduced NO synthesis. Dimethylarginine dimethylaminohydrolase-1 (DDAH1) is an FXR target gene and functions as a key catabolic enzyme of ADMA. The increased hepatic DDAH1 gene expression and concomitantly decreased ADMA have been found in Zucker diabetic fatty rats (23). A recent study by Vignozzi and associates found that INT-747, a selective FXR agonist, regulated the expression of DDAH1 and improved endothelium-dependent relaxation in metabolic syndrome-associated erectile dysfunction via upregulation of NO transmission and inhibition of RhoA/ROCK pathway (24). In addition, FXR up-regulated the expression of angiotensin II type 2 receptors, which might also be involved in vascular dilation (25). NO is a critical vasodilator and NOS derived NO plays a pivotal role in modulating vascular tone. Although the roles of eNOS and iNOS in vascular tone modulation have been confirmed, long-term inhibition of neuronal NOS (nNOS) did not change endothelium-dependent relaxation (26). In present study, CDCA-induced concentration dependent relaxation was impaired in the presence of L-NAME and also, the treatment with CDCA increased NOx content in thoracic aorta. The above evidence indicated that CDCA might dilate arteries through NO mechanism, perhaps by increasing NOS-derived NO production. The upregulated expression of eNOS by FXR ligands has been demonstrated in vascular endothelial cells (14); however, the systemic expression of eNOS, iNOS and nNOS when FXR is activated needs further investigation.

Reactive oxygen species, especially O2•−, have been indicated in the development of atherosclerosis through modulating vascular structure and function, including inflammation, apoptosis, and vascular response (28). O2•− can inactivate NO by forming peroxynitrite and induce vascular contraction (28). Local inflammation and activated neutrophils could generate O2•− and cause a marked contraction in rat aorta (29). Qin and associates (10) found that the level of ROS increased when treated the aorta with CDCA at a high concentration. However, in present study, we did not find significant redox alterations in thoracic aorta segments by detecting the level of O2•−. However, in present study, even at the 400 µmol/L, the level of O2•− was not significantly altered by CDCA. The fact that CDCA did not alter the level of O2•− in the aorta indicated that concentration-dependent relaxation in thoracic aorta when treated with CDCA might not be associated with ROS. However, additional evidence is needed to further confirm it.

Fig. 4. Nitrite and nitrate content (nmol/mg protein) in CDCA-treated thoracic aorta. Values are mean ±S.E.M. n=5 animals in each group. * P<0.05, **P<0.01 vs. DMSO.

Fig. 5. Representative fluorescence photographs of superoxide level in control (A), DMSO-treated (B) and CDCA (400 µmol/L) -treated (C) thoracic aorta. Arteries were labeled with the oxidative dye dihydroethidium, which reacted with O2•− to form ethidium and produced a red fluorescence. Original magnification, ×400; n=5 in each group.
In conclusion, we demonstrated that FXR activation by CDCA inhibited vascular contraction and induced concentration-dependent relaxation in normal aorta through NO mechanism, which could partly interpret hemodynamic changes in patients with cirrhosis and related disorders. For the high level of bile acids in the circulation and FXR expression in the vasculature, the roles of FXR in the vasculature merit further investigation.

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