Effects of Total Alkaloids in *Buxus microphylla* Leaves on Aorta Smooth Muscle of Rats and Their Mechanisms

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Abstract: **Objective** To investigate the effects of total alkaloids in *Buxus microphylla* leaves (ABML) on isolated rats thoracic aorta rings, and then to explore the possible mechanisms underlying the effects. **Methods** Thoracic aortas of Wistar rats were isolated, removed, and mounted onto an organ bath. The effects of ABML at different concentration on the contraction of isolated thoracic aorta rings (with and without endothelium) precontracted with KCl or PE were observed with organ bath technique. Dose-effect curves of $CaCl_2$ were recorded by organ bath technique. The concentration of intracellular Ca^{2+} ($[Ca^{2+}]_i$) increased by PE, KCI, and caffeine in the presence of ABML was determined using Ca^{2+} sensitive fluorescence indicator Fura-2/AM loaded thoracic aorta vascular smooth muscle (VSM) cells of rats. **Results** In aorta rings precontracted with PE and KCl, ABML produced concentration-dependent relaxation in both intact and denuded endothelium ring groups. There was no difference in the inhibition of contraction between the intact and denuded endothelium ring groups at the same concentration. Exposure of isolated thoracic aorta rings to ABML led to a significant reduction in the contracting response induced by $CaCl_2$, and shifted the cumulative concentration-response curves to right. ABML could significantly inhibit the extracellular Ca^{2+} influx induced by PE and KCl under $[Ca^{2+}]_0$ of 1.5 mmol/L, with inhibitory ratios of 40.2% and 49.9%, respectively. In the case of Ca^{2+}-free, ABML could significantly inhibit the intracellular Ca^{2+} release induced by PE, with inhibitory ratio of 72.4%. **Conclusion** ABML relaxes thoracic aorta VSM cells by suppressing influx of extracellular Ca^{2+} via voltage-dependent Ca^{2+} channel and receptor-operated Ca^{2+} channel.

Key words: Ca^{2+}; Fura-2/AM; isolated thoracic aorta rings; total alkaloids of *Buxus microphylla* leaves; vascular smooth muscle cells

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Introduction

Cyclovirobuxine D (CVB-D, Huangyangning), an alkaloid from the roots of *Buxus microphylla* Sieb. et Zucc. var. *sinica* Rehd. et Wils (also known as boxwood), is a new drug developed in China in recent years for the treatment of cardiovascular diseases. Clinical application shows that CVB-D has beneficial effects on arrhythmia, angina, coronary heart disease, heart failure, and other cardiovascular disorders (Tan, Gu, and Wu, 2005). Currently, pharmacological study on CVB-D has been conducted extensively. However, biological activities of the active ingredients (alkaloids, glycosides, and sugars) in the leaves of boxwood plants have not been reported.

B. microphylla extract from fresh leaves (BMA) and *B. microphylla* extract from dried leaves (BMB) were obtained by different extraction methods from *B. microphylla* leaves produced in Guilin, Guangxi Province, and we conducted preliminary pharmacological studies. The results show that both BMA and BMB have the significant contractions on isolated guinea pig ileum and rabbit vaginal smooth muscle (Zhang and Huang, 2005); BMA and BMB have the significant antihypertensive effects on the blood pressure

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in the anesthetized dogs and rabbits.

The aims of this paper were to test the pharmacological effects of total alkaloids in *B. microphylla* leaves (ABML) on rat thoracic aorta rings *in vitro* and to explore their mechanisms using cell culture.

Materials and methods

Drugs and reagents

Adrenal Phenylephrine Hydrochloride Injection (20060908) was purchased from Shanghai Harvest Pharmaceutical Co., Ltd.; Acetylcholine (036K0151), bovine serum albumin (BSA), and Fura-2/AM (126K1591) were purchased from Sigma; ABML was provided by Department of Pharmacology of Guilin Medical College; CVB-D (200501018) was purchased from Henan Kaifeng Pharmaceutical Co., Ltd.; FBS (91108001) and 0.25% trypsin-EDTA (500050) were from GIBCO; Caffeine (0176B030) was US production.

Experimental devices

HV—4 *in vitro* Tissue and Organ Perfusion System; HW—1000 Super-heated Water Bath System, BL—410 Biological Function Experimental System (Chengdu Thai Union Technology Co., Ltd., China); JH—2 Tension Transducer (Beijing Space Medical Engineering Institute, China), CO_2 Incubator [US NAPCO (series 5400)], SW-CJ-1F-type Clean Bench (Suzhou Antai Air Technology Co., Ltd., China), CK40 Inverted Microscope (Olympus, Japan); RF—510 Fluorescence Spectrophotometer (Shimadzu, Japan), and 550 Microplate Reader (Bio-Rad, USA) were used.

Experimental animals

Healthy male Wistar rats, weighing 150－180 g, were from Animal Center of Institute of Radiation Medicine, Chinese Academy of Medical Sciences, Animal Certificate of Conformity [SCXK (Beijing) 2005-001]. All experiments were conducted in accordance with the *National Institute of Health Guide for the Care and Use of Laboratory Animals*.

Rat thoracic aorta experiments

The dish filled with cold Krebs Henseleit (KH) solution was placed on ice and passed through 95% O_2 and 5% CO_2 gas mixture for ready. Healthy male Wistar rats were killed after cervical dislocation, the chest was opened, and the thoracic aorta was quickly removed, isolated, and cut into 3 mm wide aortic rings. Endothelial cells were removed from aortic rings by mechanical method. Specimens were fixed in the bath containing KH solution at 37 ℃, and the bath continued to pass through 95% O_2 and 5% CO_2 gas mixture, kept stable for 15 min, the basic tension was adjusted from 0 to 2 *g* and maintained, then balanced for 1.5 h, and the solution was changed with the fresh 37 ℃ KH liquid every 15 min during the whole experiment. The changes of aortic vascular smooth muscle (VSM) were recorded by BL—410 Biological Function Experimental System.

After contraction of aorta link reached the peak value in PE 10^{-6} mol/L liquid, the integrity of the aorta endothelial cells was tested by adding acetylcholine (Ach, 10^{-5} mol/L). If Ach makes the aortic rings be precontracted by PE relax more than 80%, endothelial cell is considered complete, otherwise, incomplete, that is to say, the endothelial cell has been removed.

When PE (10^{-6} mol/L) or KC1 (60 mmo1/L) reached maximum effect on precontracted aortic rings, we added ABML cumulatively (final concentration at 4, 8, 16, 32, 64, 128, 256, and 512 μg/mL), once per 10 min, and at the same time observed its effect on endothelial integrity and endothelium tension of aortic rings; Then we added KH isometric solution to replace ABML as control; Acetic acid (HAc) group: joined acetate/KH solution instead of ABML isometric.

Aortic rings were preserved for 60 min in normal KH solution, rinsed for four times with Ca^{2+}-free KH solution, then kept stable for 20 min, pretreated with ABML (64 and 256 μg/mL) and isovolumic Ca^{2+}-free KH solution for 20 min after pretreatment. Then KCI (60 mmol/L) was added to polarization, and $CaCl_2$ was added with the cumulative method (final concentration at 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, and 3 mmol/L). Thus the effects of ABML on the contraction curves of aortic rings induced by $CaCl_2$ were observed.

Cell experiments

Cultured rat VSM cells Wistar rats were killed by cervical dislocation, thoracic aorta was rapidly removed, and adventitia of artery was carefully stripped. The arteries in the film were repeatedly cut into 1 mm^2 blocks, evenly placed in the bottom of the culture bottle, with 0.5 cm tissue spacing distance. The culture bottle

was filled with 4－5 mL high glucose DMEM culture medium containing 20% FBS, then placed in 37 ℃, 5% CO_2 incubator for 2－4 h, until the tissue was dried up and attached to the bottle wall. Then the culture bottle was slowly turned flat, so was completely immersed in tissue culture medium. The tissue was cultured in CO_2 incubator in absolute static status for the first time about 4－5 d, then medium was changed, once every 3－4 d. When the cells covered bottom of the bottle about 60%－80%, the culture flasks were filled with 0.25% trypsin-EDTA. As the cells were observed changing retract and round with cell gap increasing under the inverted microscope, DMEM medium containing 20% FBS was immediately added to terminate digestion, which was put and inoculated in a new culture flask. The medium was changed the next day, and then was changed once every 3－4 d.

Fura-2/AM loading and fluorescence measurement The VSM cells in logarithmic growth phase were digested into single cell suspension, and were collected by centrifugation to 1 × 10^6/mL density re-suspended into the cell suspension. When survival rate of VSM cells was more than 90% by Taiwan phenol blue exclusion method, cells could carry out the load Fura-2 at room temperature in dark.

Cell suspension (1 mL) was pre-heated at 37 ℃ for 5 min, and was oscillated at 37 ℃ for 50 min with 5 μL Fura-2/AM (final concentration at 5 μmol/L). Cell suspension was centrifuged at 1000 r/min for 5 min and washed for three times with D-Hanks solution containing 0.2% BSA. Cells were resuspended with D-Hanks solution and adjusted the cell density to 1 × 10^6/mL. The results were measured with RF-510 Fluorescence Spectrophotometer. The $[Ca^{2+}]_i$ of VSM cell was calculated according to the formula: $[Ca^{2+}]_i = K_d \times (R-R_{min})/(R_{max}-R) \times \beta$ (nmol/L). ABML was joined and incubated at 37 ℃ for 15 min, then stimulating agents KCl (60 mmol/L), PE (10^{-6} mol/L), and caffeine (10 mmol/L) were added for 5 min before the test.

Statistical methods The results were expressed as $\bar{X} \pm s$, carried on the statistical analysis by the SPSS15.0 statistics software and the data of multiple groups were compared using ANOVA, $P < 0.05$ indicated significant difference.

Results

Influence of ABML on aortic endothelial loop tension pretreated by PE

When ABML was cumulatively added from 4 to 512 μg/mL to intact and denuded-endothelium aorta rings pretreated by PE, significant vasodilation produced ($P < 0.01$, 0.05). The effect was enhanced with the increasing of the concentration, which showed a dose-response relationship, and as compared with the control group, the HAc group had no significant difference. And there was no difference in the inhibition between the intact and denuded endothelium rings at the same concentration (Figs. 1－3).

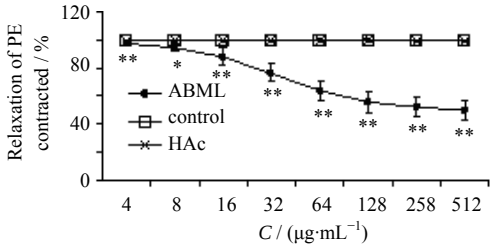

Fig. 1 Effect of ABML on intact endothelium aorta rings precontracted by PE ($\bar{X} \pm s$, n = 8)

$^*P < 0.05$ $^{**}P < 0.01$ *vs* control, same as below

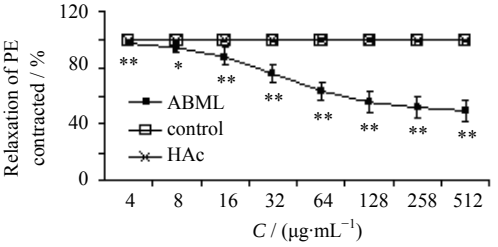

Fig. 2 Effect of ABML on denuded endothelium aorta rings precontracted by PE ($\bar{X} \pm s$, n = 8)

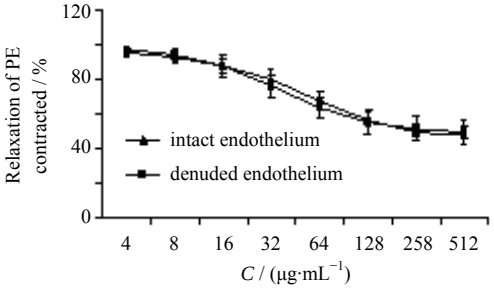

Fig. 3 Effect of ABML on intact and denuded endothelium aorta rings precontracted by PE ($\bar{X} \pm s$, n = 8)

Influence of ABML on aortic endothelial loop tension pretreated by KCl

When ABML was cumulatively added from 4 to 512 μg/mL to intact and denuded endothelium aorta

rings pretreated by KCl, significant vasodilation was produced ($P < 0.01$). The effect was enhanced with the increase of concentration, which showed a dose-response relationship, and as compared with the control group, the HAc group had no significant difference. And there was no difference in the inhibition between the intact and denuded endothelium rings at the same concentration (Figs. 4−6).

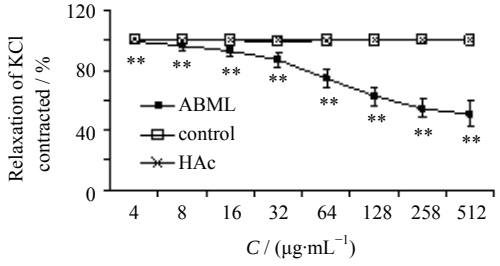

Fig. 4 Effect of ABML on intact endothelium aorta rings precontracted by KCl ($\bar{X} \pm s$, $n = 8$)

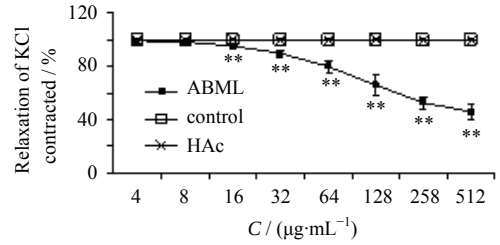

Fig. 5 Effect of ABML on denuded endothelium aorta rings precontracted by KCl ($\bar{X} \pm s$, $n = 8$)

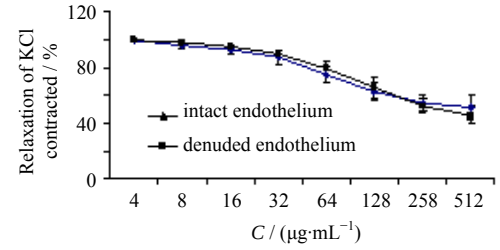

Fig. 6 Effect of ABML on intact and denuded endothelium aorta rings precontracted by KCl ($\bar{X} \pm s$, $n = 8$)

Effect of ABML on $CaCl_2$-induced contraction curves of aortic rings

In the KCl (60 mmol/L) KH depolarization Ca^{2+}-free medium, $CaCl_2$ (0.25−3.0 mmol/L) was cumulatively added, and aortic rings showed concentration-dependent contraction; After pretreated with ABML (64 and 256 μg/mL), the concentration-effect curve moved to the right. The maximum contraction amplitude decreased ($P < 0.01$); In addition to $CaCl_2$ (0.25 and 0.5 mmol/L), the effects of 256 μg/mL ABML were stronger than those of 64 μg/mL ($P < 0.05$, 0.01) (Fig. 7).

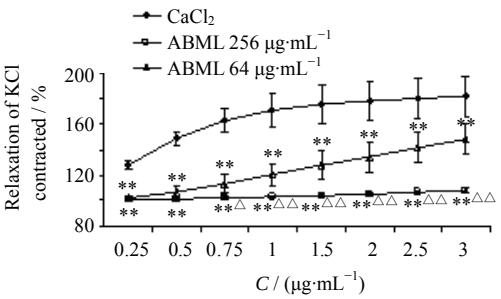

Fig. 7 Effect of ABML on $CaCl_2$ concentration-dependent curves ($\bar{X} \pm s$, $n = 8$)

$^{*}P < 0.05$ $^{**}P < 0.01$ *vs* $CaCl_2$; $^{\triangle\triangle}P < 0.01$ *vs* ABML 64 μg·mL^{-1}

Effects of ABML on Ca^{2+} influx of VSM cells induced by PE and KCl

When extracellular Ca^{2+} concentration ($[Ca^{2+}]_e$) was 1.5 mmol/L, ABML significantly inhibited the PE- and KCl-induced $[Ca^{2+}]_i$ increase, the inhibitory rates were 40.2% and 49.9%, respectively (Fig. 8).

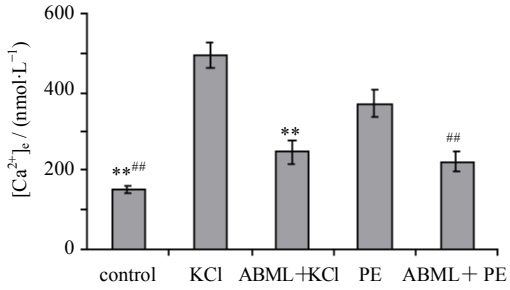

Fig. 8 Effects of ABML on $[Ca^{2+}]_e$ influx induced by PE and KCl ($\bar{X} \pm s$, $n = 8$)

$^{\#\#}P < 0.01$ *vs* PE $^{**}P < 0.01$ *vs* KCl

Effect of ABML on Ca^{2+} release in aortic VSM cells induced by PE and caffeine

When $[Ca^{2+}]_e$ was 0 mmol/L, ABML significantly inhibited PE-induced intracellular Ca^{2+} release with inhibitory ratio of 72.4%, while had no effect on the caffeine-induced release of intracellular Ca^{2+} (Fig. 9).

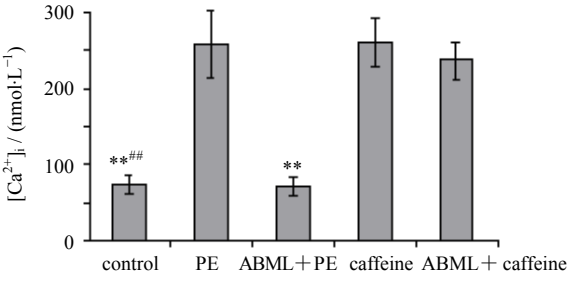

Fig. 9 Effects of ABML on intracellular Ca^{2+} release induced by PE and caffeine ($\bar{X} \pm s$, $n = 8$)

$^{**}P < 0.01$ *vs* PE $^{\#\#}P < 0.01$ *vs* caffeine

Discussion

The contraction of VSM cells and $[Ca^{2+}]_i$ are closely related. Intracellular Ca^{2+} come from the extracellular Ca^{2+} influx and Ca^{2+} release. Extracellular Ca^{2+} influx is primarily through receptor-gated Ca^{2+} channel (RGCC) and voltage-dependent Ca^{2+} channel (VDCC); And the release of intracellular Ca^{2+} is mainly from the sarcoplasmic reticulum. Within sarcoplasmic reticulum of VSM cells there are two Ca^{2+} release channels: the inositol triphosphate (IP_3) receptor and the receptor given by Renault (RyR). When these receptors are activated and open, they could lead Ca^{2+} to release from the sarcoplasmic reticulum, thereby increase $[Ca^{2+}]_i$, and cause the contraction of aortic rings (Ji, Benishm, and Pang, 1998).

When $[Ca^{2+}]_i$ of VSM cells increases to threshold after stimulation, Ca^{2+} and calmodulin (CaM) form Ca^{2+}-CaM complex, and then the complex combines with myosin light chain kinase (MLCK) and activates MLCK. Activated MLCK phosphorylates the 20-kD light chain of myosin on serine 19. Phosphorylation of myosin interacts with actin, leads to ATP in the myosin molecule activation, and results in the contraction of actin. When the Ca^{2+} concentration decreased to 10^{-7} mmol/L, CaM and MLCK would separate and the activity would disappear; And phosphorylated MLC is dephosphorylated by myosin light chain phosphatase (MLCP), separates with actin, and restores muscle relaxation state (Bitar, 2003; Christopher, 1992).

This study found that ABML had a significant concentration-dependent vasodilation on PE and KCl precontraction of intact denuded and endothelium aortic rings; And the relaxation had no statistical difference between the two.

The results show that the role of ABML on aorta relaxation is not dependent on vascular endothelial function, or may not be related to the vascular endothelium-derived relaxing factors, e.g. nitric oxide. ABML may have a direct effect on VSM resulting in vasodilation.

High potassium mainly causes VSM cells depolarization, activates VDCC in the membrane, and makes the extracellular Ca^{2+} influx also induce cell Nei Leinuo set (ryanodine)-sensitive Ca^{2+} pool release, the so-called Ca^{2+}-induced Ca^{2+} release (CICR). As a result, the intracellular Ca^{2+} increased, and the contraction was enhanced (Zhang *et al*, 2007). PE activates the phospholipase C mainly by acting on the α_1 adrenergic receptor, and generates IP_3 and diacylglycerol, which induces another IP_3-sensitive Ca^{2+} pool release intracellular Ca^{2+}, PE could also enhance cell Ca^{2+} influx by activating RGCC in membrane. It was found that ABML had significantly concentration-dependent vasodilation on aortic rings pretreated by KCl and PE, suggesting ABML might inhibit extracellular Ca^{2+} influx caused by the high K^+ and PE or inhibit the two different intracellular Ca^{2+} pools release Ca^{2+}, and play a role in vascular relaxation. That pretreatment with ABML could make $CaCl_2$ dose-response curve move to the right and further confirm that blocking Ca^{2+} influx is one of the main mechanisms.

To further explore the mechanism of ABML on vasodilation, this experiment has carried on primary culture and subculture of aorta thoracicalis blood VSM cells in rats, aiming at further exploring its vasodilatory mechanism from the cellular level. It was found that in the cell suspension if $[Ca^{2+}]_i$ was 1.5 mmol/L, ABML pretreatment could significantly reduce $[Ca^{2+}]_i$ induced by PE and KCl, which further showed that ABML vasodilatation related to the inhibition on VDCC and RGCC.

In the extracellular Ca^{2+}-free conditions, Ca^{2+}, which triggers cell contraction, mainly comes from intracellular Ca^{2+} pool. In the extracellular Ca^{2+}-free conditions, PE increased $[Ca^{2+}]_i$ mainly through inducing Ca^{2+} release from the IP_3-sensitive Ca^{2+} pool (Shin *et al*, 2005). ABML pretreatment could significantly inhibit the PE-induced intracellular Ca^{2+} release, suggesting that ABML might inhibit Ca^{2+} release from the IP_3-sensitive Ca^{2+} pool. High concentration of caffeine is a channel agent which could open ryanodine receptor, and it could make the ryanodine-sensitive Ca^{2+} pool release Ca^{2+}, resulting in shrinkage (Noguera *et al*, 1998). It was found that caffeine in the extracellular Ca^{2+}-free conditions could induce $[Ca^{2+}]_i$ significant increase, but $[Ca^{2+}]_i$ did not change obviously with ABML pretreatment. We speculate that ABML does not affect the Ca^{2+} release from the ryanodine-sensitive Ca^{2+} pool induced by caffeine, and its role in relaxing blood vessels may be unrelated to RyR.

In summary, ABML have obvious relaxant effect

on the vasoconstriction which is caused by PE and the high potassium, and assumes dose-effect relationship, and their function has nothing to do with the blood vessel burst and BMLA may cause the $CaCl_2$ quantity effect curve right lateral, then the biggest response be reduced. The vasodilatation of ABML has nothing to do with inhibition of $[Ca^{2+}]_i$, whereas the decrease of $[Ca^{2+}]_i$ was primarily caused by inhibiting the Ca^{2+} influx mediated by VDCC and RGCC, and inhibiting Ca^{2+} release from the IP_3-sensitive Ca^{2+} pool, but has nothing with ryanodine receptors. However, how ABML affects VDCC and RGCC as well as IP_3 receptors would wait to be further studied using the patch clamp technique.

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