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Ligustilide induces vasodilatation via inhibiting voltage dependent calcium channel and receptor-mediated Ca^{2+} influx and release

Yong-Xiao Cao [a,b,*,1], Wei Zhang [a,b], Jian-Yu He [a], Lang-Chong He [a], Cang-Bao Xu [a,b]

[a] *Department of Pharmacology, Xi'an Jiaotong University School of Medicine, and Key Laboratory of Environment and Genes Related to Diseases (Xi'an Jiaotong University), Ministry of Education, Xi'an, Shaanxi, 710061, P.R. China*
[b] *Division of Experimental Vascular Research, Department of Medicine, Lund University, Lund, S-221 85, Sweden*

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Abstract

The purpose of the present study was to investigate the effect of ligustilide on vasodilatation in rat mesenteric artery and the mechanisms responsible for it. Isometric tension of rat mesenteric artery rings was recorded by a sensitive myograph system in vitro. The results showed that ligustilide at concentrations more than 10 μM relaxed potassium chloride (KCl)-preconstricted rat mesenteric artery in a concentration-dependent manner. The vasodilatation effect of ligustilide was not dependent on endothelium. Ligustilide rightwards shifted concentration–response curves induced by KCl, calcium chloride ($CaCl_2$), noradrenaline (NA) or 5-hydroxytryptamine (5-HT) in a non-parallel manner. This suggests that the vasodilatation effects were most likely via voltage-dependent calcium channel (VDCC) and receptor-operated calcium channel (ROCC). Propranolol, glibenclamide, tetraethylammonium and barium chloride did not affect the vasodilation induced by ligustilide, showing that β-adrenoceptor, ATP sensitive potassium channel, calcium-activated potassium channel and inwardly rectifying potassium channel were not involved in the vasodilatation. Ligustilide concentration-dependently inhibited the vasoconstriction induced by NA or $CaCl_2$ in Ca^{2+}-free medium, indicating that the vasodilatation relates to inhibition of extracellular Ca^{2+} influx through VDCC and ROCC, and intracellular Ca^{2+} release from Ca^{2+} store. Since caffeine-induced contraction was inhibited by ligustilide, inhibition of intracellular Ca^{2+} released by ligustilide occurred via the ryanodine receptors. Our results suggest that ligustilide induces vasodilatation in rat mesenteric artery by inhibiting the VDCC and ROCC, and receptor-mediated Ca^{2+} influx and release.

Keywords: Ligustilide; Vasodilatation; Rat mesenteric artery; Calcium

1. Introduction

A lot of traditional Chinese medicines (TCM) have been widely used in clinic in lots of Asian countries for the treatment of cardiovascular diseases. In more than 10,000 different kinds of TCM, *Ligusticum chuanxiong* is a frequently used one for cardiovascular diseases to promote the circulation of blood and removing stasis (Hou et al., 2004a; Tsai et al., 2002; Gong and Sucher, 1999). The combination of *L. chuanxiong* Hort (or *Ligusticum wallichii* Franch.) (LC) and *Angelica sinensis* (Oliv.) Diels (AS) is called *Fo Shou San* (Chinese medicinal formulas) with a long history of use for treatment of atherosclerosis and hypertension (Han et al., 1995).

Many effective components have been extracted from *L. chuanxiong*, such as chuanxiongzine (Huang et al., 1998; Cui et al., 2003), organic acids (Hou et al., 2004b), phthalides (Naito et al., 1996), etc. Ligustilide, one of the phthalides, has been studied and demonstrated that it has antiasthmatic action (Tao et al., 1984), centrally acting muscle relaxant effect (Ozaki et al., 1989), effets on atria (Nakazawa et al., 1989), and effects on central noradrenergic and/or GABA(A) systems (Matsumoto et al., 1998).

In addition, the effects of ligustilide on vasculature have been noticed and studied. It is mentioned that ligustilide is a main effective component of *L. chuanxiong* as a medicine in treatment for vascular diseases. It is reported that ligustilide has a vasodilatation effect on rat abdominal aorta, in the form of inhibiting NA

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* Corresponding author. Department of Pharmacology, Xi'an Jiaotong University School of Medicine, Xi'an, Shaanxi 710061, China. Tel.: +86 29 82655140; fax: +86 29 82657764.
E-mail address: yxy@xjtu.edu.cn (Y.-X. Cao).

[1] Mailing address: No. 76, Yanta West Road, Xi'an, Shaanxi Province 710061, P.R. China.

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and $CaCl_2$-induced vasoconstriction (Liang et al., 2005). However, the mechanism of this effect remain unknown. The objective of the present study is to investigate the mechanism responsible for ligustilide-induced vasodilatation in rat mesenteric artery.

2. Materials and methods

2.1. Animals and reagents

Male Sprague-Dawley rats (weighing 250–300 g) were from Animal Center of Xi'an Jiaotong University (Xi'an, China). Ligustilide (Lig) was supplied from Prof. He Langchong (Xi'an Jiaotong University, China). Noradrenaline (NA), 5-hydroxytryptamine (5-HT), acetylcholine (ACh), Triton X-100, propranolol (Prop), glibenclamide (Glib), tetraethylammonium (TEA), caffeine and dimethyl sulphoxide (DMSO) were obtained from Sigma Aldrich (St. Louis, USA). All other reagents were analytical reagent (AR) grade. All substances were dissolved in distilled water except for ligustilide which was dissolved in DMSO and further dilutions were made in Kreb's solution.

2.2. In vitro pharmacology

The rats were anesthetized and sacrificed by decapitation. The superior mesenteric artery was removed gently and immersed in cold oxygenated Kreb's solution and dissected free of adhering tissue under a microscope. In endothelium-denuded experiments the endothelium was denuded by perfusion of the vessel for 10 s with 0.1% Triton X-100 followed by another 10 s with Kreb's solution (Adner et al., 1996; Cao et al., 2004). The vessels were then cut into 1 mm long cylindrical segments and mounted on two L-shaped metal prongs, one of which was connected to a force displacement FT-03C transducer (Grass Instruments, USA) attached to a PowerLab 8SP unit (AD Instruments, UK) for continuous recording of the isometric tension, and the other to a displacement device. The mounted artery segments were immersed in tissue baths (DMT, Denmark) containing Kreb's solution of 1 ml, which was aerated continuously with a gas

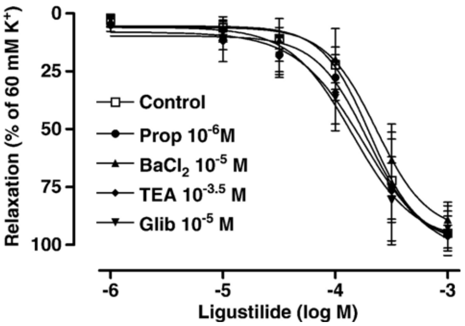

Fig. 2. Vasodilatation effect of ligustilide in endothelium-denuded artery rings in the presence of propranolol (Prop), glibenclamide (Glib), tetraethylammonium (TEA) or $BaCl_2$. No significant effects could be observed. $n=7$.

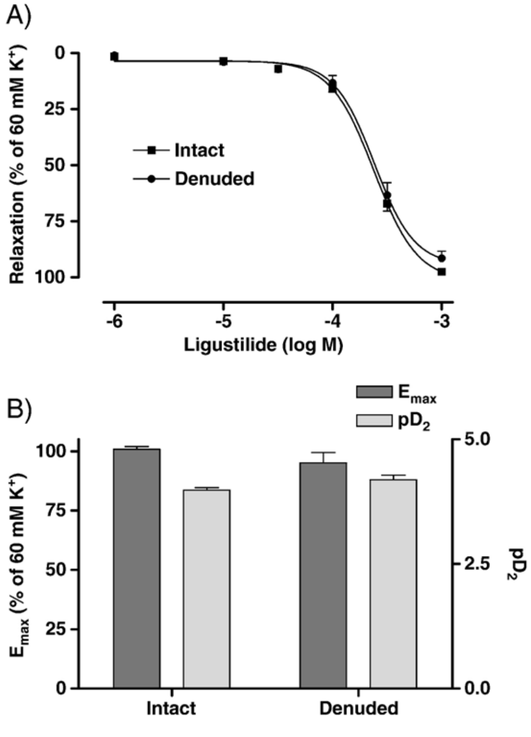

Fig. 1. (A) The concentration–response curves of ligustilide on vasodilatation effect of endothelium-intact and endothelium-denuded rat mesenteric artery precontracted by KCl. The relaxation was expressed as the percentage of the preconstriction by 60 mM K^+. (B) Maximal relaxation and pD_2 of ligustilide on rat mesenteric artery precontracted by KCl. R_{max} refers to maximal relaxation calculated as percentage of the corresponding precontraction with 60 mM K^+. pD_2 is the negative logarithm of the drug concentration that elicited 50% relaxation. $n=8$.

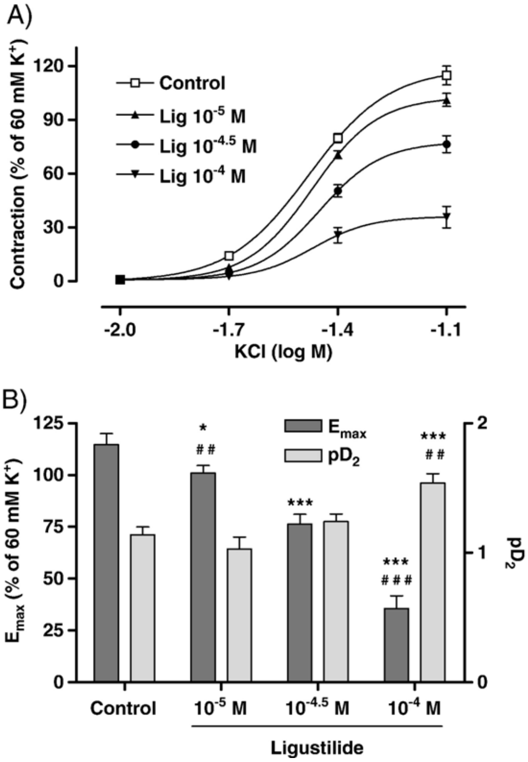

Fig. 3. (A) Effect of ligustilide on the concentration–response curves of KCl on rat mesenteric artery segments without endothelium. The concentration–response curve shifted towards right in a non-parallel manner compared with control. (B) Maximal effect (E_{max}) and pD_2 of KCl on rat mesenteric artery. $n=8$. $*P<0.05$, $***P<0.001$ vs. control. $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs. ligustilide $10^{-4.5}$ M.

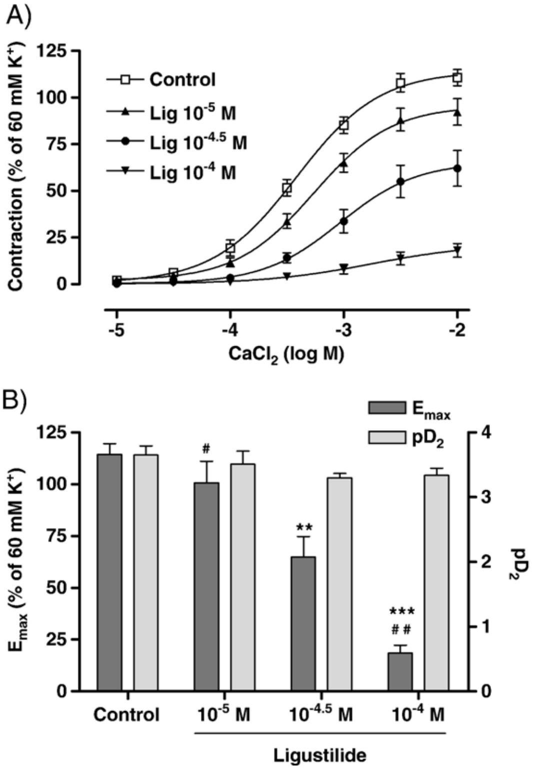

Fig. 4. (A) Effect of ligustilide on the concentration–response curve of $CaCl_2$ on rat mesenteric artery segments without endothelium. The concentration–response curve shifted towards right in a non-parallel manner compared with control. (B) E_{max} and pD_2 of $CaCl_2$ on rat mesenteric artery. $n=7$. $^{**}P < 0.01$, $^{***}P<0.001$ vs. control. $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. ligustilide $10^{-4.5}$ M.

mixture of 95% O_2 and 5% CO_2 and maintained at 37 °C. The artery segments were equilibrated for 1.5 h with a resting tension of 5 mN before the experiments were started. The contractile capacity of each vessel segment was tested by exposure to a K^+-rich Kreb's solution (with 60 mM KCl) in which NaCl was exchanged for an equimolar concentration of KCl. When two reproducible contractions had been achieved the vessels were used for further experiments. After equilibration, artery segments were exposed to K^+-rich Kreb's solution. Once the sustained tension was obtained, ligustilide (1 μM–1 mM) was added cumulatively to the baths, and the concentration–response curves to ligustilide were constructed.

In the experiment involving endothelium, the completeness of endothelium denudation was tested with acetylcholine (ACh) (10 μM) after pre-contraction with KCl. No relaxation in response to ACh in the denuded preparation indicated an effective functional removal of the endothelium. The rings with endothelium that produced less than 30% relaxation in response to ACh were discarded.

2.3. Statistical analysis

The effects of ligustilide are expressed as percentage of relaxation from the pre-contraction. Data are shown as mean ± SEM. Statistical analysis was performed with unpaired Student's *t*-test. The *P* value of less than 0.05 was regarded to be significant.

3. Results

3.1. Vasodilation effect of ligustilide on rat mesenteric artery preconstricted by KCl

Ligustilide (1 μM–1 mM) concentration-dependently relaxed the artery segments pre-contracted by KCl with endothelium or without endothelium (Fig. 1A). In the artery ring segments with endothelium the maximum relaxation effect (R_{max}) of ligustilide was 100.8±1.1%. In rings denuded endothelium, the effect of ligustilide did not change significantly, and R_{max} was 95.0±4.4% ($P>0.05$, Fig. 1B).

3.2. Vasodilation effect of ligustilide in the presence of different blockers

The endothelium-denuded artery rings were incubated in the presence of propranolol (1 μM), glibenclamide (10 μM), tetraethylammonium (300 μM) or barium chloride ($BaCl_2$, 10 μM) for 20 min. Vasodilatation effect of ligustilide on KCl-pre-contracted artery rings was recorded in order to test the effects of β-adrenoceptor, ATP sensitive potassium channel, calcium-activated potassium channel and inwardly rectifying potassium channel, which may contribute to the ligustilide-induced vasodilatation. The results (Fig. 2) showed that endothelium-denuded artery rings with propranolol, glibenclamide, tetraethylammonium or $BaCl_2$ had no significant effect on the ligustilide-induced relaxation response.

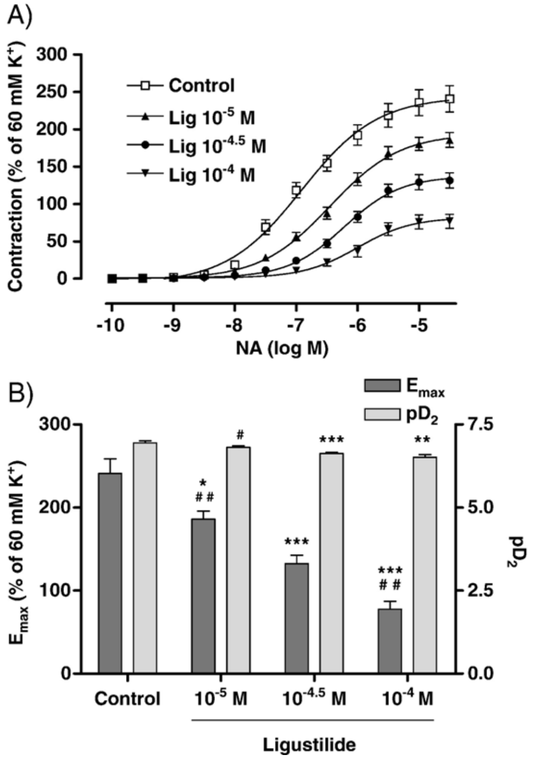

Fig. 5. (A) Effect of ligustilide on the concentration–response curve of NA on rat mesenteric artery segments without endothelium. The concentration–response curve shifted towards right in a non-parallel manner compared with control. (B) E_{max} and pD_2 of NA on rat mesenteric artery. $n=7$. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs. control. $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. ligustilide $10^{-4.5}$ M.

3.3. Effect of ligustilide on the KCl and $CaCl_2$-induced concentration–contraction curves

After removal of endothelium, artery segments were equilibrated for 1.5 h. Kreb's solutions contained KCl (10, 20, 40, 80 mM) and DMSO of the same volume as ligustilide were replaced in order and the concentration–response curves were constructed. After washout and equilibrated for 1 h, the arteries were incubated with ligustilide (10, 30, 100 μM) for 15 min. Then, the concentration–response curves of KCl were constructed again as above, while DMSO was replaced with ligustilide. The concentration–response curve of KCl in the presence of ligustilide shifted towards right in a non-parallel manner compared with control ($P<0.05$, Fig. 3A). The maximum effects (E_{max}) of KCl were reduced by different concentrations of ligustilide ($P<0.05$, Fig. 3B). The pD_2' value of ligustilide to KCl was 4.28±0.05.

After contraction, the endothelium-denuded artery segments were exposed to Ca^{2+}-free K^+-rich solution containing EDTA (100 μM) and KCl (60 mM) for 20 min. After DMSO of the same volume as ligustilide was added to the baths and incubated for 15 min, $CaCl_2$ (10 μM to 10 mM) was added cumulatively to the baths, and the concentration–response curves to $CaCl_2$ were constructed. After washout and equilibration for 1 h, DMSO was replaced with ligustilide (10, 30, 100 μM) and the concentration–response curve of $CaCl_2$ were constructed again as above. The concentration–response curve of $CaCl_2$ in the presence of ligustilide shifted towards right in a non-parallel manner compared with control ($P<0.05$, Fig. 4A). The E_{max} of $CaCl_2$ were reduced by different concentrations of ligustilide ($P<0.05$, Fig. 4B). The pD_2' value of ligustilide to $CaCl_2$ was 4.45±0.02.

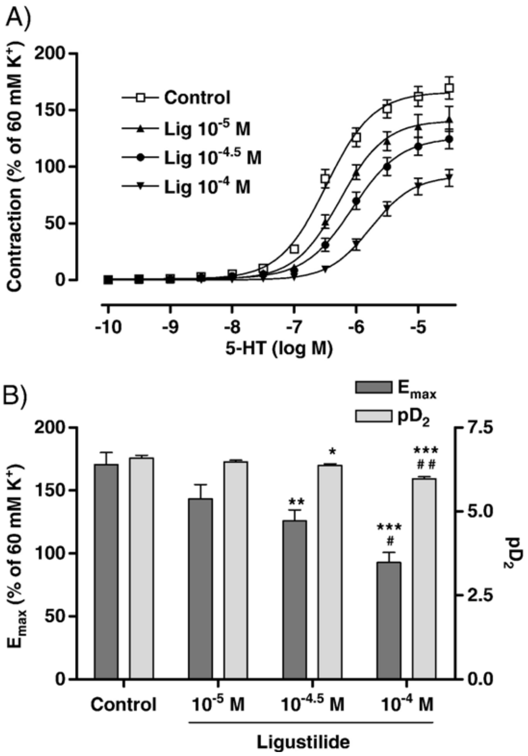

Fig. 6. (A) Effect of ligustilide on the concentration–response curve of 5-HT on rat mesenteric artery segments without endothelium. The concentration–response curve shifted towards right in a non-parallel manner compared with control. (B) E_{max} and pD_2 of 5-HT on rat mesenteric artery. $n=7$. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs. control. $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. ligustilide $10^{-4.5}$ M.

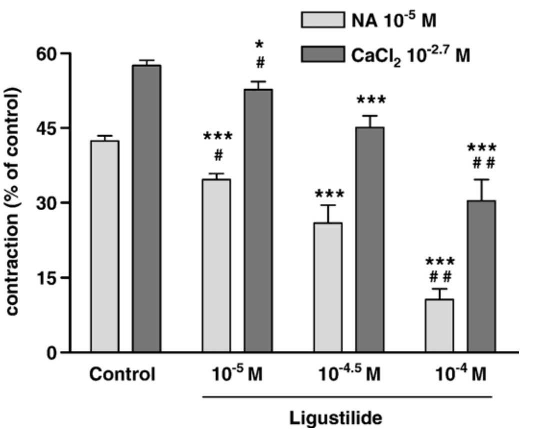

Fig. 7. Effect of ligustilide on the contraction induced by NA and $CaCl_2$ in Ca^{2+}-free Kreb's solution. $n=8$. $^*P<0.05$, $^{**}P<0.001$ vs. control. $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. ligustilide $10^{-4.5}$ M.

3.4. Effect of ligustilide on the NA and 5-HT induced concentration–contraction curve

NA and 5-HT can both induce a potent and sustained constriction of mesenteric artery segments in a concentration-dependent manner. Ligustilide (10, 30, 100 μM) was added to the baths before cumulative addition of NA or 5-HT (0.1 nM–30 μM). As a result, ligustilide potently inhibited the NA or 5-HT induced vasoconstriction and concentration-dependently shifted the concentration–contractile curves towards right on a non-parallel manner with a decreased E_{max} (Figs. 5 and 6). The pD_2' value of ligustilide to NA and 5-HT was 4.39±0.23 and 3.86±0.27, respectively.

3.5. Effect of ligustilide on the contraction of artery segments in Ca2+-free solution

After the contraction activity being tested, the endothelium-denuded artery segments were exposed to Ca^{2+}-free Kreb's solution containing EDTA (100 μM) for 10 min. Another 10-min incubation of DMSO were followed by addition of 10 μM NA. After the NA-induced contraction being sustained, $CaCl_2$ of

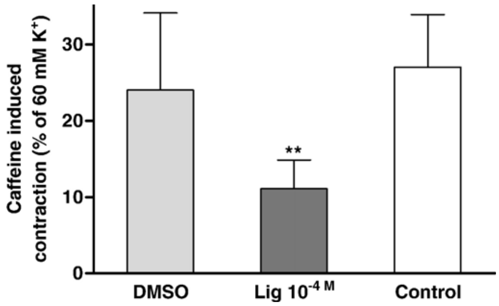

Fig. 8. Effect of ligustilide on the contraction induced by caffeine in Ca^{2+}-free Kreb's solution. The contraction was expressed as the percentage of the preconstriction by 60 mM K^+. $n=7$. $^{**}P<0.01$ vs. control.

2 mM was added to contract the segments again (Broekaert and Godfriend, 1979; Zhao et al., 1997). The segments were thereafter washed with Kreb's solution (45 min contact time for Ca^{2+} refilling of the intracellular stores) and then 3 times with Ca^{2+}-free solution (15 min contact time). The second contractile response to NA and $CaCl_2$ were tested in the presence of ligustilide (10, 30, 100 μM) for 10 min. Then the effect of ligustilide on the contraction induced by NA and $CaCl_2$ in the Ca^{2+}-free Kreb's solution was tested in endothelium-denuded preparations. Ligustilide at three different concentrations significantly inhibited the contraction induced by NA and $CaCl_2$ concentration-dependently ($P<0.05$, Fig. 7).

3.6. Effect of ligustilide on the contraction induced by caffeine in Ca2+-free solution

The endothelium-denuded artery segments were exposed to Ca^{2+}-free Kreb's solution for 10 min followed by another 10-min incubation of DMSO. Then sustained contractions of arteries to caffeine (30 mM) were obtained (Cao et al., 2005). After washout and reloading, the effect of ligustilide (100 μM) on the vasoconstriction induced by caffeine was tested as above. A blank control (without incubation) was made before the end of the experiment (Fig. 8). Compared with control, the inhibitory rate in presence of DMSO was 14.5 ± 8.1 % ($n=7$), while that in presence of ligustilide was 59.3 ± 3.2 % (n=7, $P<0.01$). The results showed that ligustilide could significantly inhibit the vasoconstriction induced by caffeine in Ca^{2+}-free solution.

4. Discussion

The present study showed that ligustilide at concentration of more than 10 μM relaxed the mesenteric artery precontracted by KCl. Removal of the endothelium did not affect the relaxant effect of ligustilide, indicating that ligustilide-induced vasodilatation was not dependent on endothelium.

Different blockers has been used to test the possible mechanisms. Propranolol (a general β-adrenoceptor antagonist), glibenclamide (an ATP sensitive potassium channel inhibitor), tetraethylammonium (a calcium-activated potassium channel inhibitor) and $BaCl_2$ (an inwardly rectifying potassium channel inhibitor) did not affect the vasodilation induced by ligustilide, showing that β-adrenoceptor, ATP sensitive potassium channel, calcium-activated potassium channel and inwardly rectifying potassium channel were not involved in the vasodilatation.

The mechanism of vascular smooth muscle contraction involves different signal transduction pathways, all of which converge to increase intracellular calcium (Broekaert and Godfriend, 1979). Both extracellular Ca^{2+} influx, through voltage-dependent calcium channel (VDCC) or receptor-operated calcium channel (ROCC), and intracellular Ca^{2+} release result in the increase of intracellular calcium level. Ligustilide shifted the concentration–response curve of KCl and $CaCl_2$ towards right in a non-parallel manner with decreased $E_{\max}$, suggesting that VDCC on smooth muscle cells accounts for the vasodilator action of ligustilide. Moreover, the concentration–response curve of NA and 5-HT also shifted in the same pattern in the presence of ligustilide, suggesting that ROCC was involved in the vasodilation effect of ligustilide.

The release of intracellular stored Ca^{2+} is mainly regulated by IP_3 receptor system (IP_3Rs) and ryanodine receptor system (RyRs). The former induces Ca^{2+} release directly when the receptors are bound to IP_3. The later may function through a Ca^{2+} induced Ca^{2+} release (CICR) mechanism when the receptors are activated by caffeine (Leijten and Van Breemen, 1984). The present results show that ligustilide concentration-dependently inhibited the contraction induced by NA and $CaCl_2$ in Ca^{2+}-free medium, which suggests that the mechanism of the vasodilatation is related to the inhibition of extracellular Ca^{2+} influx through VDCC and ROCC, and intracellular Ca^{2+} release from Ca^{2+} store. Besides, ligustilide affects the caffeine-induced contraction of artery segments, which proved the involvement of RyRs in the release of intracelluar stored Ca^{2+}.

In conclusion, ligustilide induced vasodilatation mainly by inhibiting voltage-dependent calcium channel and the receptor-mediated Ca^{2+} influx and release.

Acknowledgments

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References

Adner, M., Cantera, L., Ehlert, F., Nilsson, L., Edvinsson, L., 1996. Plasticity of contractile endothelin-B receptors in human arteries after organ culture. British Journal of Pharmacology 119, 1159–1166.

Broekaert, A., Godfriend, T., 1979. A comparison of the inhibitory effect of cinnarizine and papaverine on the noradrenaline and calcium evoked contraction of isolated rabbit aorta and mesenteric arteries. European Journal of Pharmacology 53, 281–284.

Cao, Y.X., He, L.C., Xu, C.B., Edvinsson, L., 2004. Alteration in contractile response to noradrenaline, 5-hydroxytryptamine, sarafotoxin 6c, and angiotensin II in rat mesenteric artery during organ culture. Academic Journal of Xi'an Jiaotong University 16, 155–159.

Cao, Y.X., Zheng, J.P., He, J.Y., Li, J., Xu, C.B., Edvinsson, L., 2005. Induces vasodilatation of rat mesenteric artery in vitro mainly by inhibiting receptor-mediated Ca^{2+}-influx and Ca^{2+}-release. Archives of Pharmacal Research (28, 6), 710–715.

Cui, R.X., Chen, Y.G., Gu, Y., 2003. Influence of acupuncture on brain taxis of tetramethylpyrazine in acute cerebral infarction rats. World Journal of Acupuncture-Moxibustion 13 (2), 36–40.

Gong, X.D., Sucher, N.J., 1999. Stroke therapy in traditional Chinese medicine (TCM): prospects for drug discovery and development. Trends in Pharmacological Sciences 20 (5), 191–196.

Han, Q.H., Shu, H.Y., Wang, J., 1995. Study on the effect of Fo Shou San plus danshan in preventing IUGR rats with passive smoking from peroxidation in erythrocyte lipid. Journal of TongJi Medicine University 15, 120–124.

Hou, Y.Z., Zhao, G.R., Yang, J., Yuan, Y.J., Zhu, G.G., Raimo, H., 2004a. Protective effect of *Ligusticum chuanxiong* and *Angelica sinensis* on endothelial cell damage induced by hydrogen peroxide. Life Sciences 75 (14), 1775–1786.

Hou, Y.Z., Yang, J., Zhao, G.R., Yuan, Y.J., 2004b. Ferulic acid inhibits vascular smooth muscle cell proliferation induced by angiotensin II. European Journal of Pharmacology 499 (1–2), 85–90.

Huang, R.J., Liao, C.X., Chen, D.Z., Chen, W.M., Weng, Q.Y., 1998. Effects of tetramethylpyrazine on TXA_2 and PGI_2 in congenital heart diseases with pulmonary hypertension patients during cardio-pulmonary bypass. Chinese Journal of Integrated Traditional and Western Medicine 4 (4), 263–266.

Leijten, P.A., Van Breemen, C., 1984. The effects of caffeine on the noradrenaline-sensitive calcium store in rabbit aorta. Journal of Physiology 357, 327–339.

Liang, M.J., He, L.C., Yang, G.D., 2005. Screening, analysis and in-vitro vasodilation of effective components from *Ligusticum chuanxiong*. Life Sciences 78 (2), 128–133.

Matsumoto, K., Kohno, S., Ojima, K., Tezuka, Y., Kadota, S., Watanabe, H., 1998. Effects of methylenechloride-soluble fraction of Japanese angelica root extract, ligustilide and butylidenephthalide, on pentobarbital sleep in group-housed and socially isolated mice. Life Sciences 62 (23), 2073–2082.

Naito, T., Ikeya, Y., Okada, M., Mistuhashi, H., Maruno, M., 1996. Two phthalides from *Ligusticum chuanxiong*. Phytochemistry 41 (1), 233–236.

Nakazawa, K., Fujimori, K., Inoue, K., Sekita, S., Takanaka, A., 1989. Effects of extract from a herbal drug, cnidium rhizome (senkyu), on contraction, heart rates and membrane potentials of isolated guinea pig atria. Yakugaku Zasshi 109 (9), 662–671.

Ozaki, Y., Sekita, S., Harada, M., 1989. Centrally acting muscle relaxant effect of phthalides (ligustilide, cnidilide and senkyunolide) obtained from *Cnidium officinale* Makino. Yakugaku Zasshi 109 (6), 402–406.

Tao, J.Y., Ruan, Y.P., Mei, Q.B., Liu, S., Tian, Q.L., Chen, Y.Z., Zhang, H.D., Duan, Z.X., 1984. Studies on the antiasthmatic action of ligustilide of dang-gui, *Angelica sinensis* (Oliv.) Diels. Yao Xue Xue Bao 19 (8), 561–565.

Tsai, C.C., Lai, T.Y., Huang, W.C., Liu, I.M., Cheng, J.T., 2002. Inhibitory effects of potassium channel blockers on tetramethylpyrazine-induced relaxation of rat aortic strip in vitro. Life Sciences 71 (11), 1321–1330.

Zhao, M.G., Mei, Q.B., Zhao, D.H., Jia, M., 1997. Effects of MC9204, a novel calcium channel blocker, on isolated rabbit arterial rings. Chinese Pharmacological Bulletin 13 (2), 150–152.