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Article

Xyloketal B exerts antihypertensive effect in renovascular hypertensive rats via the NO-sGC-cGMP pathway and calcium signaling

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Abstract

Xyloketal B (Xyl-B) is a novel marine compound isolated from mangrove fungus *Xylaria sp.* (No 2508). We previously showed that Xyl-B promoted endothelial NO release and protected against atherosclerosis through the Akt/eNOS pathway. Vascular NO production regulates vasoconstriction in central and peripheral arteries and plays an important role in blood pressure control. In this study, we examined whether Xyl-B exerted an antihypertensive effect in a hypertensive rat model, and further explored the possible mechanisms underlying its antihypertensive action. Administration of Xyl-B (20 $mg{\cdot}kg^{-1}{\cdot}d^{-1}$, ip, for 12 weeks) significantly decreased the systolic and diastolic blood pressure in a two-kidney, two-clip (2K2C) renovascular hypertensive rats. In endothelium-intact and endothelium-denuded thoracic aortic rings, pretreatment with Xyl-B (20 μmol/L) significantly suppressed phenylephrine (Phe)-induced contractions, suggesting that its vasorelaxant effect was attributed to both endothelial-dependent and endothelial-independent mechanisms. We used SNP, methylene blue (MB, guanylate cyclase inhibitor) and indomethacin (IMC, cyclooxygenase inhibitor) to examine which endothelial pathway was involved, and found that MB, but not IMC, reversed the inhibitory effects of Xyl-B on Phe-induced vasocontraction. Moreover, Xyl-B increased the endothelial NO bioactivity and smooth muscle cGMP level, revealing that the NO-sGC-cGMP pathway, rather than PGI_2, mediated the anti-hypertensive effect of Xyl-B. We further showed that Xyl-B significantly attenuated KCl-induced Ca^{2+} entry in smooth muscle cells *in vitro*, which was supposed to be mediated by voltage-dependent Ca^{2+} channels (VDCCs), and reduced ryanodine-induced aortic contractions, which may be associated with store-operated Ca^{2+} entry (SOCE). Taken together, these findings demonstrate that Xyl-B exerts significant antihypertensive effects not only through the endothelial NO-sGC-cGMP pathway but also through smooth muscle calcium signaling, including VDCCs and SOCE.

Keywords: hypertension; two-kidney, two-clip renovascular hypertensive rats; aortic rings; smooth muscle cells; xyloketal B; NO-sGC-cGMP pathway; SNP, methylene blue; indomethacin; KCl; ryanodine

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Introduction

Hypertension is a major risk factor that predisposes patients to cardiovascular disorders and has high rates of morbidity and mortality. Emerging data implicate increases in systemic oxidative stress[1] and vascular inflammation[2] in the pathogene-

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sis of hypertension. Endothelial dysfunction, which manifests as impaired nitric oxide (NO) bioactivity, is an important early event associated with impaired vessel diastolic function[3] and mainly results from increased nitric oxide (NO) degradation due to interactions between NO and superoxide anions. Pharmacological approaches to restore endothelial function or complement NO have been demonstrated to have antihypertensive effects.

Marine organisms are abundant natural sources of novel bioactive compounds because they can produce a variety of molecules with unique structural features and exhibit various types of biological activities. Xyloketal B (Xyl-B), obtained from the South China Sea Coast, is one of a series of novel ketal compounds isolated from the mangrove fungus *Xylaria sp* (No 2508)[4, 5]. In previous studies, multiple biomedical activities of Xyl-B have been demonstrated, including neuroprotective effects against toxicity[6], antioxidant effects on endothelial cells[7] and zebrafish[8], anti-glioma effects[9] and reduced atherosclerosis plaque formation in apolipoprotein E-deficient mice[10]. After exploring the underlying mechanisms, we have found that Xyl-B can directly scavenge DPPH free radicals, promote endothelial NO release[7] and increase eNOS phosphorylation at Ser-1177 in a concentration- and time-dependent manner[10]. Furthermore, the effect of Xyl-B on aortic tension was also evaluated, and the data indicated that Xyl-B can improve NO-dependent aortic vasorelaxation in atherosclerotic ApoE$^{-/-}$mice[10] and exert potent vasorelaxant activity on KCl-induced contractions in isolated rat aortic rings[11]. Based on these findings, we hypothesize that Xyl-B may regulate vascular tone through the endothelial NO system and thus has an effect on blood pressure control.

Therefore, in the present study, we established a 2-kidney, 2-clip (2K2C) renovascular hypertensive rat model to evaluate the effects of Xyl-B in the development of hypertension. In addition, we further explored the possible mechanisms for the antihypertensive effects of Xyl-B, including Xyl-B-mediated vasorelaxation, endothelial nitric oxide synthase (eNOS) bioactivity, the NO-sGC-cGMP pathway and the prostacyclin pathway. Abnormal calcium signaling has been demonstrated to regulate arterial tone in the development of hypertension in both human patients and animal models. Consequently, Ca^{2+} channel blockers are commonly used as anti-hypertensive agents. Because we found that Xyl-B suppressed KCl- and phenylephrine (Phe)-induced contractions in endothelium-denuded thoracic aortic rings, which could not be completely explained by the endothelium-dependent pathway, the influence of Xyl-B on calcium signaling in vascular smooth muscle cells was also investigated.

Materials and methods

Chemicals and reagents

Xyl-B was isolated by the Department of Applied Chemistry, Sun Yat-sen University (Guangzhou, China). The identity and purity of the compound were characterized by HPLC and ^{2}D-NMR as previously described[4]. Xyl-B was dissolved in dimethyl sulfoxide (DMSO) and stored at -20°C until use. The final concentration of DMSO in culture media was less than 0.1%. Phenylephrine (Phe), acetylcholine (ACh), sodium nitroprusside (SNP) and N^G-nitro-*L*-arginine methyl ester (*L*-NAME) were purchased from Sigma (St Louis, MO, USA). Methylene Blue was obtained from Merck (Massachusetts, USA). [^{3}H]*L*-arginine was obtained from Beijing Institute of Nuclear Industry (Beijing, China). All other reagents used were purchased from Sigma unless otherwise specified.

Animals and experimental design

All experimental animal procedures and protocols were approved by the Sun Yat-sen University Committee for Animal Research and were conducted in accordance with the National Research Council's guidelines. 2K2C stroke-prone renovascular hypertension was induced in male Sprague-Dawley rats (60–80 g, purchased from the Experimental Animal Center of Sun Yat-sen University) as described previously[12, 13]. All rats were randomly divided into 5 groups: (1) the Sham group: sham-operated control group; (2) the Htn group: 2K2C-operated hypertensive group; (3) the propylene glycol-treated hypertensive (Htn+Sol) group: the rats received an intraperitoneal injection of 2 mL of saline solution containing 20% propylene glycol once daily after surgery; (4) the Htn +Xyl-B group: 2K2C rats received an intraperitoneal injection of Xyl-B (20 mg·kg^{-1}·d^{-1} in 2 mL of saline solution containing 20% propylene glycol) after surgery; and (5) the Htn+Cap group: 2K2C rats were treated with captopril (0.1 mg·kg^{-1}·d^{-1}, ig). The sham-operated animals underwent sham procedures without clip placement. Body weight and SBP or DBP measured by tail cuff plethysmography[13] were recorded every two weeks until 12 weeks after operation.

Vascular reactivity experiments in aortic rings

Aortic rings were prepared and vascular reactivity experiments were performed as previously described[14]. Briefly, Sprague-Dawley rats (200–250 g) were euthanized and their aortas were quickly removed and placed into ice-cold Kreb's solution (composition in mmol/L: NaCl 119, KCl 4.7, KH_2PO_4 1.18, $MgSO_4$ 1.17, $NaHCO_3$ 25, $CaCl_2$ 2.5, EDTA 0.026, and glucose 5.5) where they were freed of fatty and connective tissues. Aortic ring segments (3–5 mm) were then cut and each segment was mounted onto separate Mulvany myograph chambers, which involved placing each ring onto two stainless hooks in an isometric myograph filled with Kreb's solution (5 mL) aerated with 95% O_2/5% CO_2 and maintained at 37 °C. Changes in isometric force were recorded online using a Multi-myograph data acquisition system with PowerLab software (AD Instruments, Inc). The rings were stretched to an optimal resting tension (1.5 g) and allowed to stabilize for 2 h. An additional equilibration period of 30 min was allotted before treatment with any drugs. The rings were stimulated with Phe (1 μmol/L) when indicated to induce contraction. To examine the possible mechanisms of the vaso-relaxant effects of Xyl-B, the NO synthase (NOS) inhibitor *L*-NAME (100 μmol/L), the soluble guanylate cyclase inhibitor methylene blue (MB, 10 μmol/L) or the cyclooxygenase inhibitor

indomethacin (IMC, 10 μmol/L) was used. The endothelium-intact aortic rings were pre-incubated with these inhibitors for 30 min before Xyl-B was added.

eNOS activity detection in human umbilical vascular endothelial cells (HUVECs)

Primary culture of HUVECs was performed using collagenase digestion of the human umbilical cord as described previously[7]. Three to six generations of identified HUVECs were used in this experiment. Cells were pre-incubated with 20 μmol/L of Xyl-B for 0, 15, 30, 45, or 60 min or with 0, 10, 20, 40, or 80 μmol/L of Xyl-B separately for 30 min, and eNOS activity was evaluated by determining the conversion ratio of [^{3}H]*L*-arginine to [^{3}H]*L*-citrulline at 15 min or 45 min with a liquid scintillation counter (Packard Inc, CT, USA)[15].

Measurement of cyclic GMP in primary cultured VSMCs

Vascular smooth muscle cells (VSMCs) were isolated from adult rat thoracic aortas by the explants method as previously described[16]. Passages 3 to 6 showing >99% positive immunostaining against α-smooth muscle–actin (α-SMA) were used for the experiments, and cells at 80% confluence were arrested by serum-starvation for 24 h. Cyclic GMP (cGMP) levels in VSMCs were determined using Parameter ^{TM}Cyclic GMP Assay kits (R&D Systems, Minnesota, USA) according to the manufacturer's instructions. The cells were divided into a VSMC mono-culture group and a VSMC-HUVEC co-culture group. Each group of cells was treated with DMSO, SNP (10 μmol/L), or Xyl-B (20 and 40 μmol/L) and the incubation time was 30 min. None of the drugs exhibited cytotoxicity at any dose used (evaluated by MTT assay, data not shown).

Determination of single-cell intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in VSMCs

Intracellular Ca^{2+} was detected by Fluo-3/AM staining as described previously[17]. Cells plated in 35-mm petri dishes were loaded with Fluo-3/AM (10 μmol/L, Sigma) in Kreb's solution at 37 °C for 30 min and then exposed to KCl (60 mmol/L). Xyl-B was added 5 min prior to KCl stimulation. The dishes were washed twice with Kreb's solution to remove unhydrolyzed indicator before transfer to a chamber where the drugs were added. Cellular real-time fluorescence ($E_{x488\ nm}$, $E_{m522\ nm}$) was measured using a confocal microscope (FV500-IX 81, OLYMPUS, Japan). A change in fluorescence was expressed as F/F_0, where F represents the fluorescence intensity (F) of each pixel in the original fluorescence image and F_0 is defined as the F value at the beginning of the images when the cell was assumed to be in a resting state.

Statistical analysis

All values are expressed as the means±SEM. The data were analyzed by a two-tailed unpaired Student's *t*-test between 2 groups and by one-way ANOVA followed by the Bonferroni *post hoc* test for 3 or more groups. The analyses were performed using GraphPad Prism Software (GraphPad Software Inc, La Jolla, CA, USA). A P value <0.05 was considered statistically significant.

Results

Xyl-B decreases blood pressure in 2K2C hypertensive rats

First, we assessed the antihypertensive effects of Xyl-B using the 2K2C hypertensive rat model and blood pressures were measured 0, 2, 4, 6, 8, 10 and 12 weeks after the operation. As shown in Figure 1A and 1B, systolic blood pressure (SBP) and diastolic blood pressure (DBP) in the hypertension (Htn) group increased progressively at 2, 4, 6, 8, 10 and 12 weeks after the operation, which is consistent with our previous report[18, 19]. Xyl-B at 20 mg·kg^{-1}·d^{-1} was administered to the 2K2C rats intraperitoneally; saline solution containing 20%

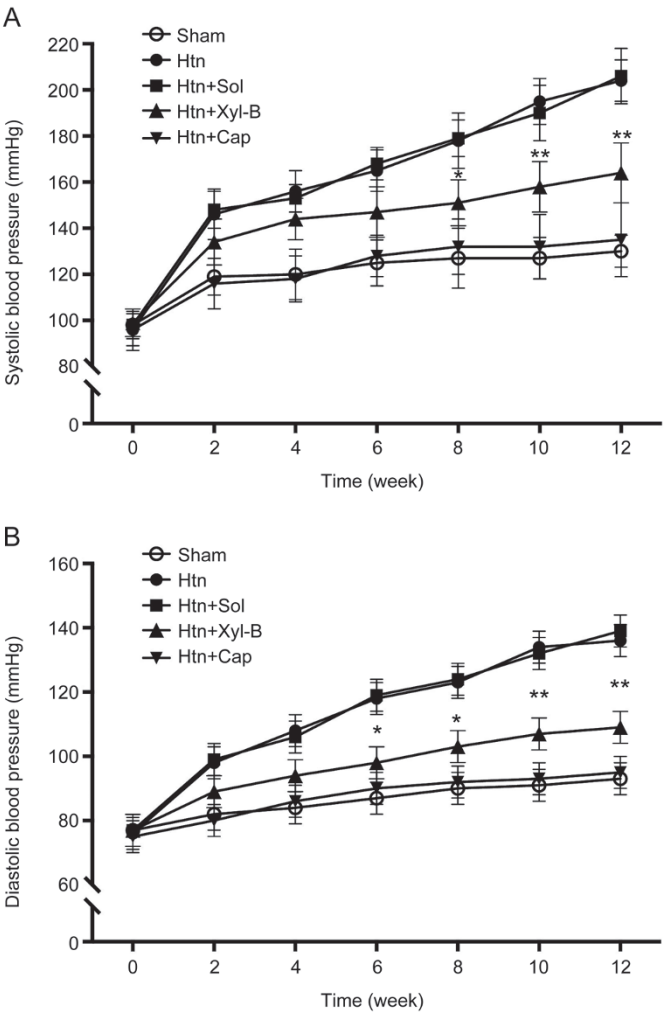

Figure 1. Effect of Xyl-B administration on blood pressure of 2-Kidney 2-Clip (2K2C) hypertensive rats. 2K2C hypertensive rat model was established and 20 mg·kg^{-1}·d^{-1} of Xyl-B was administered intraperitoneally everyday while saline solution containing 20% propylene glycol and captopril were given as solvent and positive control respectively for 12 weeks. Systolic pressure (A) and diastolic pressure (B) were measured at 0, 2, 4, 6, 8, 10 and 12 weeks after operation. Htn: 2K2C hypertension; Sol: saline solution containing 20% propylene glycol (2 mL, ip); Cap: captopril (0.1 mg·kg^{-1}·d^{-1}, ig). n=12–14. $^*P$<0.05, $^{**}P$<0.01 *vs* Htn+Sol group.

propylene glycol (2 mL, ip) and captopril (0.1 mg·kg^{-1}·d^{-1}, ig) were used as the solvent and the positive control, respectively. The solvent control (Htn+Sol) group maintained high SBP and DBP values, showing no significant differences from the age-matched Htn group (n=12−14, P>0.05). Xyl-B significantly reduced the SBP and DBP of the 2K2C hypertensive rats at 8, 10 and 12 weeks, with decreases of 59.57%/65.63%, 55.17%/64.10%, 59.15%/68.18% relative to the corresponding values in the positive control (Htn+Cap) group (SBP/DBP, n=12−14, P<0.05 *vs* the Htn group).

Xyl-B reduces aortic tension in response to phenylephrine

Xyloketals have been demonstrated to exert vaso-relaxant activities in KCl-induced vasoconstriction that were stronger in endothelium-intact rings than in denuded rings, suggesting that the vaso-relaxant effect of these compounds may be mediated by endothelium-dependent mechanisms[11]. Hypertension is closely related to vascular tone, and our previous studies have suggested that Xyl-B plays an important role in endothelial NO synthesis, which mediates vessel relaxation. To further confirm whether Xyl-B protects against hypertension by affecting vascular tension, thoracic aorta constriction in response to Phe was measured. As shown in Figure 2, Xyl-B significantly inhibited Phe-induced vasoconstriction at concentrations of 10^{-6} mol/L and 10^{-5} mol/L. Interestingly, the inhibition effect was partly reversed by *L*-NAME, the inhibitor of endothelial nitric oxide synthetases (eNOS); however, a significant difference was observed in vessel tension between the Xyl-B+*L*-NAME group and the solvent control group at 10^{-5} mol/L.

Xyl-B inhibits aortic vasoconstriction through the NO-sGC-cGMP pathway

To identify the pathway through which Xyl-B exerts its antihypertensive effect, we first detected Xyl-B's influence on acetylcholine (ACh)- and sodium nitroprusside (SNP, an exogenous NO provider)-induced vessel diastole in Phe precontracted vessels. As shown in Figure 3, Xyl-B enhanced the vaso-relaxant effect of ACh, but not SNP, indicating that Xyl-B decreases vessel tension through the endothelium-dependent NO-related pathway, which is consistent with Figure 2.

Endothelium-dependent vasodilation is regulated primarily by NO but also by prostacyclin (PGI_2) and an unidentified endothelium-derived hyperpolarizing factor. NO and PGI_2 are the predominant endogenous endothelial vaso-relaxant substances[20]. The NO-sGC-cGMP signaling cascade plays an essential role in vascular smooth muscle relaxation, and clinical studies indicate that endothelium-derived NO is involved in normal and pathological blood pressure regulation. PGI_2 is produced by the cyclooxygenases, COX-1 and COX-2, which form the prostaglandin endoperoxide PGG_2. This is converted to PGH_2, which is then transformed enzymatically into PGI_2 by prostacyclin synthase. To determine whether the PGI_2 pathway is also altered by Xyl-B, indomethacin (IMC, 10 μmol/L) was used to inhibit PGI_2 production by depressing COX-1, while methylene blue (MB, 10 μmol/L) was applied to inhibit sGC to interrupt the NO-sGC-cGMP pathway. As shown in Figure 3B, IMC did not alter the inhibitory effect of Xyl-B on vasocontractions provoked by Phe (1 μmol/L), which was significantly reversed by MB, indicating that the NO-sGC-cGMP pathway mediates the anti-hypertensive effect of Xyl-B rather than PGI_2.

In addition, we detected eNOS activity in HUVECs, which was measured as the conversion% of [^{3}H]*L*-citrulline transformed from [^{3}H]*L*-arginine. As shown in Figure 4A, Xyl-B increased endothelial eNOS bioactivity in a dose- and time-dependent manner. Moreover, Xyl-B increased the cGMP level of co-cultured VSMC-HUVECs, but not mono-cultured VSMCs (Figure 4B).

Xyl-B attenuates smooth muscle cell (SMC) Ca^{2+} signaling

As shown above, Xyl-B inhibited vascular constriction in response to KCl and Phe and this effect could not be completely eliminated by pre-incubation with *L*-NAME, suggesting that Xyl-B worked through other pathways or factors in addition to NO. As PGI_2 production in the endothelium was excluded (Figure 5), the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) of smooth muscle cells, which plays a critical role during vasoconstriction, was speculated to be involved in this process. KCl and Phe elevate the $[Ca^{2+}]_i$ in different manners: KCl induces depolarization of SMCs, resulting in voltage-dependent Ca^{2+} channel activation, while Phe increases the $[Ca^{2+}]_i$ by stimulating Ca^{2+} release from the ER and triggering store-operated Ca^{2+} entry. Our previous data indicated that some xyloketals, including xyloketal B, can attenuate the L-type Ca^{2+} channel current in cultured newborn mouse hippocampal neurons[21]. Therefore, to investigate whether Xyl-B has an effect on the $[Ca^{2+}]_i$ in SMCs, we first detected the effect of Xyl-B on KCl-induced Ca^{2+} entry in SMCs and found that Xyl-B significantly suppressed the Ca^{2+} entry stimulated by KCl (60 mmol/L), as shown in Figure 5A. Then, we determined the influence of Xyl-B on Ca^{2+} release from the ER using ryanodine (10^{-7} mol/L). As shown in Figure 5B, Xyl-B pre-incubation obviously decreased ryanodine-induced vasoconstriction.

Discussion

This study demonstrates the antihypertensive effects of Xyl-B and the main findings are as follows: (1) 20 mg·kg^{-1}·d^{-1} of Xyl-B significantly reduced SBP and DBP in the 2K2C hypertensive rat model, providing evidence for Xyl-B's *in vivo* antihypertensive effect for the first time. (2) Xyl-B decreased vascular tension in response to KCl and phenylephrine in thoracic aortas, which was partly reversed by incubation with *L*-NAME. (3) Xyl-B inhibited aortic vasocontractions through the endothelial NO-sGC-cGMP pathway rather than the PGI_2 pathway. (4) Xyl-B regulated smooth muscle Ca^{2+} signaling by affecting both voltage-dependent Ca^{2+} entry (VDCC) and store-operated Ca^{2+} entry (SOCE) in vascular smooth muscle cells (Figure 6).

The 2K2C model is a widely used renovascular hypertension model[12] with significant value for assessing whether a drug has potential antihypertensive effects. Therefore, in this study, 2K2C hypertensive rats were used to evaluate Xyl-B's

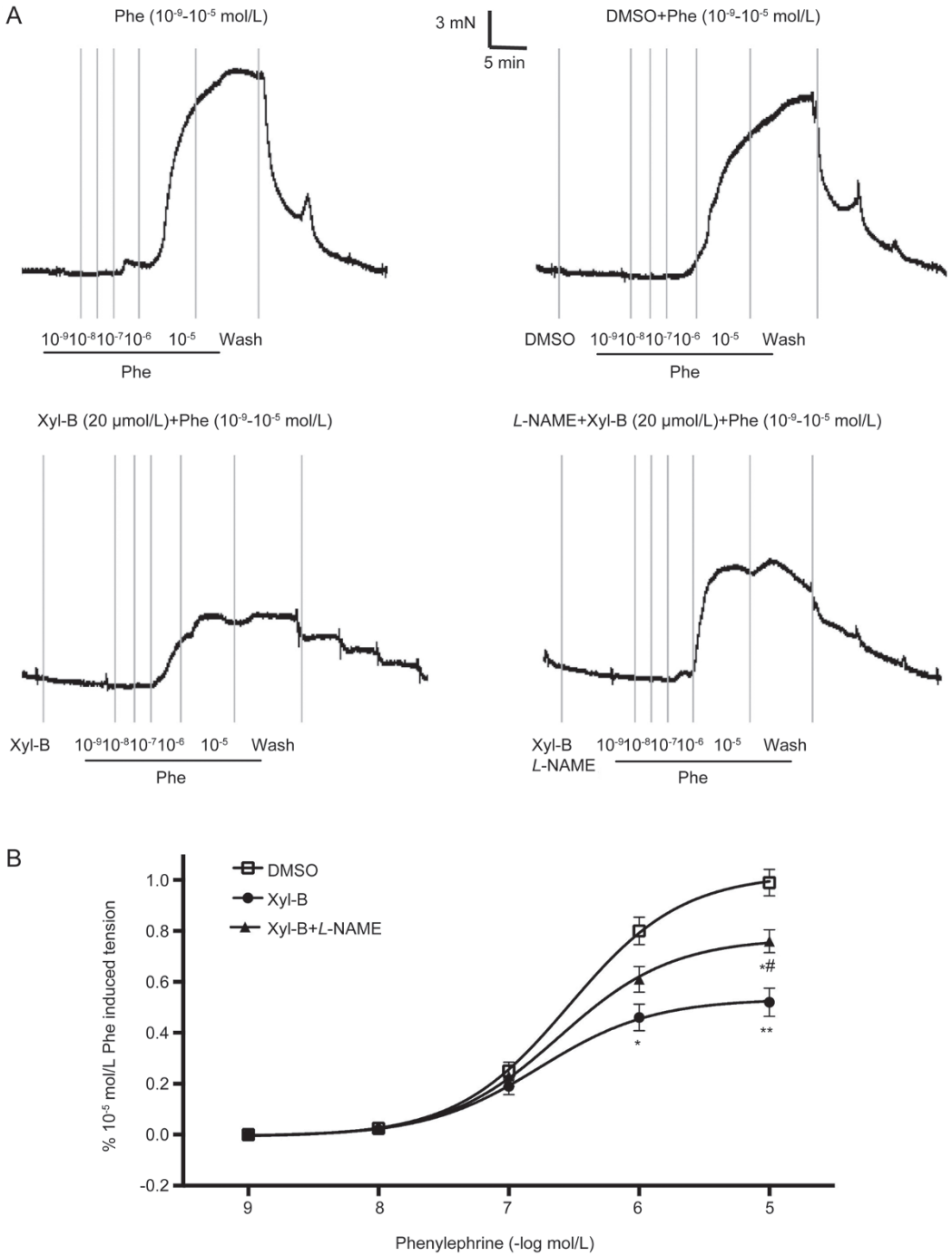

Figure 2. Effect of Xyl-B on phenylephrine (Phe) concentration-response curve for contraction in isolated rat thoracic aorta. Cumulative addition of Phe (10^{-9}–10^{-5} mol/L) induced a stepwise increase in aortic contraction. (A) Representative tension graph of Phe-induced aorta constriction with or without Xyl-B (20 μmol/L) pre-treatment (10 min) and the influence of *L*-NAME (10 μmol/L). Vascular tension caused by various concentrations (10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} mol/L) of Phe were recorded. (B) Statistical graph shows Xyl-B decreased Phe-induced tension at 10^{-6} mol/L and 10^{-5} mol/L, and this reduction was partly reversed by *L*-NAME but not totally abolished. Data are expressed as mean±SEM. $^{*}P<0.05$, $^{**}P<0.01$ *vs* DMSO group. $^{\#}P<0.05$ *vs* Xyl-B group. $n=6$.

antihypertensive effects, and the results showed that Xyl-B could significantly decrease SBP and DBP in this model. Our previous studies demonstrated that Xyl-B can directly scavenge free radicals, promote endothelial NO release[7] and protect the functions of mitochondria and the endothelium[6]. We suppose that these endothelium-dependent mechanisms may contribute to the antihypertensive effects of Xyl-B.

The endothelium regulates vascular homeostasis by modulating vasomotor tone through the production of several vasoactive mediators, including NO and prostacyclin[22, 23]. In

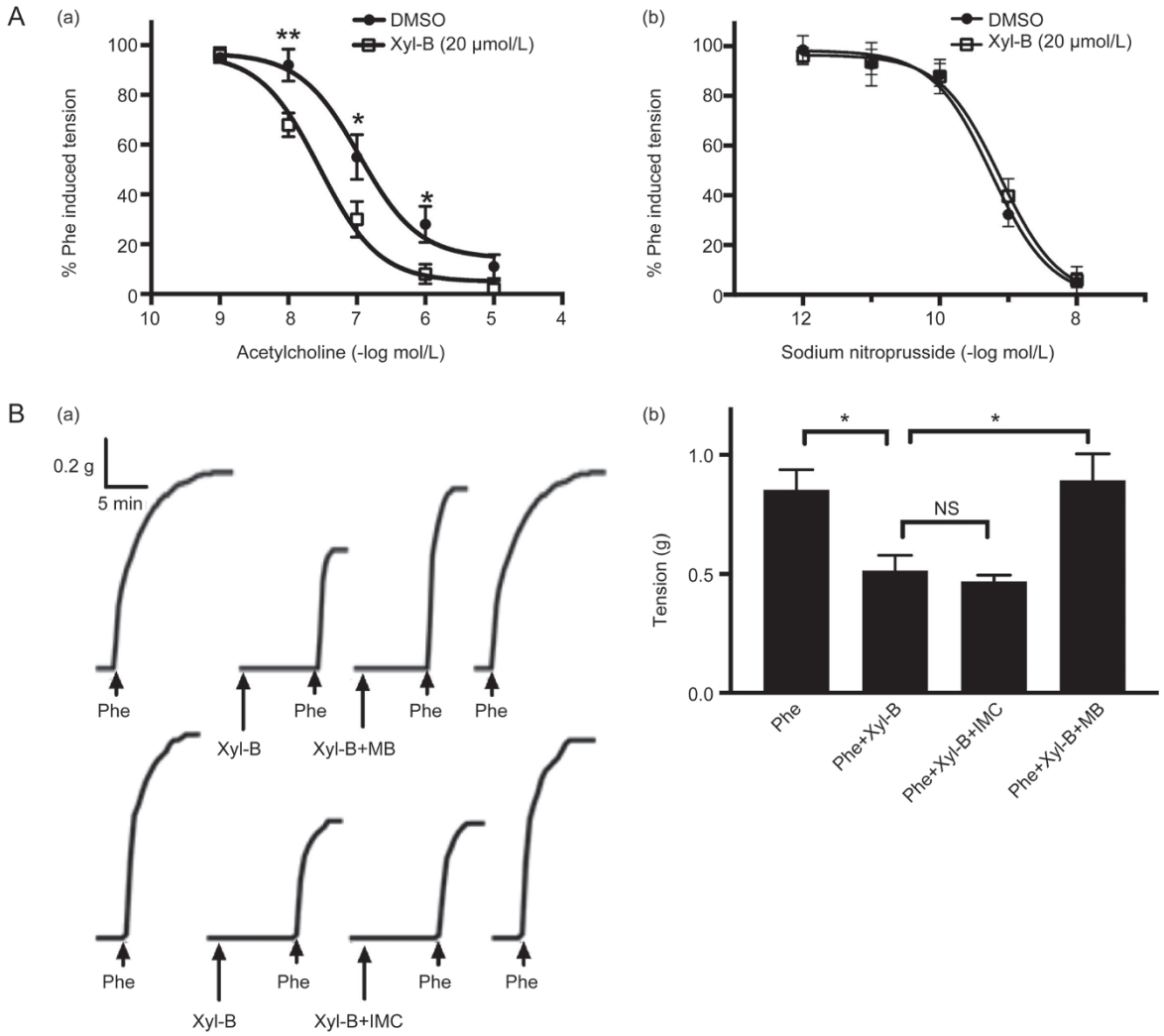

Figure 3. (A) Effect of Xyl-B on acetylcholine (ACh) or sodium nitroprusside (SNP) induced vaso-relaxation. Thoracic aorta rings were pre-contracted by Phe (1 μmol/L), then different concentration of (a) ACh (10^{-9}–10^{-5} mol/L) and (b) SNP (10^{-12}–10^{-8} mol/L) were cumulatively added. Data were expressed as mean±SEM in concentration-response curve. $^{*}P<0.05$, $^{**}P<0.01$ *vs* DMSO group. $n=6$. (B) Xyl-B regulates vascular tone through endothelial NO-sGC-cGMP pathway but not PGI_2 pathway. (a) Original data show the influence of Indomethacin (IMC 10 μmol/L) and methylene blue (MB, 10 μmol/L) on the inhibition effect of Xyl-B on Phe-induced vasoconstriction. (b) Bar graph showed that IMC did not affect the inhibition effect of Xyl-B on vasocontraction provoked by Phe (1 μmol/L), which was reversed by MB. Data were expressed as mean±SEM. $^{*}P<0.05$. $n=6$.

the development of hypertension, endothelial dysfunction precedes increases in blood pressure and predisposes patients to structural vascular changes[24, 25]. Endothelial dysfunction is characterized by reduced NO levels or bioavailability[26]. Classically, NO is generated from *L*-arginine by the binding of Ca^{2+}-calmodulin to eNOS[27]. NO released from endothelial cells stimulates soluble guanylate cyclase (sGC), leading to a sequential increase in the cGMP level in VSMCs[28], which in turn activates cGMP-dependent protein kinase and leads to increased extrusion of Ca^{2+} from the cytosol in VSMCs and inhibition of the contractile machinery[28, 29]. In the present study, both the eNOS inhibitor *L*-NAME and the sGC inhibitor MB suppressed the endothelium-dependent vasorelaxation induced by Xyl-B, but the cyclooxygenase blocker (indomethacin) could not. In addition, the cGMP-enhancing effect of Xyl-B was observed in VSMCs co-cultured with HUVECs, but not in the VSMC mono-culture. These data demonstrated that the endothelium-dependent vaso-relaxant activities of Xyl-B are attributed to the NO-sGC-cGMP signaling pathway rather than PGI_2.

eNOS activity is known to be regulated by various intracellular signals, resulting in phosphorylation of eNOS[30], and eNOS phosphorylation by the upstream PI3K/Akt pathway is required for efficient NO production[31, 32]. Xyl-B has been shown to promote NO release and eNOS bioactivity in HUVECs[8], which was confirmed in the present study through detection of the conversion ratio of [^{3}H]*L*-arginine into [^{3}H]*L*-citrulline. Our recent study showed that Xyl-B could increase eNOS activity by regulating phosphorylation of Akt and eNOS at Ser^{1177} and Thr^{495}[10]. Although many aspects of Xyl-B as a novel drug candidate for protecting the vascular endothelium have been identified and found to be effective for

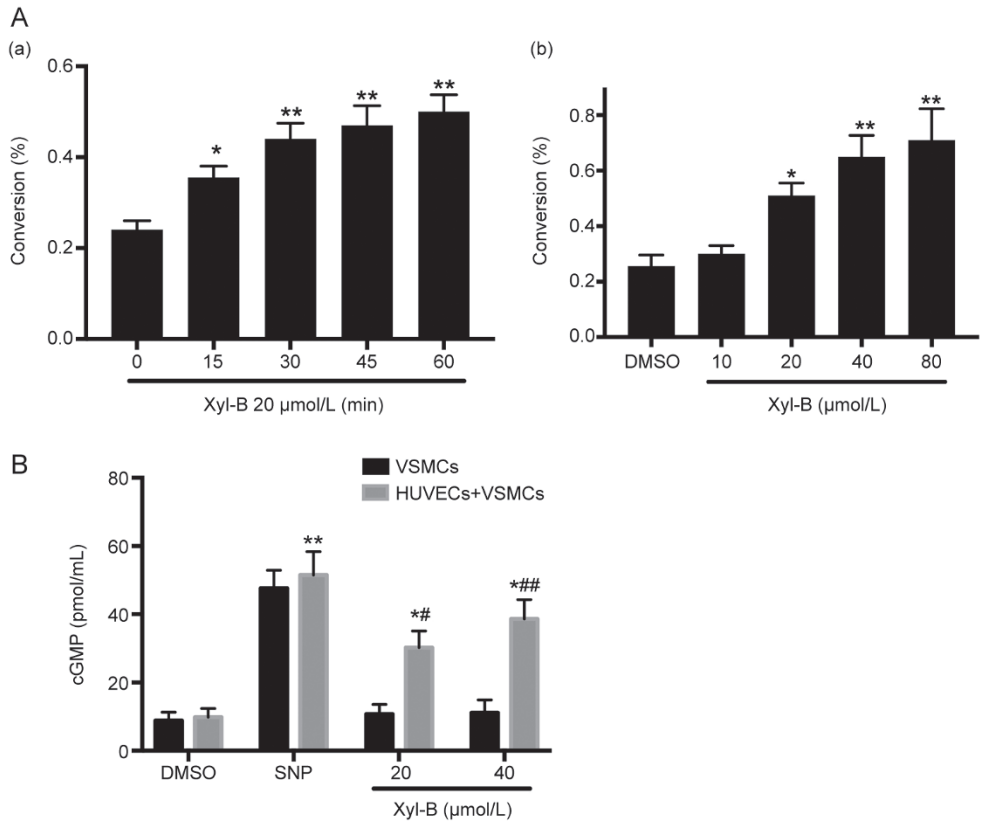

Figure 4. Effects of Xyl-B on eNOS activity in HUVECs and cGMP levels in VSMCs. (A) HUVECs were incubated in with 20 μmol/L of Xyl-B for 0, 15, 30, 45 and 60 min, or incubated for 30 min with Xyl-B of 0, 10, 20, 40, 80 μmol/L. Then eNOS activity was detected by measuring the formation of [^{3}H]*L*-citrulline from [^{3}H]*L*-arginine. eNOS activity was represented by conversion %. (B) Vascular smooth muscle cells (VSMCs) were cultured alone or co-cultured with HUVECs. Both mono-cultured VSMCs and co-cultured VSMCs-HUVECs were incubated in the absence (DMSO) or presence of Xyl-B (20 and 40 μmol/L) for 30 min. SNP (10 μmol/L) served as positive control, then cGMP level was detected. Values are mean±SEM. $^*P<0.05$, $^{**}P<0.01$ *vs* DMSO group. $^{\#}P<0.05$, $^{\#\#}P<0.01$ *vs* corresponding mono-cultured VSMCs. $n=6$.

the treatment of cardiovascular diseases such as atherosclerosis[7, 8, 10, 11], further experiments are required to confirm the role of the eNOS-dependent mechanism in the antihypertensive action of Xyl-B.

Our previous reports showed marked differences between endothelium-intact (EC_{50}=19 μmol/L) and endothelium-denuded (EC_{50}=41 μmol/L) aortic rings after Xyl-B treatment[10, 11], suggesting that Xyl-B may exert vaso-relaxant activities by endothelium-dependent mechanisms and by endothelium-independent mechanisms to a lesser extent. Interestingly, when we investigated the endothelial NO pathway using *L*-NAME, *L*-NAME could only partly reverse Xyl-B's vaso-relaxant effect on Phe-stimulated pre-contracted aortic rings, but it could not eliminate this effect as a significant difference remained between the solvent control group and the Xyl-B+*L*-NAME group, indicating that other mechanisms independent of the NO system may be involved.

In addition to endothelial NO, vascular smooth muscle dysfunction is another important cause of hypertension[33]. Abnormal regulation of intracellular Ca^{2+} in VSMCs contributes to the pathogenesis of hypertension and also contributes to vascular and cardiac remodeling secondary to hypertension. Two types of Ca^{2+} channels exist in VSMCs: VDCCs and voltage-independent Ca^{2+} channels (including receptor-operated Ca^{2+} channels and store-operated channels)[34]. Ca^{2+} channel blockers (CCBs) are clinically useful vasodilators and are widely used in the treatment of hypertension and related cardiovascular diseases[35]. CCBs lower blood pressure through a well-characterized mechanism of blocking L-type voltage-dependent Ca^{2+} channels in vascular cells to restore Ca^{2+} homeostasis[36]. Our previous data indicated that some xyloketals, including Xyl-B, can attenuate L-type Ca^{2+} currents in primary cultured hippocampal cells from newborn rats using patch-clamp experiments[21], and the current study indicates that Xyl-B dilated Phe- or KCl-induced precontracted thoracic aortic rings. Phe-induced contractions in aortic rings are mainly mediated by Ca^{2+} entry via voltage-independent Ca^{2+} channels, such as SOCE, while KCl mainly activated the L-type Ca^{2+}channels (VDCCs). In the present study, we detected the effect of Xyl-B on KCl-induced Ca^{2+} entry in SMCs and on ryanodine-induced aortic contraction. The results not only further confirmed the L-type Ca^{2+} channel-blocking effect of Xyl-B in smooth muscle cells but also indicated that Xyl-B may affect SOCE, warranting further investigation of the exact

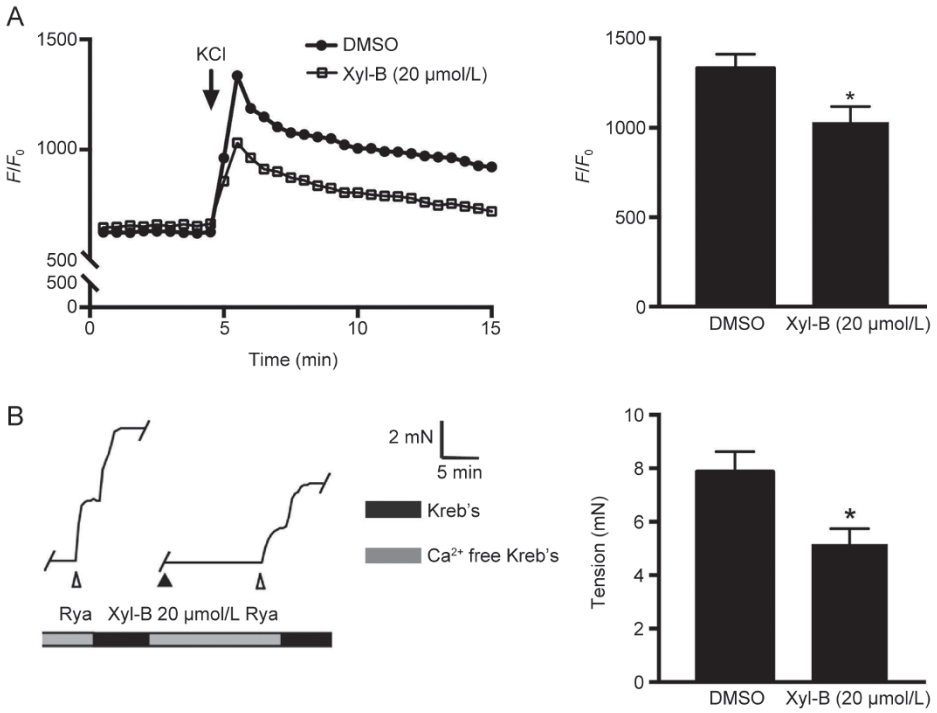

Figure 5. Xyl-B attenuates smooth muscle cells (SMCs) calcium signaling. (A) Xyl-B inhibits KCl-induced calcium entry in VSMCs. VSMCs were pre-treated with Xyl-B (20 μmol/L) or DMSO for 10 min before stimulation with isotonic 60 mmol/L KCl. Realtime $[Ca^{2+}]_i$ was determined using Fluo-3/AM staining and calculated every 30 s (left panel). Peak $[Ca^{2+}]_i$ of both Xyl-B and DMSO treated cells were shown in bar graph. $^*P<0.01$ *vs* DMSO group. Data were obtained from 6 independent experiments. (B) Aortic rings were placed in calcium-free solution with or without Xyl-B (20 μmol/L), then ryanodine (10^{-7} mol/L) and calcium containing solution were added in order, aortic tension was recorded during this process. Bar graph shows that Xyl-B decreased ryanodine-induced vasoconstriction. Data are expressed as mean±SEM. Results were obtained from 6 independent experiments.

Ca^{2+} signaling mechanism. Additionally, new xyloketals such as xyloketal F and xyloketal A have shown strong inhibitory effects on VDCC in primary cultured rat hippocampal cells (50.33% and 21.47%, respectively)[21], but they had no effects on KCl-induced contractions of rat thoracic aortic rings and their effects could be completely inhibited by nifedipine (data not shown). Among the screened xyloketal compounds, only Xyl-B showed significant antihypertensive effects with minor inhibitory effects on VDCCs in newborn rat hippocampal cells (12.05%). These data suggest that the influence of Xyl-B on Ca^{2+} signaling in different tissues and cell types should be interpreted with caution. Based on the current results, whether regulation of Ca^{2+} signaling by Xyl-B is the predominant mechanism of its effects in hypertension remains unknown.

Furthermore, during the development of renovascular hypertension, major structural alterations in the heart include left ventricular (LV) hypertrophy and fibrosis[13]. The cardiovascular remodeling associated with hypertension involves oxidative stress and enhanced matrix metalloproteinase (MMP) expression/activity[13]. CCBs shows beneficial effects on left ventricular (LV) hypertrophy and fibrosis via antioxidative mechanisms[37]. The data observed in our current study show that Xyl-B can reverse cardiac remodeling and fibrosis in 2K2C rats (unpublished data). Therefore, Xyl-B not only attenuates the high BP level in hypertension but may also ameliorate the complications of hypertension.

In summary, this is the first study to demonstrate that Xyl-B can reduce blood pressure in 2K2C renovascular hypertensive rats. Our results suggest that Xyl-B induces relaxation in rat aortic rings through an endothelium-dependent pathway mediated by the NO-sGC-/cGMP pathway and through an endothelium-independent pathway involving VDCC blockade mediated by Ca^{2+} entry in VSMCs. One study limitation is that neither the NO-sGC-/cGMP pathway nor the Ca^{2+} signaling mechanisms underlying Xyl-B's antihypertensive effects were verified in VSMCs. Therefore, the mechanisms of the antihypertensive action of Xyl-B should be interpreted with caution, and further *in vivo* studies are warranted to determine the precise mechanism. Taken together, the findings of this study support Xyl-B as a potential new drug candidate for the treatment of hypertension.

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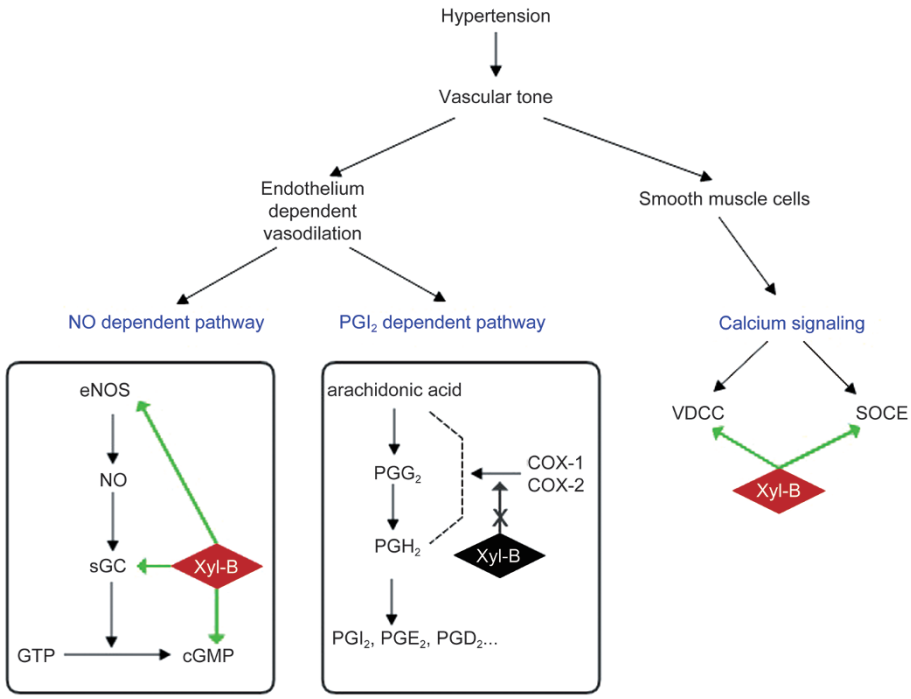

Figure 6. Summary of the vasoconstriction signal transduction pathways showing points of intervention by Xyl-B. Vascular tone is the result of joint regulation of endothelium and smooth muscle and endothelium-dependent vasodilation is regulated primarily by NO-sGC-cGMP signaling cascade and prostacyclin (PGI_2) in vascular smooth muscle cells. PGI_2 is produced by the cyclooxygenases, COX-1 and COX-2, which form the prostaglandin endoperoxide PGG_2, PGG_2 is converted to PGH_2 which is then transformed enzymatically into PGI_2 by prostacyclin synthase. Our present study demonstrated that Xyl-B affected eNOS activity and cGMP level, and sGC was also involved but PGI_2 pathway was not. Additionally, as Xyl-B's inhibitory effect on vasoconstriction could not be completely explained by endothelial pathway, we furtherly found that Xyl-B regulated vascular tone by affecting both voltage dependent calcium entry (VDCC) and store-operated calcium entry (SOCE) in vascular smooth muscle cells.

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Author contribution

Yao-min DU and Yong-cheng LIN conceived and designed the experiments; Li-yan ZHAO, Jie LI, Wei-feng MENG, Guo-hao WANG and Wen-liang CHEN performed the experiments; Li-yan ZHAO, Hong-shuo SUN and Xiong-qing HUANG analyzed the data; Xiao-fei LV and Ji-yan PANG contributed reagents and materials; Li-yan ZHAO and Guan-lei WANG wrote the paper.

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