α-Isocubebene modulates vascular tone by inhibiting myosin light chain phosphorylation in murine thoracic aorta

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ABSTRACT α-Isocubebene (ICB) is a dibenzocyclooctadiene lignin contained in Schisandra chinensis (SC), a well-known medicinal herb that ameliorates cardiovascular symptoms, but the mechanism responsible for this activity has not been determined. To determine the role played by ICB on the regulation of vascular tone, we investigated the inhibitory effects of ICB on vascular contractile responses by adrenergic α-receptor agonists. In addition, we investigated the role on myosin light chain (MLC) phosphorylation and cytosolic calcium concentration in vascular smooth muscle cells (VSMC). In aortic rings isolated from C57BL/6J mice, ICB significantly attenuated the contraction induced by phenylephrine (PE) and norepinephrine (NE), whereas ICB had no effects on KCl (60 mM)-induced contraction. In vasculatures pre-contracted with PE, ICB caused marked relaxation of aortic rings with or without endothelium, suggesting a direct effect on VSMC. In cultured rat VSMC, PE or NE increased MLC phosphorylation and increased cytosolic calcium levels. Both of these effects were significantly suppressed by ICB. In conclusion, our results showed that ICB regulated vascular tone by inhibiting MLC phosphorylation and calcium flux into VSMC, and suggest that ICB has anti-hypertensive properties and therapeutic potential for cardiovascular disorders related to vascular hypertension.

INTRODUCTION
Hypertension is closely associated with the development and presence of cardiovascular diseases, and may result in heart attack, stroke, kidney failure, and disability [1]. Among various risk factors for cardiovascular diseases, vascular tone is an important determinant of peripheral resistance and blood pressure, and essential hypertension is characterized by an abnormal increase in peripheral vascular resistance [2,3]. Furthermore, prolonged vasoconstriction of a resistant artery is the main driver of vascular remodeling in hypertension [4]. Thus, compounds with resistant artery dilating effects are viewed as being potentially useful for treating hypertension. However, although many different anti-hypertensive drugs are used clinically, new types of medications are required to treat hypertension. Accordingly, much research effort has been directed over recent years to identify novel anti-hypertensive compounds that regulate vascular tone.

It is well known that several mechanisms are involved in the regulation of vascular tone, among which increase in cytoplasmic calcium and myosin light chain (MLC) phosphorylation are important mechanistic features [5]. MLC phosphorylation is determined by the activities of Ca²⁺-dependent myosin light chain kinase [6,7]. Several authors have shown that MLC phosphatase also contributes to MLC phosphorylation [8-10]. Furthermore, in smooth muscle, contractile agonists such as adrenergic alpha-1 agonists increase force generation even when the intracellular concentration of Ca²⁺ ions is constant, in which MLC phosphatase plays an

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important role [11,12].

*Schisandra chinensis* has a long history of use as a medicinal herb, and thus, is a component in oriental medicines [13,14]. Several researchers have suggested that it may have beneficial effects in patients with cardiovascular diseases, as its aqueous extract induced vasodilation in rat thoracic aorta [15,16]. In our previous studies, gomisin A and gomisin J isolated from *Schisandra chinensis* relaxed vascular smooth muscle, which suggested potential therapeutic use in hypertensive patients [17]. In addition, α-iso-cubebeene (ICB), a dibenzocyclooctadiene lignin found in *Schisandra chinensis* has been suggested as a potential therapeutic intervention to ameliorate the symptoms of cardiovascular disease via its antioxidant property.

Although ICB is known to ameliorate cardiovascular symptoms, but little is known of its effect on the vascular tone of resistant arteries, which is main determinant of vascular hypertension. To determine the effects of ICB on the modulation of vascular tone, the role of ICB on vascular contractile responses by adrenergic alpha-receptor agonists, that is phenylephrine and norepinephrine. Also, the vasodilatory effects of ICB in aortic rings pre-contracted with phenylephrine was determined. In a mechanistic study, we evaluated the role played by ICB on MLC phosphorylation and changes in cytosolic calcium concentration in vascular smooth muscle cells.

**METHODS**

**Purification of α-iso-cubebeene**

α-iso-cubebeene (ICB) was purified from the dried fruits of *Schisandra chinensis* (SC) as described previously [18]. Briefly, SC (2.5 kg) fruit was dried, ground to a fine powder, and sequentially extracted at room temperature with n-hexane, chloroform (CHCl3), and methanol (MeOH). The hexane extract (308 g) was evaporated in vacuo and chromatographed on a 40 μm silica gel (J.T. Baker, Phillipsburg, NJ, USA) column (100×10 cm) by step gradient elution (0%, 5%, and 20% ethyl acetate in hexane and 5% methanol MeOH in CHCl3 to obtain 38 fractions). Fraction 1 (KH1PA, 3,689 mg) was separated on a silica gel column (100×3.0 cm) using 15% acetone in dichloromethane (CH2Cl2) to obtain nine fractions, and the second fraction (KH1PAIB, 999 mg) was separated on a silica gel column (100×3.0 cm) using 15% acetone in CH2Cl2 to yield ICB (316 mg). The purity of ICB was determined by HPLC (high-performance liquid chromatography) using a Phenomenex Luna C18 column (150×4.6 mm internal diameter; 5 μm particle size) and an acetonitrile-water-alcohol gradient at a flow rate of 1.0 ml/min and found to be >99%.

**Ethics statement and animals**

All animal procedures conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, 2011 revision), and all experimental protocols were approved by the Pusan National University Institutional Animal Care and Use Committee. Wild-type control mice (C57BL/6J) were purchased from Jackson Laboratories. All animals were housed in an air-conditioned room at 22–25°C and kept under a 12-h light/dark cycle. Food and water were provided *ad libitum*.

**Chemicals and antibodies**

Phenylephrine hydrochloride (PE), acetylcholine chloride, and EGTA were purchased from Sigma-Aldrich (St. Louis, MO). MTT working solution was from EZ-Cytox (Daeil Laboratories, Seoul), and antibodies for MLC (sc-12896), p-MLC (sc-19848, sc-293109), and anti-β-actin (sc-47778) were from Santa Cruz Biotechnology. Fluo-3/AM was purchased from Thermo Fisher Scientific (Rockford, IL). All other chemicals were of reagent grade. The solid form of ICB was dissolved in 100% DMSO at a concentration of 100 mg/ml to produce a stock solutions and subsequently added to media as required.

**Preparation and tension recording of mouse aortic rings**

C57BL/6J mice (20–25 g) were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and thoracic aortas were rapidly removed. Aortic rings (2–3 mm thick) were suspended in 10-ml organ chambers filled with Krebs’ solution (37°C) at a resting tension of 2 g. After an equilibration period of 90 min, aortic rings were constricted with KCl (60 mM) solution to stimulate the tissue. They were then washed with Krebs’ solution to restore basic tension. After aortic rings had been allowed to equilibrate, sustained and stable contraction was induced by treating them with phenylephrine or norepinephrine. Changes in isometric tension were recorded using a force-displacement transducer (Grass FT 0.3, Quincy, MA, USA) connected to a Power Lab system 400 (ML 118, PowerLab, AD Instruments, Medford, MA, USA).

In this study, aortic rings were incubated with various concentrations of ICB for 10 min, and then phenylephrine (PE) or norepinephrine (NE) was added to induce transient vasoconstriction. The vasodilatory potency of ICB was studied using cumulative additions of ICB at concentrations of 1–10 μg/ml. Involvement of endothelium in ICB-induced relaxation was examined by comparing the relaxation magnitudes of endothelium-denuded and endothelium-intact specimens. Relaxations are expressed as percentage of relaxation of PE-induced tone.

**Vascular smooth muscle cell culture**

Sprague-Dawley rats (Charles River Breeding Laboratories, Kingston, NY, USA) were sacrificed by CO2 inhalation, and then
primary VSMCs obtained from thoracic aorta were cultured. Briefly, excised aortas were cut into ~1 mm² segments, and placed as explants in a cell culture dish containing DMEM (Gibco BRL, Grand Island, NY) with 10% FBS (Gibco BRL). Cells were maintained in DMEM containing 10% FBS and antibiotic-antimycotic (Gibco BRL) at 37°C. An MTT assay was used to determine the viability of VSMCs. Briefly, cells (a total of 1×10⁵ cells) were treated with MTT working solution (EZ-Cytox, Daeil Laboratories, Seoul, Republic of Korea), and incubated at 37°C for 1 h. OD values of solution were obtained at a wavelength of 450 nm by ELISA.

**Measurement of cytosolic calcium levels**

Cytosolic calcium levels were measured as described elsewhere [19]. Briefly, VSMC was loaded with Fluo-3/AM in normal Tyrode’s solution (pH 7.4) containing (in mM): 140 NaCl, 5.4 KCl, 1 MgCl₂, 2 CaCl₂, 5.5 glucose, and 5 HEPES. To deplete intracellular Ca²⁺ stores, cells were treated with 4 μM thapsigargin in Ca²⁺-free Tyrode’s solution containing 0.2 mM ethylene glycol tetraacetic acid (EGTA). Real-time fluorescent images were captured every 10 s and analyzed using MetaFluor imaging software (Molecular Devices, Sunnyvale, CA).

**Western blot assay**

VSMC lysates were prepared in ice-cold lysis buffer, and equal amounts of proteins were separated on 8-10% polyacrylamide gel under reducing conditions, and then transferred to nitrocellulose membranes (Amersham-Pharmacia Biotech, Piscataway, NJ). Membranes were blocked with 5% skim milk in TBST, incubated overnight with primary antibodies to total MLC and phosphorylated MLC (at Ser 19, Thr18, and Thr18/Ser19) in 5% skim milk. Blots were then washed with TBST, incubated with HRP-conjugated secondary antibody for 2 h, and developed using ECL Western blot detection reagents (Amershams). Membranes were re-blotted with anti-β-actin antibody (Santa Cruz Biotechnology), which was used as the internal control.

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**Fig. 1. Effects of α-isocubebene (ICB) on phenylephrine (PE)- and norepinephrine (NE)-induced contractions in mouse thoracic aorta.** Under pretreatment with vehicle or ICB (1-10 μg/ml), PE (10⁻⁷-3×10⁻⁶ M) (A) or NE (10⁻⁸-3×10⁻⁷ M) (B) was added in a step-wise manner to elicit vasorelaxation. Tracings are representative of 4-5 independent experiments. Bottom: percentage vasorelaxation represents contraction relative to that elicited by 60 mM KCl. Results were presented as the means±SEM of 4-5 independent experiments. *p<0.05; **p<0.01 vs. corresponding value in Vehicle.
Statistical analysis

Results are presented as means±S.E.M. One-way analysis of variance followed by Tukey’s multiple comparison test was used to determine the significances of intergroup differences. The analysis was conducted using Prism version 3.03 software (GraphPad Software, San Diego, CA, USA) and p-values <0.05 considered significant.

RESULTS

ICB attenuated the contraction induced by α-agonists

The contractility of aortic preparations with intact endothelium was increased dose-dependently by stimulation with phenylephrine (PE, $10^{-7}$-3×$10^{-6}$ M) or norepinephrine (NE, $10^{-8}$-3×$10^{-7}$ M). As shown in Fig. 1, pre-treatment with ICB (1-10 μg/ml) significantly and dose-dependently attenuated contractile responses to PE or NE. However, ICB alone did not affect the basal tension (2.0 g) of aortic rings.

ICB induced vasodilation in endothelium-intact or –denuded aortic preparations

To determine the effects of ICB on endothelium-dependent or nitric oxide-dependent vasodilation, we investigated the role of ICB on vascular relaxation induced by acetylcholine or sodium nitroprusside in aortic preparations with intact endothelium. As shown in Fig. 2, both acetylcholine and sodium nitroprusside markedly relaxed aortic rings pre-contraction with PE, and this relaxation was not affected by pretreating ICB. These results suggested that the inhibitory effects of ICB on vasocontraction induced by PE might not due to an endothelium-dependent mechanism.

To further investigate the involvement of vascular endothelium on the vascular action of ICB, the vasorelaxing effects of ICB were examined in endothelium-intact or -denuded aortic preparations pre-contraction by PE. As shown in Fig. 3, endothelium did not exhibit a significant influence on the vasodilatory role of ICB, suggesting ICB acted directly on vascular smooth muscle with high potency. Therefore, we subsequently investigated the mechanism underlying the vasodilatory effects of ICB using vascular smooth muscle cells (VSMC).

ICB inhibited MLC phosphorylation in VSMC stimulated with α-agonists

To determine whether ICB attenuated vascular contraction by acting on thick filament, we investigated the role of ICB on MLC phosphorylation at Ser19 and Thr18 sites of MLC, based on the facts that Ser19 and Thr18 are known as MLCK-preferred phos-
**α-isocubebene modulates vascular tone**

Fig. 3. Effects of α-isocubebene (ICB) on vascular tension in mouse thoracic aorta pre-contracted with phenylephrine (PE, 1 μM). In mice thoracic aorta with (ED+) or without (ED-) endothelium, ICB (3-30 μg/ml) was added cumulatively to elicit vasodilation. Tracings are representative of 6 independent experiments (A). Percentage vasodilation represents relative value to vasodilation induced by SNP (0.1 μM). Data was presented as the means±SEM of 6 independent experiments (B).

Fig. 4. Inhibitory effect of α-isocubebene (ICB) on MLC phosphorylation in phenylephrine (PE) (A)- or norepinephrine (NE) (B)-treated VSMC. After pretreating cells with vehicle or ICB (10 μg/ml), PE (1 μM) or NE (1 μM) was added to elicit MLC phosphorylation. Blots are representative of 5-6 independent experiments. Densitometric results of Western blot are shown in the bottom panel. The p-MLC to t-MLC ratios were presented as the means±SEM of 5-6 independent experiments. **p<0.01 vs. corresponding value in control. ***p<0.01 vs. corresponding value in vehicle.
phorylation sites in MLC [20]. As shown in Fig. 4, although total MLC expression was unaffected by PE or NE, the phosphorylation of MLC at Ser19, Thr18, and Thr18 plus Ser19 was markedly increased in VSMC stimulated with PE or NE. However, these increases were markedly suppressed in cells treated with ICB at concentration of 10 μg/ml. On the other hand, ICB did not change basal MLC phosphorylation levels in VSMC.

**ICB attenuated cytosolic Ca\(^{2+}\) increases in VSMC stimulated with \(\alpha\)-agonists**

To examine whether ICB attenuates transient increases in cytosolic Ca\(^{2+}\) induced by PE or NE, rat VSMC were used in this study. When PE (1 μM) or NE (0.1 μM) was added to VSMC, the fluorescence emitted from cells was markedly and rapidly increased, indicating an increase in intracellular calcium concentration. However, these increases were significantly attenuated by pretreating VSMC with ICB at concentration of 10 μg/ml (Fig. 5).

**DISCUSSION**

Our findings show that \(\alpha\)-iso-cubebene (ICB) attenuated vascular contraction by inhibiting the Ca\(^{2+}\)-myosin light chain (MLC) signaling pathway. ICB attenuated cytosolic calcium increases and MLC phosphorylation in VSMC stimulated with adrenergic \(\alpha\)-receptor agonists. In isolated aortic ring preparations, ICB significantly attenuated agonist-induced contraction, and also induced marked relaxation in pre-contracted aortic rings with or without endothelium. These observations suggested that ICB plays a pivotal role in the modulation of vascular tone by directly acting on VSMC.

*Schisandra chinensis* (SC) has long been used as a tonic, sedative, astringent, anti-aging agent, and as a treatment for cardiovascular symptoms in Korea, China and Japan [21,22]. In our previous study, hexane extracts of SC were found to cause vasorelaxation in endothelium (ED)-intact vasculature and in ED-denuded rat thoracic aortas [16]. Furthermore, the relaxant effect of these extracts on ED-intact vasculature was more prominent

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**Fig. 5. Effects of \(\alpha\)-isocubebene (ICB) on cytosolic Ca\(^{2+}\) levels in vascular smooth muscle cells.** After pretreating cells with vehicle or ICB (10 μg/ml), phenylephrine (PE, 1 μM) (A) or norepinephrine (NE, 0.1 μM) (B) was added to induce calcium influx. Tracings are representative of 4-5 independent experiments (A). Bottom panel: Relative fluorescence ratio was presented as the means±SEM of 4-5 independent experiments. **p<0.01 vs. vehicle.
than that on ED-denuded aorta [16], which suggested the importance of vascular endothelium in vascular relaxation evoked by SC extracts.

The major bioactive components of SC fruits are lignans such as ICB, schizandrin and gomisins, such as, gomisin J, N and A [23,24]. Several researchers have reported the beneficial bioactivities of ICB, such as its anti-inflammatory, anti-septic and immunomodulatory activities [25,26]. Also, ICB has neuroprotective [25,27,28] and anti-inflammatory effects, the latter of which was attributed to the inhibition of endothelial expression of adhesion molecules [26]. In our previous study, ICB has been suggested to be therapeutically useful in proliferative vascular diseases by inhibiting VSMC proliferation [29]. Although ICB has been suggested to have beneficial effects on various cardiovascular symptoms, the mechanisms underlying its potential protective effects have not yet been fully investigated.

Vascular tone is an important determinant of peripheral resistance and blood pressure, and essential hypertension is characterized by abnormal increases in peripheral vascular resistance [30,31]. Thus, compounds with vasodilatory effects would be expected to be useful for the treatment of vasospasm, hypertension, and the other conditions associated with hypercontractility of various vasculatures. In the present study, we investigated the effects of ICB on vascular contraction induced by adrenergic $\alpha$-receptor agonists to determine the role played by ICB on the modulation of vascular tone. In isolated preparations of mouse thoracic aorta, ICB dose-dependently attenuated the vasoconstriction induced by PE or NE. Vascular contraction in response to PE is known to involve $Ca^{2+}$ release from intracellular stores, and $Ca^{2+}$ movement into cytosol due to store-operated $Ca^{2+}$ influx and/or receptor operated $Ca^{2+}$ channels [32,33]. However, it was reported in a recent study in which PE failed to elicit significant intracellular $Ca^{2+}$ release [34], thus it would appear that PE- or NE-induced vascular contraction occurs as a result of $Ca^{2+}$ influx through receptor operated channels and voltage-dependent channels [34,35].

In the present study, ICB attenuated increases in cytosolic calcium levels in VSMC stimulated with adrenergic $\alpha$-agonists. Moreover, the contraction induced by PE and NE in isolated aortic rings of mice was markedly attenuated by ICB, whereas ICB has no effects on high K$^+$ (60 mM)-induced vasoconstriction. Because high K$^+$-induced vasoconstriction is mediated by membrane depolarization of VSMC through an opening of voltage-gated $Ca^{2+}$ channels [36], these observations suggested that the inhibitory effects of ICB on $\alpha$-agonist-induced vasoconstriction might be due, at least in part, to $Ca^{2+}$ influx through receptor operated channels, but not to voltage-dependent channels.

Several intracellular mechanisms are involved in the modulation of vascular tone, and increased cytoplasmic $Ca^{2+}$ and phosphorylation of MLC might be the most crucial factors [5]. MLC phosphorylation is determined by the relative activities of $Ca^{2+}$-dependent myosin light chain kinase, and is directly linked with smooth muscle contraction [6]. Furthermore, several studies have shown that MLC phosphatase also significantly contributes to MLC phosphorylation [8,10,37]. In the case of vascular smooth muscle, contractile agonists such as adrenergic $\alpha$-agonists increase force generation even when $Ca^{2+}$ concentration remain constant, in which MLC phosphatase plays an important role [11,37]. In the present study, we evaluated the role of ICB on MLC phosphorylation in VSMC as a mechanistic study. Among various sites in MLC including Ser1, Ser8, Ser19, Thr9, and Thr18 are known as MLCK-preferred phosphorylation sites [20]. Thus, we identified the effects of ICB on MLC phosphorylation at Ser19 and Thr18 sites. In the VSMC, adrenergic $\alpha$-agonists including PE and NE increase MLC phosphorylation in association with an increase in cytosolic calcium. Both MLC phosphorylation and changes in cytosolic calcium induced by PE or NE were significantly attenuated by ICB. Thus, it was suggested that ICB regulated vascular tone by inhibiting MLC phosphorylation and calcium flux. However, further studies are required to clarify the detail signaling mechanism, in which ICB regulates MLC phosphorylation.

Arterial hypertension is one of the most common cardiovascular disorders and is characterized by altered vascular tone and increased vascular contractility resulting in high blood pressure [38-40]. In our present study, the contractile agonists such as PE and NE provoked $Ca^{2+}$ mobilization and increased cytosolic $Ca^{2+}$, which in turn increased MLC phosphorylation, thus increasing vascular contraction. Furthermore, the vascular contraction induced by adrenergic $\alpha$-agonists was attenuated by ICB via the inhibition of MLC phosphorylation and cytosolic $Ca^{2+}$ fluxes into VSMC. These results encourage us to suggest that ICB is considered as a potential therapeutic intervention for the treatment of cardiovascular disorders, such as cerebral and coronary vasospasm, hypertension, and the other conditions associated with hypercontractility of the various vasculatures.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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