Full Length Research Paper

Vasomotor effect of cyclovirobuxine D and its underlying mechanisms

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To investigate the vasomotor effects of Cyclovirobuxine D (CVB) on the rat aortic rings *in vitro* and underlying mechanisms, rat aorta was obtained, and aortic rings with or without endothelium were pre-treated with potassium chloride (KCl) or phenylephrine (PE). Then, CVB was added and its vasomotor effects were observed. In addition, the influence of other drugs on the CVB induced vasomotor effects was detected. The effects of CVB and verapamil (VER) on the dilation of the aortic rings were observed. After incubation in Ca^{2+} free solution containing CVB, the KCl pre-treated aortic rings were treated with $CaCl_2$ aiming to investigate the effects of CVB on the influx of extracellular calcium, or with PE and caffeine to observe the effects of CVB on the release of intracellular calcium. In aortic rings precontracted with PE or KCl, CVB produced concentration-dependent relaxation in both endothelium-intact and denuded rings. But the dilation of rings with intact endothelium was more potent than that of rings without endothelium. In intact rings, L-NAME (100 µmol/L) or indomethacin (10 µmol/L) reduced the degree of CVB-induced relaxation. CVB (3.2×10^{-4} mol/L) and VER (1 µmol/L) could induce the dilation of KCl precontracted rings and these effects could be partially reversed by $CaCl_2$ (1.25 mmol/L). In Ca^{2+} free solution, calcium-dependent KCl contractions were inhibited by CVB (3.2×10^{-4} mol/L). Furthermore, CVB (3.2×10^{-4} mol/L) inhibited the PE (1 µmol/L) induced contraction of aortic rings, but had no effect on the caffeine (10 mmol/L) induced contraction. CVB vasodilates by endothelium-dependent and endothelium-independent mechanism, Endothelium-dependent involving nitric oxide or prostaglandin released from the endothelium. Its endothelium-independent vasodilation probably occurs via the suppression of voltage- sensitive Ca^{2+} channel and inhibition of IP3-sensitive Ca^{2+} released in vascular smooth muscles, but has no effect on ryanodine receptor.

Key words: Cyclovirobuxine D, vasomotor effects, Ca^{2+}.

INTRODUCTION

Cyclovirobuxine D (CVB) is an active compound extracted from Buxus microphylla and a derivative of steroid alkaloid. Its molecular formula is $C_{26}H_{46}N_2O$. Clinically, it has dual-directional regulation on blood pressure. Animal study showed CVB could dilate coronary artery in pigs (Grossini et al., 1999). To date, the exact mechanisms underlying the vasomotor effects of CVB have not been studied deeply. The present study aimed to investigate the mechanisms involved in the direct

vasomotor effects of CVB on rat aorta.

MATERIALS AND METHODS

Main reagents

CVB was purchased from Nanjing Xiaoying Pharmacology Co. Ltd. It is white crystalline powder, odorless, and bitter in taste and the purity is about 99.47%. CVB was dissolved in Krebs-Henseleit (K-H) solution containing 5 l/ml acetic acid at a concentration of 250 mmol/L immediately before use. Phenylephrine (PE), acetycholine (ACh), N -nitro-L-arginnine-methyl-ester, (L-NAME), Indomethacin, and Verapamil were purchased from Sigma, USA and Caffeine from Hangzhou Mingsheng Pharma. The K-H solution included 118.3 mmol/L NaCl, 4.74 mmol/L KCl, 1.18 mmol/L K_2HPO_4, 1.2 mmol/L

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MgSO$_4$, 24.9 mmol/L NaHCO$_3$, 1.25 mmol/L CaCl$_2$, and 11.1 mmol/L glucose. In the Ca^{2+} free K-H solution, the components were identical to those in complete K-H solution except the CaCl$_2$ was replaced with 50 µmol/L EGTA.

Experimental animals

Forty male Sprague-Dawley (SD) rats (specific pathogen free) weighing 220 to 260 g were purchased from the Animal Center of Zhejiang College of Traditional Chinese Medicine. This study was approved by the Ethics Committee of Sir Run Run Shaw Hospital.

Preparation of rat thoracic aortic rings

The animals were sacrificed by cervical dislocation followed by thoracotomy. The thoracic aorta was obtained and placed in 4°C K-H solution. The fat and connective tissues on the thoracic aorta were removed and then the thoracic aorta was cut into segment (aortic rings) 2 to 3 cm in length. For the preparation of rings without endothelium, the tips of a forcep were rotated several circles in the segment to remove the endothelium. These rings were placed in 10-ml organ baths containing K-H solution. The bath fluid was ventilated continuously with a mixture 5% of CO$_2$ and 95% O$_2$, and the temperature was maintained at 37°C. One end of the segment was fixed and the other end connected to a tension transducer which was then connected to the MedLab bio-signal acquisition and processing system (Nanjing Medease Science and Technology Co., Ltd). This system was used to record the tone. The load was initiated with 0 g and the segment was allowed to equilibrate for a period of 15 min. Then, the load was changed into 2 g and the segment was allowed to equilibrate for a period of 1 h during which K-H solution was refreshed every 15 min. After the end of equilibration period, KC1 (60 mmol/L) was used to test the responsiveness of these rings. When a steady state in the response of contraction was reached, the fluid was refreshed with K-H solution and this procedure was repeated three times to maximize the contraction and achieve the more steady state. When the steady state of contraction was reached, PE (1 µmol/L) was used to induce the contraction and then ACh (10 µmol/L) to induce the dilation of aortic rings to test the integrity of endothelium. Endothelium-intact rings responding to ACh by relaxation more than 80% were considered to have functionally intact endothelium and complete lack of relaxation response to ACh confirms the removal of endothelium.

Contractile and diastolic function of rat thoracic aortic artery rings

After confirmation of endothelial integrity, the aortic rings were washed with K-H solution several times until a return to the resting tone of the arterial rings was achieved. Then, PE (1 µmol/L) or KCl (80 mmol/L) was used to induce the maximal contraction (maximal tone). The ratios of vascular tension after treatments with other drugs to the maximal tension after PE or KCl treatment were used to delineate dose-response curve of vascular activity. The vascular tone after treatments with other drugs was normalized to that after the third KCl treatment and expressed as percentages.

Direct vasomotor effects of CVB

Vasomotor effects of CVB on the aorta with intact endothelium

Vasomotor effects of CVB on the aorta at baseline: (a) CVB group: Cumulative dosing procedure was performed when a steady state

was reached. CVB was added at an interval of 10 min at concentrations of 10^{-5}, 2×10^{-5}, 4×10^{-5}, 8×10^{-5}, 1.6×10^{-4}, 3.2×10^{-4} and 6.4×10^{-4} mol/L, and then the vasomotor effect was observed. (b) Control group: The CVB solution was replaced with K-H solution of equal volume.

Vasomotor effects of CVB on the PE pre-treated aorta: (a) CVB group: When a steady state was reached, PE (1 µmol/L) was added to induce the contraction. Then, CVB was administrated at the concentrations of 10^{-5}, 2×10^{-5}, 4×10^{-5}, 8×10^{-5}, 1.6×10^{-4}, and 3.2×10^{-4} mol/L and the vasomotor effect was detected. (b) Acetic acid group: After PE treatment, the CVB was replaced with 5 µl/ml acetic acid in K-H solution of equal volume. (c) Control group: The CVB was replaced with K-H solution of equal volume.

Vasomotor effects of CVB on the KCl pre-treated aorta: (d) CVB group: When a steady state was reached, KCl (80 mmol/L) was added to induce the contraction. Then, CVB was administrated at the concentrations of 10^{-5}, 2×10^{-5}, 4×10-5, 8×10^{-5}, 1.6×10^{-4}, and 3.2×10^{-4} mol/L and the vasomotor effect was detected. (e) Acetic acid group: After KCl treatment, the CVB was replaced with 5 µl/ml acetic acid in K-H solution of equal volume. (f) Control group: The CVB was replaced with K-H solution of equal volume.

Vasomotor effects of CVB on the aorta with endothelium

Vasomotor effects of CVB on the PE pre-treated aorta: (a) CVB group: When a steady state was reached, PE (1 µmol/L) was added to induce the contraction. Then, CVB was administrated at the concentrations of 10^{-5}, 2×10^{-5}, 4×10^{-5}, 8×10^{-5}, 1.6×10^{-4}, 3.2×10^{-4} and 6.4×10^{-4} mol/L and the vasomotor effect was detected. (b) Acetic acid group: After PE treatment, the CVB was replaced with 5 µl/ml acetic acid in K-H solution of equal volume. (c) Control group: The CVB was replaced with K-H solution of equal volume. Vasomotor effects of CVB on the KCl pre- treated aorta: (d) CVB group: When a steady state was reached, KCl (80 mmol/L) was added to induce the contraction. Then, CVB was administrated at the concentrations of 10^{-5}, 2×10^{-5}, 4×10^{-5}, 8×10^{-5}, 1.6×10^{-4}, 3.2×10^{-4} and 6.4×10^{-4} mol/L and the vasomotor effect was detected. (e) Acetic acid group: After KCl treatment, the CVB was replaced with 5 µl/ml acetic acid in K-H solution of equal volume. (f) Control group: The CVB was replaced with K-H solution of equal volume.

Factors affecting the vasomotor effects of CVB

For aortic rings with intact endothelium: (a) L-NAME group: Aortic segment was pre-treated with nitric oxide synthase (NOS) inhibitor L-NAME (100 µmol/L) for 20 min. When a steady state was reached after KCl (80 mmol/L) treatment, CVB was administrated at the concentrations of 10^{-5}, 2×10^{-5}, 4×10^{-5}, 8×10^{-5}, 1.6×10^{-4}, 3.2×10^{-4} and 6.4×10^{-4} mol/L and the vasomotor effect was detected. (b) Indomethacin group: Aortic segment was pre- treated with prostaglandin synthetase inhibitor indomethacin for 20 min. When a steady state was reached after KCl (80 mmol/L) treatment, CVB was administrated at the concentrations of 10^{-5}, 2×10^{-5}, 4×10^{-5}, 8×10^{-5}, 1.6×10^{-4}, 3.2×10^{-4} and 6.4×10^{-4} mol/L and the vasomotor effect was detected.

Role of calcium in the vasomotor effects of CVB on rat aortic rings (removal of endothelium)

Aortic rings were pre-treated with 80 mmol/L KCl to induce contraction. When a steady state was reached, (a) aortic rings were treated with 3.2×10^{-4} mol/L CVB, and then with CaCl$_2$ (2.5 mmol/L) in K- H solution. The vasomotor effects were observed; (b) Aortic rings were treated with 1 µmol/L verapamil and then with CaCl$_2$. The vasomotor effects were observed. When a steady state was
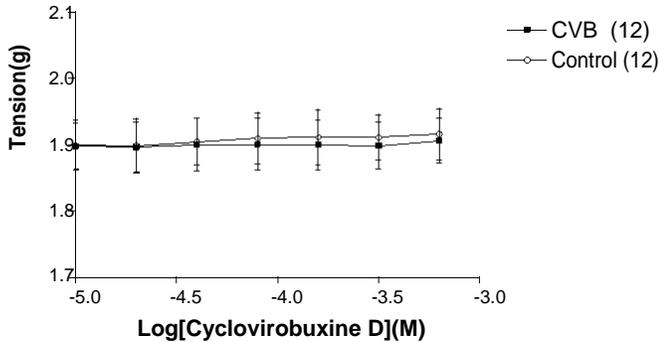
Figure 1. Vasomotor effect of CVB on the aortic rings at baseline. CVB was added to the organ bath at an interval of 10 min with an accumulation method. There was no significant difference between CVB group and control group (P>0.05).

reached, aortic rings were washed with Ca^{2+} free K-H solution and immersed in Ca^{2+} free K-H solution for 20 min for equilibration. (a) These rings were pre-treated with 3.2×10^{-4} mol/L CVB and then with KCl in Ca^{++} free K-H solution. Finally, $CaCl_2$ was added to the solution at a final concentration of 1.25 mmol/L to induce contraction; (b) control group: The CVB was replaced with K-H solution.

When a steady state was reached, aortic rings were washed with Ca^{2+} free K-H solution. Then, (a) these rings were pre-treated with 3.2×10^{-4} mol/L CVB for 20 min and then with 1 μmol/L PE; (b) Control group: These rings were pre-treated with K-H solution for 20 min and then with 1 μmol/L PE; (c) The rings were pre-treated with 3.2×10^{-4} mol/L CVB and then with 10 mmol/L caffeine; (d) Control group: The rings were pre-treated with K-H solution and then with 10 mmol/L caffeine.

Statistical analysis

Data were expressed as means ± standard deviation ($\bar{x}$ ±s) and comparisons between multiple groups were done with analysis of variance (ANOVA). A value of P <0.05 was considered statistically significant.

RESULTS

Direct vasomotor effects of CVB

Direct vasomotor effects of CVB at baseline

For the aortic rings with intact endothelium, treatment with CVB of different concentrations (10 to 640 μmol/L) did not affect the vascular tone at baseline (Figure 1).

Vasomotor effects of CVB on PE or KCl pre-treated aortic rings without intact endothelium

For the 1 μmol/L PE pre-treated aortic rings, CVB could induce the dilation in a dose dependent manner. However, the vascular tones were not significantly changed in acetic acid group and control group (Figure

2A). For the 80 mmol/L KCl pre-treated aortic rings, CVB also induced evident dilation in a dose dependent manner. But, the vascular tones were not markedly changed in acetic acid group and control group (Figure 2B).

Vasomotor effects of CVB on PE or KCl pre-treated aortic rings with removal of endothelium

For the 1 μmol/L PE pre-treated aortic rings with removal of endothelium, CVB could induce the dilation in a dose dependent manner. However, the vascular tones were not significantly changed in acetic acid group and control group (Figure 3A). For the 80 mmol/L KCl pre-treated aortic rings with removal of endothelium, CVB also induced obvious dilation in a dose dependent manner. But, the vascular tones were not markedly changed in acetic acid group and control group (Figure 3B).

Comparisons of vasomotor effects of CVB in aortic rings with or without endothelium

For PE or pre-treated aortic rings, CVB could induce the dilation in a dose dependent manner regardless of the presence of endothelium. However, the vasomotor effects of CVB in aortic rings with intact endothelium were stronger than those without endothelium (Figures 4A and B).

Vasomotor effects of CVB on L -NAME or indomethacin in combination with KCl pre-treated aortic rings with intact endothelium

After pre-treatment with L-NAME or indomethacin, the dilation of aortic rings with intact endothelium by CVB was decreased (Figure 4B).

Effects of CVB on the calcium channel

Effects of CVB and verapamil on the KCl induced contraction of aortic rings

Both CVB (3.2×10^{-4} mol/L) and verapamil (1 μmol/L) could dilate the contracted rings by KCl. But this effect was partially reversed by $CaCl_2$ (2.5 mmol/L) (Figure 5).

Effect of CVB on the influx of calcium after supplementing with calcium

After incubation with 3.2×10^{-4} mol/L CVB, the KCl treated aortic rings could contract after supplementing with 1.25 mmol/L $CaCl_2$ in the Ca^{2+} free solution. However, the contraction in control group was more potent than that in
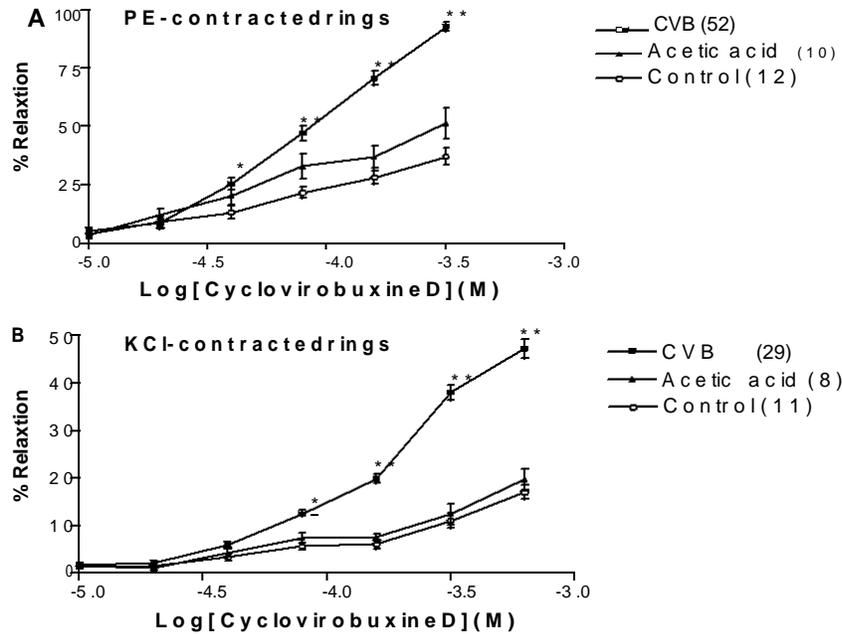
Figure 2. Vasomotor effect of CVB on the PE or KCl pre-treated aortic rings with intact endothelium. Aortic rings with endothelium were pre-treated with 1 mmol/L PE (A) or 80 mmol/L KCl (B). The maximal tones induced by PE or KCl were expressed as 100%. *P<0.05, ** P<0.01 vs control group.
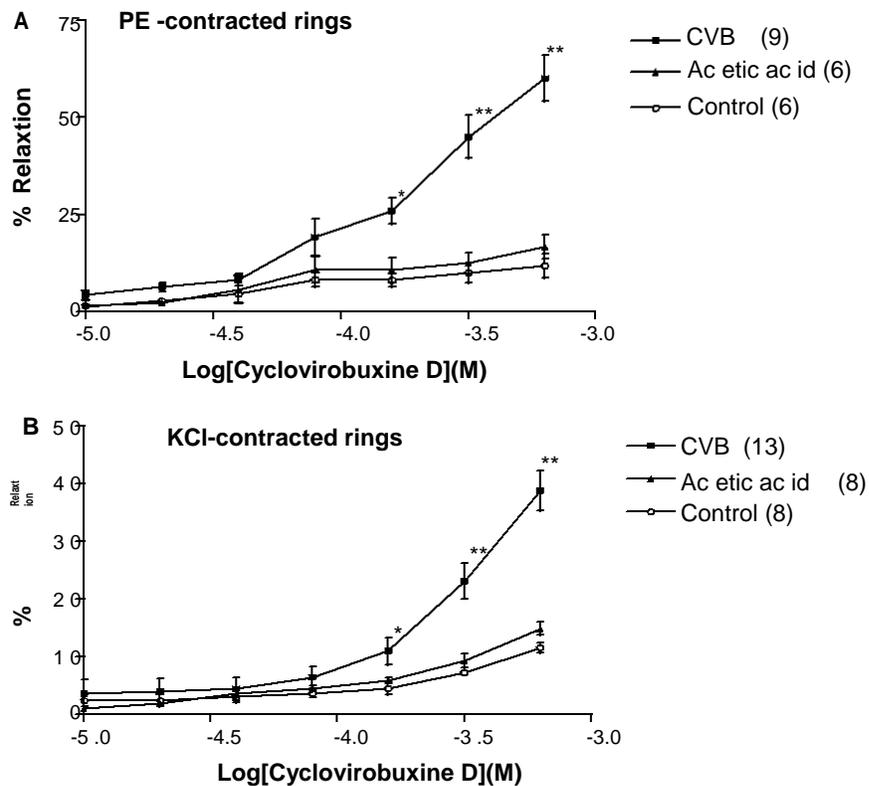
Figure 3. Vasomotor effect of CVB on the PE or KCl pre-treated aortic rings with removal of endothelium. Dose-response curves of the 1 mmol/L PE (A) or 80 mmol/L KCl (B) pre-treated aortic rings with the removal of endothelium. The maximal tones induced by PE or KCl were expressed as 100%. *P<0.05, **P<0.01 vs control group.
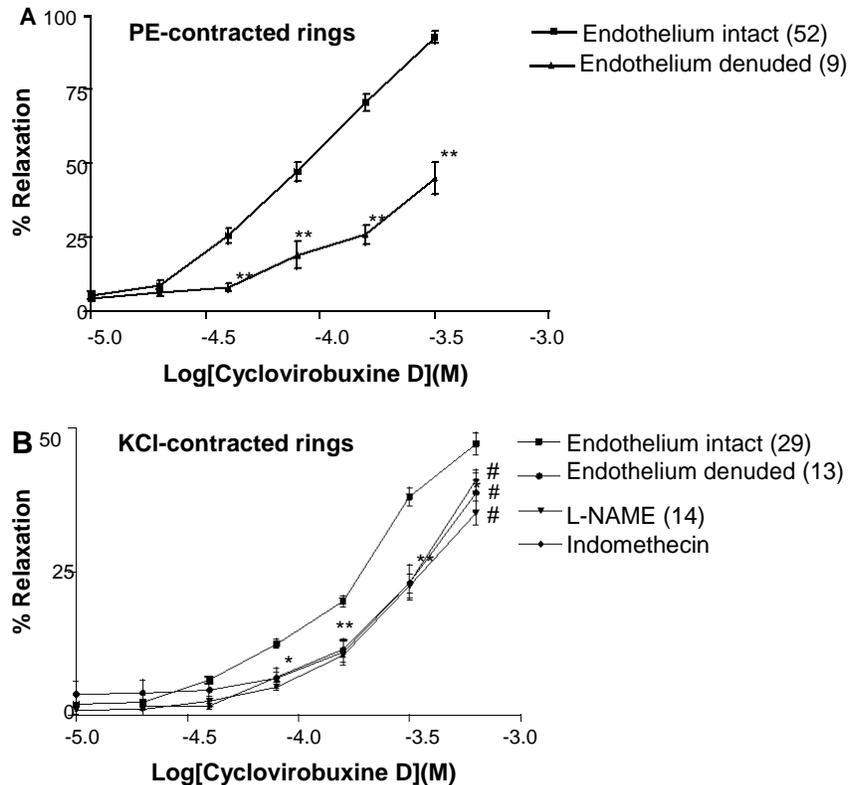
Figure 4. Vasomotor effects of CVB on the L-NAME or indomethacin pre- treated aortic rings with or without intact endothelium. Pre-treatment with 1 mmol/L PE (A) or 80 mmol/L KCl (B).
*P<0.05, **P<0.01 vs intact endothelium group. #P<0.05 vs intact endothelium group.

CVB pre-treated group (Figure 6).

Effect of CVB on the influx of calcium after supplement with PE or caffeine

The PE induced contraction was inhibited by the CVB in Ca^{2+} free solution in a CVB dose dependent manner (Figure 7). The caffeine induced contraction was not affected by the CVB in Ca^{2+} free solution (Figure 8).

DISCUSSION

CVB is a new drug that has been developed for the treatment of cardiovascular disease. Clinical trials have confirmed the dual-directional regulation on blood pressure and can exert anti-anginal effect. In the *in vivo* and *in vitro* experiments, Grossini et al. (1999) demonstrated that intravenous CVB could dilate the coronary vessels and increase the blood flow in a CVB dose dependent manner when the heart rate and aortic blood pressure remained stable. In addition, CVB could inhibit the KCl induced contraction of *in vitro* coronary

vessels, and the CVB induced dilation of coronary vessels was found to be related to the release of nitric oxide (NO) by endothelial cells. Hu et al. (2007) also indicated that cyclovirobuxine D significantly protected rat aorta endothelial cells against hypoxia and enhanced nitric oxide (NO) release from endothelial cells. The relaxation of vascular smooth muscle can be induced by endothelium dependent and independent relaxing factors. The later factors include cAMP, cGMP, prostaglandin, etc. In addition, blocking the calcium channel can also cause the relaxation of vascular smooth muscle due to influx of extracellular calcium or reduced or suppressed release of intracellular calcium.

The contraction of vascular smooth muscle is closely related to the intracellular calcium. High potassium level may lead to the depolarization of smooth muscle cells and activate the voltage-gated calcium channels (VOC) on the cells resulting in influx of extracellular calcium and release of intracellular calcium from ryanodine- sensitive calcium pool (calcium- induced calcium release, [CICR]), which finally increases the intracellular calcium level and subsequent contraction (Takeuchi et al., 2000; Leblanc et al., 1991). PE mainly acts on the $_1$-adrenergic receptor and then activates phospholipase C leading to the
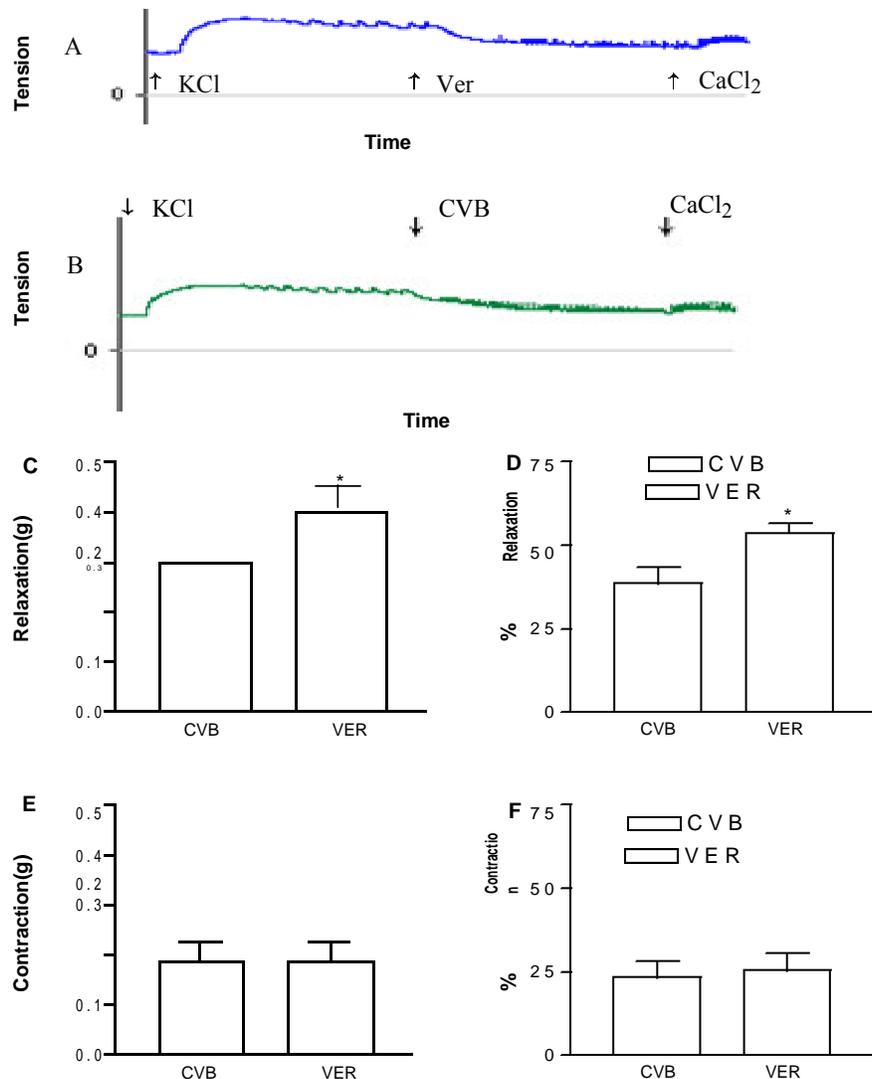
Figure 5. Response to verapamil (A) and CVB (B) in the aortic rings pre-treated with KCl, and then with $CaCl_2$. C and D showed the maximal relaxation induced by verapamil and CVB and E and F showed the maximal contraction induced by $CaCl_2$. Data in C and E are expressed in grams of developed tension. Data in D and F are expressed as percent of the maximal contractile response in normal K-H solution induced by KCl (80 mmol/L).

production of triphosphate inositol (IP_3). Subsequently, the release of calcium from IP_3 sensitive calcium pool increases leading to increased intracellular calcium level (Eckert et al., 2000; Kobayashi et al., 1991). Additionally, PE can also activate receptor-gated calcium channel (ROC) on the cells and phosphorylate VOC resulting in influx of extracellular calcium (Ford and Broadley, 1999).

The present study investigated the effects of CVB on the *in vitro* rat aortic rings. Results showed CVB did not affect the vascular tone at baseline. For PE or KCl pre-treated aortic rings with and without endothelium, CVB could induce the dilation of these rings. But the dilation of rings with intact endothelium was more potent than that of rings with removal of endothelium (Furchgott and Zawadzki,

1980). This result suggests the endothelium is not necessary for the CVB induced dilation but can increase this dilation. Thus, vascular endothelial cells partially contribute to the regulation of vascular tone by CVB. The suppression of NO release is a main cause of reduction of endothelium related dilation (Palmer et al., 1987, 1988; Moncada et al., 1976). Furthermore, after NOS inhibitor (L-NAME) treatment, the CVB induced dilation was partially reduced. Our results were consistent with those of Grossini et al. (1999) who also demonstrated the role of NO release from endothelial cells in the CVB induced dilation of coronary vessels. The reduction of endothelium dependent dilation may be also attributed to suppressed production of prostaglandin by endothelial cells (Moncada
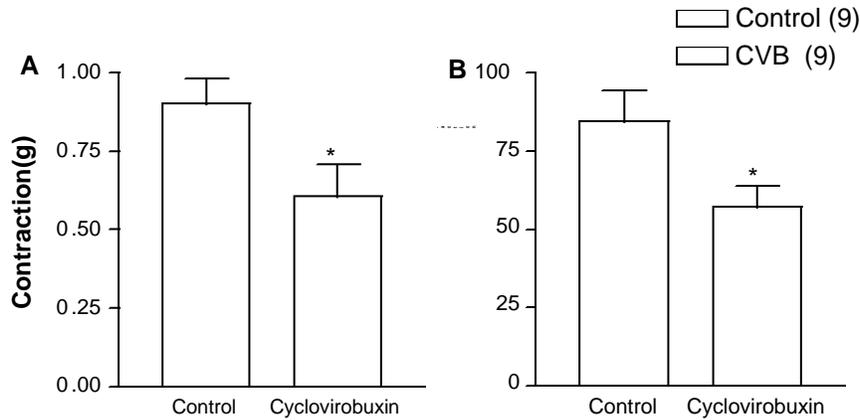
Figure 6. Maximal contraction induced by KCl (80 mmol/L) in the absence (control) and in the presence of CVB. Data in A are expressed in grams of developed tension. Data in B are expressed as percent of maximal contractile response in normal K-H solution induced by KCl (80 mmol/L).
*P<0.05, and **P<0.01 vs control group.
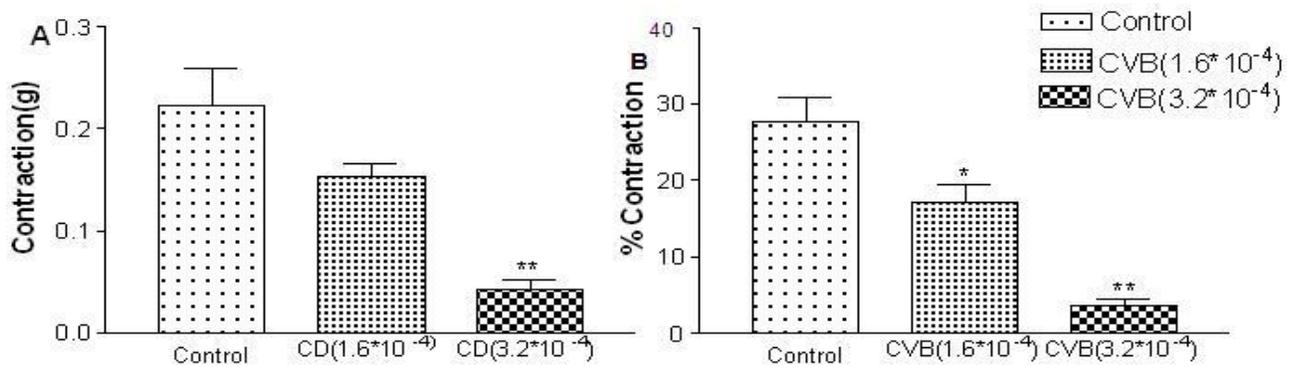
Figure 7. Maximal contraction induced by PE (1 mmol/L) in the absence (control) and in the presence of CVB (1.6×10^{-4} and 3.2×10^{-4} M) in Ca^{2+} free K-H solution. Data in A are expressed in grams of developed tension. Data in B are expressed as percent of maximal contraction in normal K-H solution induced by PE (1 mmol/L).
*P<0.05, **P<0.01 vs control group.
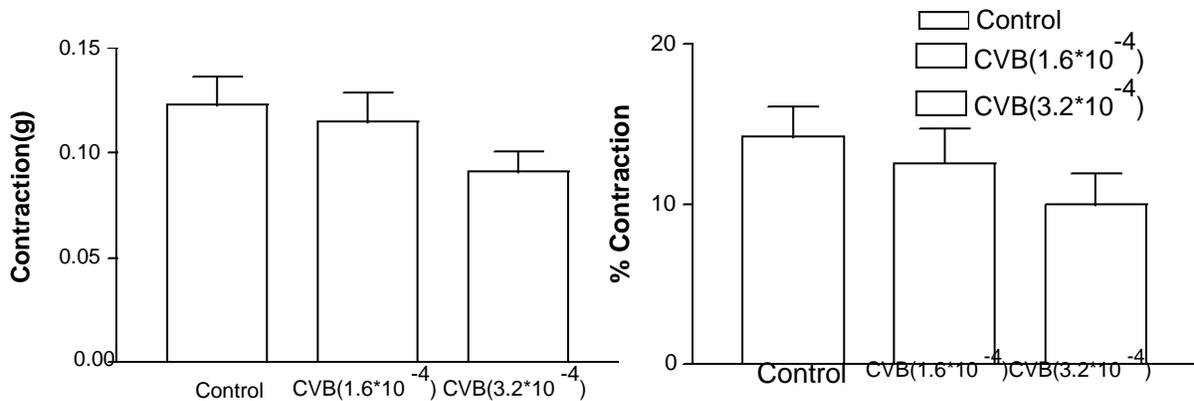
Figure 8. Maximal contraction induced by caffeine (10 mmol/L) in the absence (control) and in the presence of CVB (1.6×10^{-4} and 3.2×10^{-4} M) in Ca^{2+} free K-H solution. Data in A are expressed in grams of developed tension. Data in B are expressed as percent of the maximal contraction in normal K-H solution induced by PE (1 mmol/L). There was no significant difference between CVB group and control group (P>0.05).

et al., 1976; Unlügen et al., 2003). Study showed, in aortic rings with intact endothelium, the CVB induced dilation could be inhibited by the prostaglandin synthetase inhibitor (indomethacin). Therefore, we speculate prostaglandin also involves in the CVB induced dilation. Smooth muscle cells have several ion channels and the opening of calcium channel is closely associated with the excitation of smooth muscle. The influx of extracellular calcium through the calcium channels increases the intracellular calcium level which subsequently leads to the opening of calcium release channels on the membrane of calcium pool. The calcium in the calcium pool then enters the cytoplasm which further increases the intracellular calcium level resulting in subsequent contraction of smooth muscle cells. The increase of intracellular calcium depends on the influx of extracellular calcium and/or release of calcium from the calcium pool. The calcium release channels include Ryanodine-sensitive calcium release channel (RyR) and IP$_3$-sensitive calcium release channel (IP$_3$R). Studies have demonstrated adenosine and caffeine can increase the activity of RyR channel (Rossier and Putney, 1991; Glossmann and Striessnig, 1990).

Our study showed, in aortic rings with removal of endothelium, CVB could dilate the KCl or PE pre-treated rings in a dose dependent manner but did not affect the vascular tone at baseline. The KCl induced voltage-dependent contraction depends on extracellular calcium (Fleckenstein, 1977), but PE induced contraction relies on the -adrenergic receptor dependent calcium release from endoplasmic reticulum and the receptor-gated calcium channel dependent influx of extracellular calcium (Bolton, 1979). Experiment indicated CVB could inhibit the KCl or PE induced influx of extracellular calcium or suppress the release of calcium from different calcium pools resulting in dilation. CVB pre- treatment could shift the dose-response curve of CaCl$_2$, which confirms the role of influx of extracellular calcium in the CVB induced dilation. In addition, our results also revealed CVB and verapamil could combat with the KCl induced contraction of aortic rings, which could partially reversed by increase of extracellualr calcium level. Therefore, we postulate CVB has similar effects to verapamil and both of them can counteract with the effects of calcium. After calcium was supplied into the Ca^{2+} free K-H solution, the contraction depended on the influx of calcium through voltage-gated calcium channels on the smooth muscle cells (Durate et al., 1997; Godfraind et al., 1986). In the Ca^{2+} free solution, the PE induced rapid contraction may be dependent on intracellular calcium. Supplement with CaCl$_2$ further increased the contraction suggesting the dependence of extracellular calcium (Rembold, 1992). Vascular smooth cells have two types of calcium channels: VOC and ROC which can be activated by KCl and PE, respectively. After incubation with 3.2×10^{-4} mol/L CVB, the contraction of KCl pre-treated rings in Ca^{2+} free K-H solution was significantly inhibited after the addition

of 1.25 mmol/L CaCl$_2$ when compared with the contraction of rings without incubating with CVB. This result suggests CVB can exert inhibitive effect on the extracellular calcium dependent contraction. In addition, the PE induced contraction could be divided into intra-cellular calcium dependent contraction (calcium release) and extracellular calcium dependent contraction (opening ROC). Both contractions were suppressed after incubation of rings with CVB.

In the absence of extracellular calcium, the calcium that induces the contraction is mainly from the calcium pool. In the Ca^{2+} free K-H solution, the PE induced contraction is attributed to the release of intracellular calcium (Broekaert, 1979). Cyclovirobuxine D markedly affects intracellular Ca2+ homeostasis in endothelial cells by both promoting a discharge of intracellular pools and by interfering with the operation of store-dependent channels via plasma membrane depolarization (Grossini et al., 2005). In the present study, our results showed CVB could compromise the PE induced contraction which implies PE may affect the calcium release from the IP$_3$ sensitive calcium pool. Ryanodine receptor specifically acts on the calcium channels on the sarcoplasmic reticulum (Meissner, 1986; Sorrrentino and Volpe, 1993). Caffeine is an opener of ryanodine receptor channels and can induce the release of calcium from the ryanodine sensitive calcium pool resulting in contraction (Noguera et al., 1998) . Our results showed CVB has no obvious effect on the caffeine induced contraction, which indicates CVB can not affect the calcium release from the ryanodine sensitive calcium pool.

Taken together, CVB can significantly dilate the PE and KCl pre-treated aortic rings in endothelium dependent and endothelium independent manners. Endothelial cells can release NO or prostaglandin which involve in the CVB induced contraction. In addition, CVB can block the voltage sensitive calcium channel and inhibit the calcium release from the IP$_3$ sensitive calcium pool involve in the CVB induced contraction which however is not associated with ryanodine receptor.

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