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VASOCONSTRICTIVE EFFECT OF *XINMAILONG* INJECTION IN RAT AORTA

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Abstract

Background: Cockroach has been traditionally utilized in China for the therapy of cardiovascular disorders, such as heart failure. The present study was aimed to assess the vasoconstrictive effect of *Xinmailong* Injection (XML), a bioactive composite from American cockroach.
Methodology: The isometric tensions of rat aortic rings were measured after acutely treated with XML. Meanwhile, the systemic blood pressures (SBPs) were recorded and the levels of the endothelium-derived cytokines in blood samples were detected after rats were administered with XML for 3 days. Protein expression for the L-type Ca^{2+} channels ($Ca_{v1.2}$) was also determined in rat thoracic aorta.
Results: XML induced vasoconstrictions in rat aortic rings with or without endothelium. In addition, the vasoconstrictions due to the extracellular Ca^{2+} influx and the intracellular Ca^{2+} release were also elevated by XML. After treatment with XML for 3 days, the level of prostacyclin (PGI_2) was markedly increased whereas the levels of nitric oxide (NO) and endothelin-1 (ET-1) were not significantly changed in rats. Furthermore, expression of the $Ca_{v1.2}$ protein was significantly enhanced in aorta but the SBPs of rats were not influenced.
Conclusion: XML plays an important role in regulating vascular tone. The increases of the extracellular Ca^{2+} influx and the intracellular Ca^{2+} release may contribute to the vasoconstriction induced by XML. Our findings pave the ways to the better understandings of the therapeutic effects of XML on cardiovascular system.

Key words: *Xinmailong* Injection; American cockroach; Vasoconstriction.

Introduction

Cockroach, also named as *Zhang Lang* or *Fei Lian* in Chinese, was first recognized as one insect drug in *Shennong Bencao Jing*, the oldest Chinese book on pharmacology (a compilation of oral traditions written between about 300 BC and 200 AD). In the Ming Dynasty, *Li Shizhen*, a well-known medical specialist and pharmacologist of ancient China, described cockroach as an effective drug which can promote blood circulation and urination in *Bencao Gangmu* (Compendium of Materia Medica).

As the largest and one of the most widely distributed species of cockroach, *Periplaneta americana* (*P. americana*, also known as American cockroach) has been found to exert a wide range of biological functions, such as enhancing tissue repair, anti-tumor and antibacterial activities (He et al., 2007). Over 20 years ago, corazonin was isolated from *P. Americana* and was proved to stimulate heart beat at a very low concentration (0.2 nM) (Veenstra, 1989). This finding reveals the potential roles of *P. Americana* in the regulation of the cardiovascular functions. In the following decades, this hypothesis has been confirmed by researchers, especially Chinese researchers. As a bioactive composite extracted from dried *P. americana*, *Xinmailong* Injection (XML) was authorized to be produced by Tengchong Pharmaceutical Factory of China in 2006 (State Medical Permitment No. Z20060443). After the production, XML was shown to protect cardiovascular cells from the hypoxia-ischemic myocardial injury in rat (Huang et al., 2009) and ameliorate human heart failure by markedly increasing left ventricular ejection fraction and decreasing the left ventricular end systolic volume index (Ma et al., 2013). However, the effects of XML on vascular system have not been

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systematically investigated yet. In the present study, we sought to investigate the effect of XML on vascular tone in rat aorta, as well as the underlying mechanisms which may be involved.

Materials and Methods

Chemicals and Drugs

XML (Lot.: 120228) was provided by Tengyao Pharmaceutical Company Limited by Shares Yunnan. Acetylcholine (ACh) and phenylephrine (PE) were purchased from Sigma Aldrich Inc. (St. Louis, MO, USA). The other reagents were of analytical purity.

Experimental Animals Used

Male Sprague-Dawley rats (200-300 g) were supplied from the Laboratory Animal Unit of Kunming Medical University (Kunming, China). All experiments performed in this study were approved by the Committee on the Use of Live Animals in Teaching and Research of Yunnan Minzu University.

Tissue Preparation and Isometric Tension Measurement

Rats were anesthetized with pentobarbitone sodium (50 mg/kg, i.p.) and then sacrificed by stunning and cervical dislocation. The thoracic aorta were dissected out and immediately transferred to a dish filled with Krebs solution (mM: 118 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$, and 11.1 glucose). The aorta were cleaned of adhering fat and connective tissue and then cut into 3 mm wide rings. Care was taken to avoid abrading the intimal surface in order to maintain the integrity of the endothelial layer. The aortic rings were aerated with 5% CO_2/95% O_2 and connected to a force transducer (HV-4, TME Technology Co, Ltd., Chengdu, China). The rings were stretched progressively to their optimal resting tension (2.5 g; determined in preliminary experiments) and allowed to equilibrate for 60 min. The absence of vasodilatory responses to ACh was taken as evidence that vessel segments were functionally denuded of endothelium.

Protocol 1*: Effect of XML on Vascular Tone in Rat Aorta.* In this series of experiments, the aortic rings with or without endothelium were administrated with the cumulative addition of XML (0-500 mg/L) to obtain the concentration-response curve.

Protocol 2: *Mechanisms Underlying the XML-induced Vasoconstriction in Rat Aorta.* In order to verify the mechanisms involved in the vasoconstrictive effect of XML, the following three studies were performed. In the first set of program, aortic rings were washed with Ca^{2+}-free Krebs solution for five times before the application of PE and the following cumulative administration of XML. In the second set of program, an attempt was made to verify that Ca^{2+} influx is involved in the XML-induced vasoconstriction. Aortic rings were washed with Ca^{2+}-free Krebs solution for five times before the application of PE, and then Ca^{2+} was added cumulatively to obtain a concentration-response curve (10 μM-3 mM). XML (500 mg/L) was administrated 10 min before the addition of Ca^{2+}. In the third set of experiment, the aim was to clarify whether the vasoconstriction induced by XML relates to the activation of the intracellular Ca^{2+} release. The rings were exposed to Ca^{2+}-free solution with 50 μM EGTA for 15 min before the application of PE to induce the first transient contraction (Con1). The rings were then washed with normal Krebs solution for three times and incubated for at least 40 min for refilling of the intracellular Ca^{2+} stores. Subsequently, the medium was rapidly replaced with Ca^{2+}-free solution again and the rings were incubated for another 15 min. The second contraction (Con2) was then induced by 1 μM PE in the absence or presence of XML (500 mg/L) which was added 10 min before PE application. The ratio of the second contraction over the first contraction (Con2/Con1) was calculated.

Systemic Blood Pressure (SBP) and Cytokines Measurements

XML was administered to rats via tail vein by intravenous injection at a volume of 0.4 ml under sterile conditions daily. The control groups

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received normal saline only. After 3 days of treatment, rats were anesthetized by an injection of pentobarbitone sodium (50 mg/kg, i.p.) and then silastic catheters (0.05-mm ID, Dow Corning, Midland, MI) were inserted into the carotid arteries of the rats to record SBPs. Then blood samples were collected from the thoracic aortas. The levels of NO, endothelin-1 (ET-1) and PGI_2 in serum samples were measured with ELISA kits (Cusabio Biotech Co., Ltd, Wuhan, China) according to the manufacturer's instructions.

Proteins Extractions

After blood samples were collected, the thoracic aorta were dissected out from rats and then cut into small pieces and homogenized on ice in lysis buffer (containing 0.02 M Tris-HCl, 1 % Triton X-100, 0.15 M NaCl, 1 mM ethylenediamine tetraacetic acid, 1 mM ethylene glycol tetraacetic acid, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate) supplemented with a cocktail of protease inhibitors. The mixture was centrifuged at 5000 rpm for three minutes at 4°C and the supernatant was kept at -80°C until use. The protein concentration was determined spectrophotometrically using the Bradford protein assay reagent (Bio-rad, Hercules, CA, USA) with serial dilution of bovine serum albumin as the standard.

Western Blot Analysis

For gel electrophoresis, 20 μg of proteins were used. The samples were separated by SDS-PAGE (7.5%) at 200 V, 300 mA for 50 min. After transferring the proteins onto polyvinylidene fluoride membranes, the blotting was performed at 200 V, 300 mA for 45 min. Blocked with 5% dry milk at room temperature for 1 h, the membranes were then incubated with polyclonal L-type Ca^{2+} channels ($Ca_{v1.2}$, Cacna1c) antibody (1:1000, Cell Signaling Technology, Inc., Beverly, Mass, USA) in TBS overnight at 4°C. Then, the membranes were incubated with HRP-conjugated anti-rabbit antibody (1:2000 in TBS, room temperature, 1 h, Amersham Biosciences, Piscataway, NJ, USA). Bound secondary antibody was detected by chemiluminiscence (Amersham Biosciences) and exposed to X-ray film.

Statistical Analysis

In isometric tension recordings assay, changes in tension were expressed as an increasing percentage of the basal level. In western blotting assay, changes in protein levels were described as the ratio to β-actin. Data are means±S.E.M. Comparison between two groups was analyzed using Student's *t*-test. Comparison among three or more groups was analyzed using one-way ANOVA, $P<0.05$ was considered significant.

Results

Vasoconstrictive Effect of XML in Rat Aorta

XML induced 56.71±6.14% and 74.26±8.52% contraction in endothelium-intact and endothelium-denuded aortic rings, respectively (Fig. 1A). No significant difference was observed between the two groups.

Mechanisms Underlying the Vasoconstrictive Effect of XML

As showed in Fig. 1B, the vasoconstriction induced by XML in rat aortic rings was notably decreased in Ca^{2+}-free Krebs (18.42±5.97% induced by XML 500 mg/L, $P<0.05$ compared with control group: 69.63±9.42%). In Ca^{2+}-free Krebs solution, after PE induced a stable contraction Ca^{2+} was cumulatively (10 μM to 3 mM) added to the chamber and generated the increased tension in the rat aortic rings. After the aortic rings were pre-incubated with XML (500 mg/L) for 30 min before the application of PE the Ca^{2+}-dependent contractions were significantly enhanced (Fig. 1C, $P<0.05$ compared with the control group). Similar results were observed when studied the influence of XML on the intracellular Ca^{2+} release. As demonstrated in Fig. 1D, PE induced the first contraction in Ca^{2+}-free solution with 50 μM EGTA and subsequently induced the second contraction after the refill of the intracellular Ca^{2+} in normal Krebs solution followed by a replacement of the Ca^{2+}-free solution. The ratio of the second contraction over the first contraction was significantly increased after treatment with XML (500 mg/L,

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P<0.05, compared with the control group).

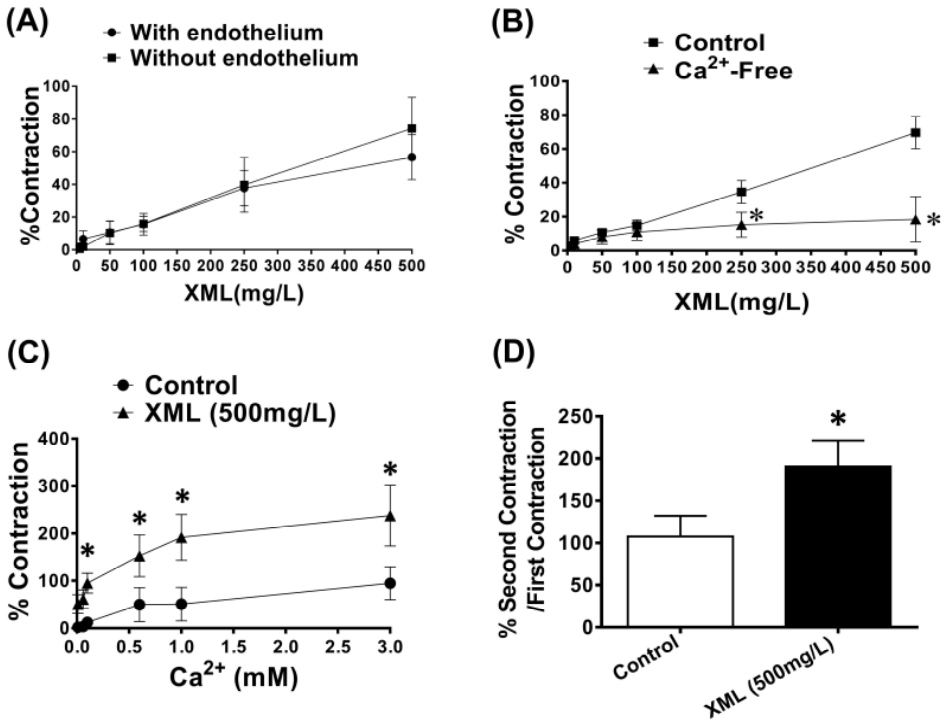

Figure 1: Vasoconstrictive effect and underlying mechanisms of XML on rat aorta. **(A)** Effects of XML on vascular isometric tension in rat aortic rings with or without endothelium. **(B)** Effects of XML on vascular isometric tension of rat aorta in Ca^{2+}-free Krebs. (C) Effect of XML on Ca^{2+} influx in rat aorta. In Ca^{2+}-free Krebs solution, after PE induced a stable aortic contraction Ca^{2+} (10 μM to 3 mM) was cumulatively added to the chamber and generated the increased tension in the rat aortic rings the absence (Control) or presence of XML (500 mg/L). **(D)** Effect of XML on intracellular Ca^{2+} release. PE induced the first contraction in Ca^{2+}-free solution with 50 μM EGTA and subsequently induced the second contraction after the refill of the intracellular Ca^{2+} in normal Krebs solution followed by a replacement of the Ca^{2+}-free solution. The ratio of the second contraction over the first contraction was measured in the absence (Control) or presence of XML (500 mg/L). Values are means ± S.E.M. (n = 6). **P*<0.05 compared with control group.

After rats were administrated with XML for 3 days, the levels of NO and ET-1 in blood samples were not significantly influenced (Fig. 2A and B). However, the levels of PGI_2 in blood samples were markedly elevated after treatment with XML (317.70±48.01 pg/ml, *P*<0.01, compared with control, Fig. 2C). As demonstrated in Fig. 3A and B, result of western blotting showed that the expressions of $Ca_{v1.2}$ in aorta were significantly increased after rats were treated with XML (500 mg/L) for 3 days.

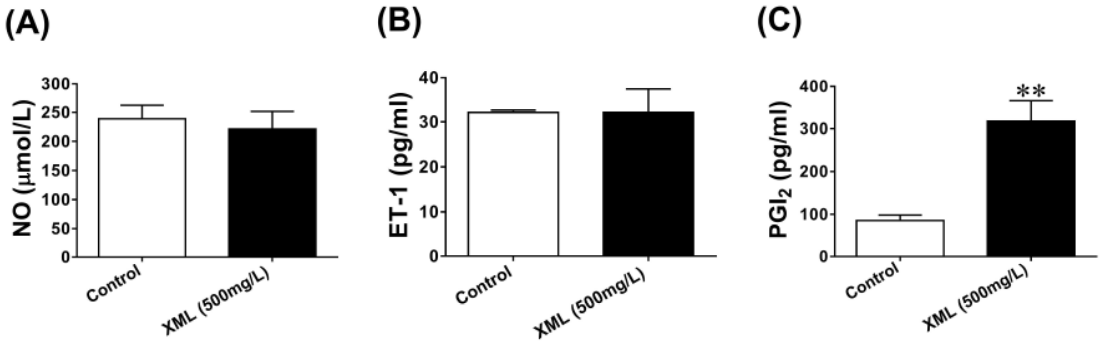

Figure 2: Effect of XML on the levels of the endothelium-derived cytokines in blood samples. Rats were intravenously administered with XML for 3 days. **(A)** Effect of XML on the level of NO. **(B)** Effect of XML on the level of PGI_2. **(C)** Effect of XML on the level of ET-1. Values are means±S.E.M. (n=6). ***P*<0.01 compared with control.

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Effect of XML on SBP in Rats

As shown in Fig. 3C, after the treatment of rats with XML (500 mg/L) for 3 days, the SBPs of rats were not notably influenced.

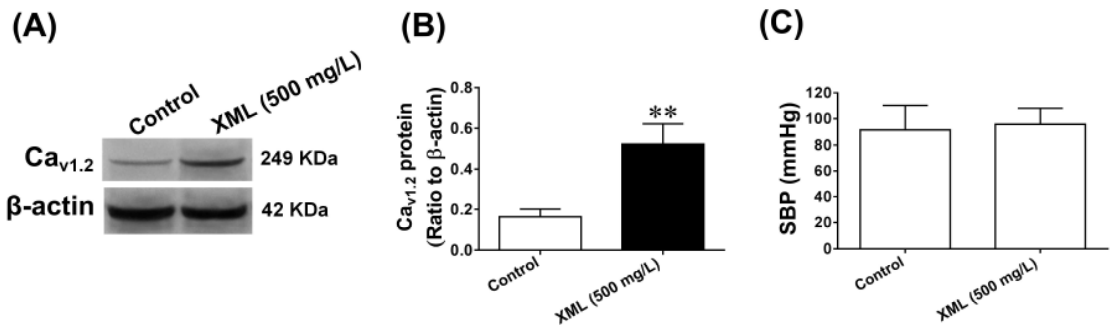

Figure 3: (A) Effect of XML on the protein expression of the α_1 subunit, the pore-forming subunit, of L-type Ca^{2+} channels ($Ca_{v1.2}$) in rat aorta. The bands present a representative result of five independent experiments. β-actin was used as a reference. **(B)** The amount of $Ca_{v1.2}$ protein as normalized to β-actin. Values are means±S.E.M. (n=5). $^{**}P<0.01$ compared with the control group. **(C)** Effect of XML on the systolic blood pressure (SBP) after rats were intravenously administered with XML for 3 days. Values are means±S.E.M. (n=6).

Discussion

The present study reveals a vasoconstrictive effect of XML in rat aorta. The extracellular Ca^{2+} influx and the intracellular Ca^{2+} release may play pivotal roles in the vasoconstrictive effect of XML. Ca^{2+} is a critical factor in excitation-contraction coupling in smooth muscle cells (Lohn et al., 2000; Wellman et al., 2003). There are two kinds of Ca^{2+} channels in vascular smooth muscle: receptor-operated Ca^{2+} channels (ROCCs) and voltage-dependent Ca^{2+} channels (VDCCs), which can be activated by PE (Xiong et al., 1995). As an α-adrenoreceptor agonist, PE causes aortic contraction through the release of Ca^{2+} from sarcoplasmic reticulum and by the Ca^{2+} influx through ROCCs. XML enhanced the Ca^{2+}-dependent contractions in rat aorta. This finding suggests that XML promotes the influx of Ca^{2+} through Ca^{2+} channels. Furthermore, XML increased Con2/Con1 caused by PE in Ca^{2+}-free solution, indicating that XML enhances the release of Ca^{2+} from sarcoplasmic reticulum. Influx of extracellular Ca^{2+} through Ca^{2+} channels and the release of Ca^{2+} from sarcoplasmic reticulum result in the increase of intracellular Ca^{2+} concentration. Subsequently, Ca^{2+} binds to calmodulin and activates a Ca^{2+}/calmodulin-dependent protein kinase known as myosin light chain kinase, which in turn induces the phosphorylation of myosin light chain. The phosphorylated myosin light chain promotes the interaction between myosin and actin, leading to the contraction of vascular smooth muscle (Rembold et al., 1988). Therefore, the activation of the extracellular Ca^{2+} influx through Ca^{2+} channels and the increase of the intracellular Ca^{2+} release may play pivotal roles in XML-induced vasoconstrictions.

Our results are in consistent with the findings of clinical study which showed XML greatly improved the left ventricular ejection fractions in patients with chronic heart failure (Ma et al., 2013). Currently, it is widely recognized that the force of muscle contraction generated in heart mostly depends on the Ca^{2+} influx as well as the release of Ca^{2+} from sarcoplasmic reticulum. Meanwhile, it is also well known that Ca^{2+} enters cell cytoplasm from extracellular space is mainly through L-type Ca^{2+} channels (Harvey et al., 2013; Treinys et al., 2008). In the present study, we also observed the up-regulatory effect of XML on α_1 subunit, the pore-forming subunit, of L-type Ca^{2+} channels in rat aorta. Therefore, it is reasonable to presume that XML could activate the L-type Ca^{2+} channels in cardiac muscle, which subsequently induces the Ca^{2+} influx and improves the cardiac functions. Endothelium is currently considered a central player for cardiovascular homeostasis by generating numerous biologically active autacoids with opposite effects, such as NO, PGI_2 and ET-1 (Yang et al., 2008). The endothelium-dependent vasodilatation is achieved by combined effects of endothelium-derived vasodilators, NO, PGI_2, and endothelium-derived hyperpolarizing factor (EDHF) (Garland et al., 2011). EDHF plays a significant contribution to the vasodilatations in smaller resistance arteries whereas NO, PGI_2 play greater roles in

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large-conduit arteries (Feletou et al., 2004). Meanwhile, the elevation of PGI_2 level is crucial in the management of pulmonary arterial hypertension (Buckley et al., 2013; Montani et al., 2014), which increases the afterload of the right ventricle and often induces the right-heart failure (Bogaard et al., 2009; Ryan et al., 2014). Since the subchronic treatment of XML (3d) significantly enhanced the level of PGI_2 in rat, our present study provides an important possibility that XML might exert it therapeutical effect on the right heart failure through affecting PGI_2 level of the patients.

Another interesting finding of the present study is that although XML significantly elevated the tensions of rat aorta, the SBPs of rats were not notably influenced after the rats were intravenously administered with XML. We presume that the counterbalance between the XML-induced elevation of PGI_2 level and the XML-produced vasoconstriction keeps the maintenance of SBP. This finding provides another possible interpretation why XML is considered as a "safe drug" to treat the heart failure (Ma et al., 2013).

Conclusion

We demonstrated an important role of XML in regulating vascular tone. The activations of the Ca^{2+} influx and the intracellular Ca^{2+} release may contribute to the vasoconstriction induced by XML. Our findings provide assistance to the better understandings of the mechanisms underlying the therapeutical effect of XML on heart failure. Meanwhile, the present study provided the preliminary data which may benefit the forthcoming investigation which will reveal the effective components of XML on vascular functions.

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