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Original Article

Magnesium lithospermate B dilates mesenteric arteries by activating BK_{Ca} currents and contracts arteries by inhibiting K_V currents

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Aim: To examine the involvement of K^+ channels and endothelium in the vascular effects of magnesium lithospermate B (MLB), a hydrophilic active component of *Salviae miltiorrhiza* Radix.
Methods: Isolated rat mesenteric artery rings were employed to investigate the effects of MLB on KCl- or norepinephrine-induced contractions. Conventional whole-cell patch-clamp technique was used to study the effects of MLB on K^+ currents in single isolated mesenteric artery myocytes.
Results: MLB produced a concentration-dependent relaxation in mesenteric artery rings precontracted by norepinephrine (1 μmol/L) with an EC_{50} of 111.3 μmol/L. MLB-induced relaxation was reduced in denuded artery rings with an EC_{50} of 224.4 μmol/L. MLB caused contractions in KCl-precontracted artery rings in the presence of *N*-nitro-*L*-arginine methyl ester (*L*-NAME) with a maximal value of 130.3%. The vasodilatory effect of MLB was inhibited by tetraethylammonium (TEA) in both intact and denuded artery rings. In single smooth muscle cells, MLB activated BK_{Ca} currents (EC_{50} 156.3 μmol/L) but inhibited K_V currents (IC_{50} 26.1 μmol/L) in a voltage- and concentration-dependent manner.
Conclusion: MLB dilated arteries by activating BK_{Ca} channels in smooth muscle cells and increasing NO release from endothelium, but it also contracted arteries precontracted with KCl in the presence of *L*-NAME.

Keywords: magnesium lithospermate B; big-conductance Ca^{2+}-activated K^+ channels; mesenteric artery; endothelium

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Introduction

Salviae miltiorrhiza Radix (Danshen) is a traditional Chinese herbal medicine used mainly to treat cardiovascular diseases. In recent decades, attention has focused on its water-soluble ingredients, the main efficacious components in decoctions of Danshen[1]. Among these, magnesium lithospermate B (MLB, Mw: 741) is the most abundant active component[2] (Figure 1).

MLB exhibits free radical–scavenging[3–5], hypotensive[6], renal function-improving[7], and angiotensin-converting enzyme-inhibiting[8] activities. Previous findings on the pharmacologic mechanisms of MLB are inconsistent. It was reported that MLB induced endothelium-dependent vasodilatation *in vitro*[9] and decreased blood pressure in rats *in vivo*[6]. However, others reported that the vasodilator effect of Danshen crude extract was not affected by *L*-NAME or mechanical removal of the endothelium in rat isolated femoral artery rings[10]. In guinea pig single ventricular myocytes, MLB was reported to inhibit voltage-dependent *L*-type Ca^{2+} channels, with no significant effects on other ion channels[11], whereas another study showed that MLB activated iberiotoxin-sensitive BK_{Ca} channels in porcine coronary artery smooth muscle cells[12].

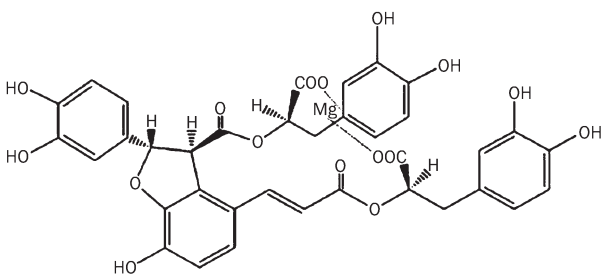

Figure 1. Structure of magnesium lithospermate B.

In the present study, we investigated the effects of MLB on vascular functions *in vitro* and the involvement of K^+ channels

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and endothelium in the vascular response to MLB.

Materials and methods

Reagents and solutions

Magnesium lithospermate B (MLB, brown powder with 99.7% purity) was obtained from the Research Center of Traditional Chinese Medicine Modernization, Shanghai Institute of Materia Medica. Norepinephrine, ACh, *L*-NAME, papain, dithiothreitol, bovine serum albumin (BSA), EGTA, taurine, sodium deoxycholate, 4-aminopyridine (4-AP), iberiotoxin, and tetraethylammonium chloride were from Sigma-Aldrich China Inc. MLB was dissolved in the appropriate external solutions to produce the desired concentrations just before experiments. Krebs solution for perfusion of artery rings contained the following (in mmol/L): 118 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.18 KH_2PO_4, 25 $NaHCO_3$, 10 glucose. The composition of the dissociation medium for enzymatic cell isolation and the external solution for patch-clamp studies was as follows (in mmol/L): 130 NaCl, 4.2 KCl, 0.5 $MgCl_2$, 10 $NaHCO_3$, 1.8 (or 0.16) $CaCl_2$, 1.2 KH_2PO_4, 10 HEPES, and 5.5 glucose (pH 7.4 with NaOH), with an osmolality of 298±2 mOsm/L. The pipette solution contained the following (in mmol/L): 100 K gluconate, 30 KCl, 5 NaCl, 1 $MgCl_2$, 1 $CaCl_2$, 3 (or 0.3 for BK_{Ca}) EGTA, 10 HEPES, 10 glucose (pH 7.2, titrated with KOH), with an osmolality of 303±2 mOsm/L.

Rats

Male Sprague-Dawley rats, weighing 250–300 g, were purchased from Shanghai Experimental Animal Center (SPF, Certificate No SCXK 2007-0005, conferred by Animal Management Committee, Chinese Academy of Sciences).

Isolation of rat mesenteric artery and tone recording

Male Sprague-Dawley rats were killed by injecting a lethal dose (80 mg/kg) of sodium pentobarbitone. The superior mesenteric arteries were carefully removed and placed in Krebs solution. Adherent adipose and connective tissue were then removed. Vessel rings of about 1.5 mm in length were cut from each artery and mounted in 20-mL bath chambers of an integrated myograph system (AD instrument PowerLab 4/20) for tone recording. Bath chambers were filled with Krebs solution at 37 °C and aerated with 95% O_2+5% CO_2 to maintain a pH of 7.4. Tone signals were relayed to a PowerLab 4 amplifier and saved in a computer (sampling rate, 100 Hz). To remove vascular endothelium, artery rings were perfused with 1.80 mg/mL sodium deoxycholate in saline for 30 s. Artery rings were then rinsed with sodium deoxycholate–free Krebs solution for 40 min. Chemical removal of the endothelium was assessed by the lack of a relaxant response to 1 nmol/L acetylcholine.

During the initial 1-h equilibration, artery rings were stretched until the resting tension remained steady at 1.5 g. Artery rings were evaluated for a reproducible contractile response to 1 μmol/L norepinephrine and were washed several times. Next, 30 mmol/L KCl or 1 μmol/L norepinephrine was applied to establish a stable contractile tone. Subsequently, cumulative concentration–response curves of MLB were constructed by cumulative application of MLB to artery rings at 8-min intervals. In the experiment investigating the involvement of nitric oxide (NO) in artery dilation, artery rings were incubated with *L*-NAME (100 μmol/L), an inhibitor of nitric oxide synthase (NOS), for 30 min before applying KCl. In the experiment investigating the involvement of potassium channels, tetraethylammonium (TEA, 1 mmol/L) was used to incubate the artery rings for 10 min before applying norepinephrine.

Isolation of vascular smooth muscle cells

Single mesenteric artery cells were isolated using an enzymatic dissociation method as described previously, with slight modifications[13]. The mesenteric artery was dissected and placed in dissociation medium containing a low concentration of Ca^{2+} (160 μmol/L). Connective tissue was carefully removed. Next, the cleaned vessel was cut along its longitudinal axis and cut into small strips. Muscle strips were allowed to stay in fresh dissociation medium for 6–10 min at room temperature, and then they were transferred into enzyme solution in a tightly capped glass bottle and stored overnight at 4 °C. The enzyme solution contained 1% papain (type IV, 14.5 units/mg protein) and 0.02% BSA (type V, essentially fatty acid–free) in 5 mL dissociation medium. The following morning, dithiothreitol (DDT, 0.1 mmol/L) was added to the bottle, and the strips were incubated at 37 °C for 2–4 min in a shaking water bath. Strips were subsequently transferred to fresh dissociation medium and gently triturated with a wide-bore (2–3 mm) pipette. Long and relaxed single cells were obtained and then stored in dissociation medium at 4 °C. Approximately 70% of the cells remained relaxed for several hours after Ca^{2+} recovered to physiological levels.

Electrophysiological recording

Whole-cell K^+ currents were measured with the conventional patch-clamp technique[14]. A small aliquot of vascular smooth muscle cells was placed in a 3-mL chamber mounted on the stage of a microscope (Optiphot-2; Nikon, Japan) and superfused with external solution via a PBS-8 solution exchange system (ALA Scientific Instruments Inc, USA) at 3 mL/min. Patch pipettes were pulled using a P-97 microelectrode puller (Shutter Instruments Co, USA) with a tip resistance of 1–5 MΩ. The pipette tip was positioned near the center of mesenteric smooth muscle cells using an oil-based hydraulic micromanipulator (Narishige Scientific Instruments, Japan). After gigaseal formation (seal resistance >1 GΩ), the membrane was ruptured with gentle suction to obtain whole-cell voltage-clamp configuration. Voltage command protocols were provided by the pClamp 6.0.4 software package (Axon Instruments, USA) via a DigiData-1200 interface. Capacitance compensation was routinely optimized, and series resistance was compensated by 40%–80%. Linear leaks were subtracted digitally online. Currents were filtered at 1 kHz and sampled at 3 kHz. Cell capacitance was measured using a short hyperpolarizing ramp pulse (5 mV for 5 ms) from a holding potential of -60 mV. The

membrane capacitance of smooth muscle cells ranged from 8 to 20 pF. Currents during the last 400 ms in each step of two or three voltage-clamp trials were sampled and averaged before analysis. Currents were normalized to cell capacitance to obtain the current densities. Allowing for equilibration of the pipette solution with the cell interior, all recordings were initiated 5 min after establishing the whole-cell configuration. Most experiments were performed within 40 min after attaining the whole-cell configuration. During this time, the macroscopic K^+ current amplitude of the control cells remained stable. To separate BK_{Ca} and K_V from total currents, I_K was recorded from different holding potentials (-20 to +80 mV for BK_{Ca} and -60 to +40 mV for K_V).

Statistical analysis

Artery responses were measured as a reduction or increment in grams upon the norepinephrine- (or KCl-) preconstricted tones and are expressed as means±SD. Patch-clamp data are presented as means±SEM. Data analyses were performed using Clampfit 9.0 (Axon Instruments, USA). The concentration of MLB yielding a 50% effect (EC_{50} or IC_{50}) was obtained by fitting the concentration–response relationship to the equation $X=1/\{1+[(C)/IC_{50}]_n\}$, where X is the normalized response, (C) is the concentration of MLB and n is the Hill coefficient. Differences were compared using the Student's t-test or one-way ANOVA, followed by Bonferroni *post-hoc* test, as appropriate. All tests were two-tailed, and a value of $P<0.05$ was considered as statistically significant.

Results

MLB enhances KCl-induced vasoconstriction in the presence of *L*-NAME

The addition of MLB (400 μmol/L) to the bath solution produced a relaxation response in the artery rings precontracted by 30 μmol/L KCl (Figure 2A). However, in the presence of *L*-NAME (100 μmol/L), MLB produced a substantial contraction in a concentration-dependent manner, with a maximal contraction amplitude of 130.3% (Figure 2A). When *L*-NAME was applied to artery rings before KCl, tones increased slightly and were readjusted to baseline.

MLB relaxes norepinephrine-induced vasoconstriction

Tones produced by 1 μmol/L norepinephrine (1.97±0.55 g) were sustained over the course of the experiment. Cumulative application of MLB induced concentration-dependent relaxation, with an EC_{50} of 111.3 μmol/L (95% confidence interval: 97.6–126.3 μmol/L). Meanwhile, in denuded artery rings the vasodilator response was reduced (EC_{50} 224.4 μmol/L, 95% CI: 198.1–248.0 μmol/L) (Figure 2B). TEA was used to examine whether K^+ channels are involved in the MLB-induced vasodilation. Application of TEA induced a slight increase in tones, which was adjusted to baseline. In the presence of TEA, the vasodilation induced by MLB was largely inhibited in either intact or denuded artery rings: 400 μmol/L MLB dilated intact mesenteric arteries by 92.3% in the absence of TEA but only by 25.8% in the presence of 1 mmol/L TEA (Figure 2B).

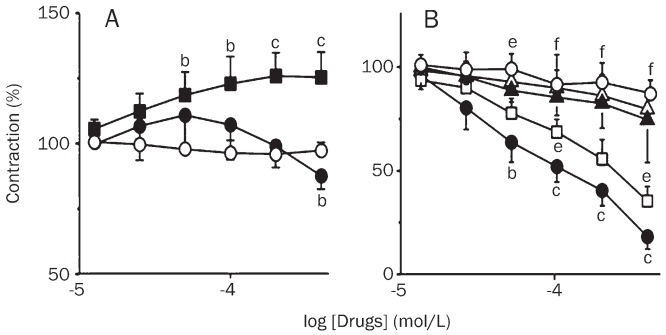

Figure 2. MLB causes relaxation or constriction of isolated artery rings. (A) Artery rings were precontracted by KCl. ○ Saline water was applied to intact artery rings as a control; ● MLB was applied to intact artery rings; ■ MLB was applied to artery rings pretreated with *L*-NAME. (B) Artery rings were precontracted by norepinephrine. ○ Saline water was applied to intact artery rings as a control; ● MLB was applied to intact artery rings; □ MLB was applied to denuded artery rings; ▲ MLB was applied to intact artery rings pretreated with TEA; △ MLB was applied to denuded artery rings pretreated with TEA. The maximal contraction induced by KCl or norepinephrine before applying saline water or MLB was taken as 100. Cumulative concentration of MLB: 12.5, 25, 50, 100, 200, or 400 μmol/L. $^bP<0.05$, $^cP<0.01$ *vs* control (○); $^eP<0.05$, $^fP<0.01$ *vs* MLB (●) (n=10).

MLB activates large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) currents

In single smooth muscle cells from rat mesenteric arteries, 500-ms voltage steps from a holding potential of -20 mV to test potentials in the range of -10 to +80 mV were adopted to activate BK_{Ca} currents. As the availability of K_V channels is voltage-dependent, I_K measured from a holding potential of -20 mV was primarily determined by BK_{Ca} channels, where the contribution of K_V channels to whole-cell I_K was negligible. A family of voltage-dependent, high-amplitude, high-noise outward K^+ currents were elicited, which were inhibited by 1 mmol/L TEA (n=5) and 1 nmol/L iberiotoxin (n=5) (Figure 3D). MLB produced a gradual voltage-dependent increment of currents, which reached a plateau within approximately

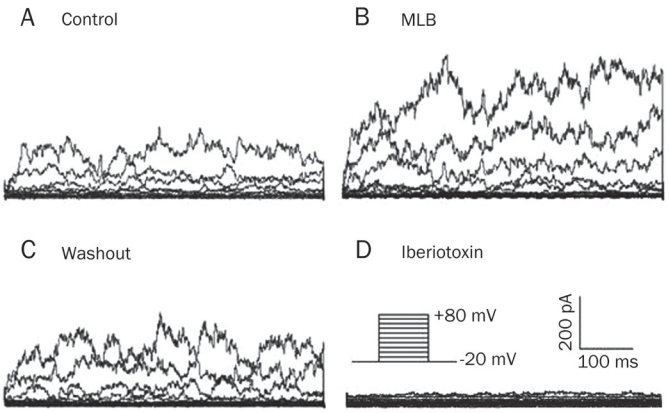

Figure 3. MLB reversibly activates BK_{Ca} currents. Representative BK_{Ca} currents before (A) and after applying 100 μmol/L MLB (B), after washout (C) and after applying 1 nmol/L iberiotoxin (D).

3 min. The densities of currents recorded at +80 mV were 9.57±3.6 pA/pF, which increased to 15.95±4.09 pA/pF after exposure to 100 μmol/L MLB (n=8, P<0.01). This MLB-induced increment of currents was partially recovered upon washout (Figure 3C). The outward currents were sustained during the depolarization pulse. The I–V relationship was plotted (Figure 4A). BK_{Ca} currents at +70 and +80 mV were significantly larger in the presence of MLB than in controls. A single depolarization pulse from -20 to +70 mV was adopted to obtain the concentration-response curve, and the results showed that the stimulatory effect of MLB on BK_{Ca} was concentration-dependent, with an EC_{50} of 156.3 μmol/L (95% CI: 136.9–175.3 μmol/L).

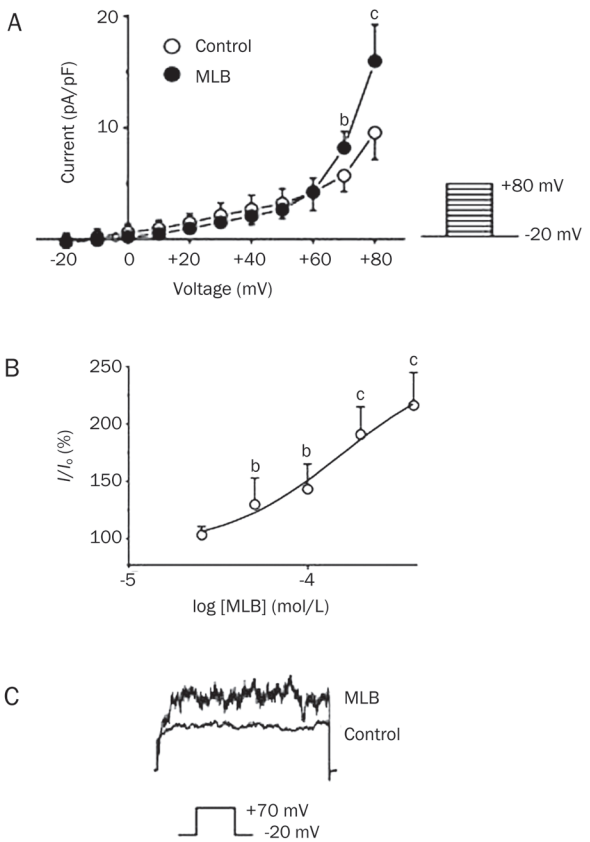

Figure 4. MLB activates BK_{Ca} currents in a concentration- and voltage-dependent manner. (A) Current–voltage (I–V) curves before and after applying 100 μmol/L MLB. (B) Concentration–response curve of BK_{Ca} currents to MLB. Cumulative concentration of MLB: 25, 50, 100, 200, or 400 μmol/L (n=8). (C) Representative BK_{Ca} currents before and after applying MLB. ^bP<0.05, ^cP<0.01 *vs* control (○).

MLB inhibits K_V currents

A family of voltage-dependent outward K^+ currents was elicited by depolarization from a holding potential of -60 mV to a series of command potentials from -50 to +40 mV. The representative currents in response to MLB (100 μmol/L) are illustrated (Figure 5A). Application of 3 mmol/L 4-AP suppressed the currents almost completely, which is characteristic of K_V currents[15, 16]. The I_K was sampled between 450 and 490 ms (steady state) to exclude any possibility of A-current contribution to the measured amplitude. The I–V curve shows that MLB inhibited K_V significantly (Figure 5B).

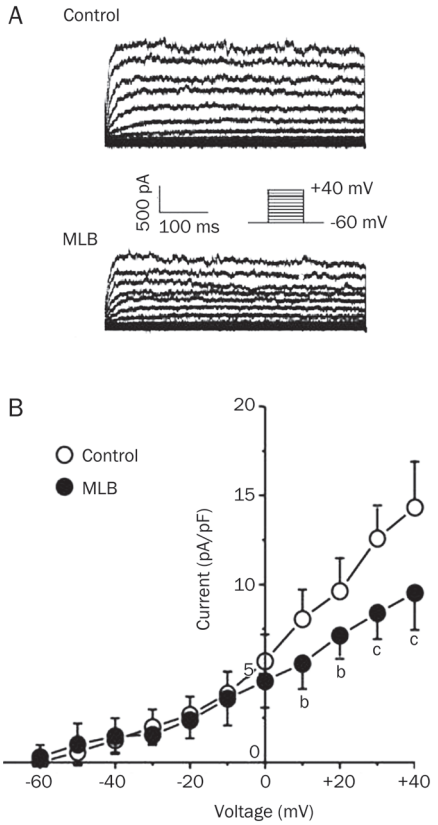

Figure 5. MLB inhibits K_V currents in a voltage-dependent manner. (A) Representative K_V currents of control and with MLB (100 μmol/L). (B) Current–voltage (I–V) curves (MLB, 100 μmol/L) (n=7). ^bP<0.05, ^cP<0.01 *vs* control (○).

The time-course of response to MLB showed that inhibition of K_V currents was reversible (Figure 6B). The depolarizing pulse of 500 ms from a holding potential of -60 mV to a test potential of +30 mV was used to obtain the concentration–response relationship (Figure 6C). The IC_{50} was 26.1 μmol/L (95% CI: 20.4–34.8 μmol/L) (n=8).

Discussion

Force and membrane potential are closely coupled in arterial smooth muscle, and K^+ conductance plays a major role in determining membrane potential[15]. Low concentrations (≤1 mmol/L) of TEA selectively block BK_{Ca}[17]. In the present study, the relaxant responses of both the denuded and the intact artery rings to MLB were inhibited almost completely by pre-incubation with TEA (1 mmol/L), suggesting the involvement of K^+ channels. We also found MLB activated BK_{Ca} currents in a reversible, concentration- and voltage-dependent manner in whole-cell patch-clamp experiments. The results from artery rings and patch-clamp experiments were consistent, indicating BK_{Ca} currents are involved in the vasodilator process and

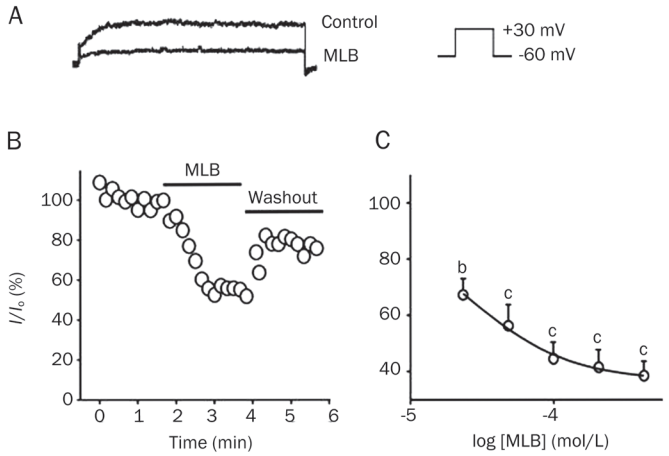

Figure 6. MLB inhibits K_V currents in a reversible and concentration-dependent manner. (A) Representative K_V currents before and after applying MLB (100 μmol/L). (B) Time-course of the response to application and washout of MLB (100 μmol/L). (C) Concentration–response curve of K_V currents to MLB (25, 50, 100, 200, or 400 μmol/L) (n=8). $^bP<0.05$, $^cP<0.01$ *vs* control (○).

activation of BK_{Ca} is the primary vasodilator mechanism in rat mesenteric arteries.

The vasoconstriction in response to MLB in artery rings precontracted with KCl is a novel finding of this study. BK_{Ca} currents are much smaller than K_V currents within the physiological membrane potential range, and activation of K_V channels is the initial inhibitory mechanism upon depolarization in arteriolar smooth muscle cells[18]. The vasocontraction induced by KCl is due to depolarization of smooth muscle cells. Our patch-clamp experiments showed that MLB inhibited K_V currents. Inhibition of K^+ currents would cause depolarization and vasoconstriction[16, 19]. Therefore, it is reasonable to presume that in artery rings precontracted with KCl, MLB caused further vasocontraction by inhibiting K_V currents. Without precontraction with KCl, artery rings were not contracted by MLB (data not shown), implying the vasoconstriction to MLB might be situation-dependent.

Although K_V channels are dominant in the artery, the inhibitory effects of Ca^{2+} on K_V channels lead to a shift of dominance to BK_{Ca} channels once the intracellular Ca^{2+} levels rise substantially[20, 21]. This could be the reason that MLB relaxed artery rings precontracted with norepinephrine but contracted artery rings precontracted with KCl.

In cardiovascular disease, K^+ channels functionally change[22–24]. Hypertension develops, with functional down-regulation of K_V channels but up-regulation of BK_{Ca} in smooth muscle[25]. Activating BK_{Ca} but inhibiting K_V channels might have important pharmacologic and therapeutic implications.

Previous findings indicate MLB causes endothelium-dependent vasodilatation *in vitro*[9]. Meanwhile, another study demonstrated that its vasodilatory action is not endothelium-dependent but primarily by works by inhibiting Ca^{2+} inflow[26]. In this study, the involvement of endothelium was investigated using both denuded artery rings and *L*-NAME. In denuded artery rings precontracted with norepinephrine, the vasodilatory effect of MLB was reduced significantly compared with intact artery rings. In artery rings incubated with *L*-NAME, an inhibitor of NOS, and precontracted by KCl, MLB induced vasoconstriction. Conversely, without *L*-NAME, application of MLB dilated artery rings. These results demonstrate that endothelium and endothelial NO participate in the vasodilation process of MLB. By contrast, compared with the effect of TEA, the removal of endothelium caused a relatively minor effect. NO stimulates smooth muscle soluble guanylate to produce cGMP, which consequently activates BK_{Ca} and induces vasodilatation[7, 27]. Furthermore, NO can activate BK_{Ca} directly[27]. So far, however, it is still unknown how NO participates in the vasodilatory action of MLB.

In conclusion, our data provide evidence that MLB, a hydrophilic constituent of *S miltiorrhiza*, dilates artery rings primarily by activating iberiotoxin-sensitive BK_{Ca} channels in smooth muscle cells and increasing NO release from endothelium. MLB also contracts arteries precontracted with KCl in the presence of *L*-NAME.

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Author contribution

Yi-ping WANG designed the research; Guo-yuan HU and Xue-qing CHEN provided equipment and technical support; Hai-fei ZHANG performed the research and analyzed the data; Yi-ping WANG and Hai-fei ZHANG wrote the paper.

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