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## Vasodilatory effects of betaine on isolated rat pulmonary artery rings

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The aim of this study was to investigate the vasodilatory effects of betaine, an alkaloid isolated from *Lycium barbarum*, on isolated pulmonary artery rings in rats and its possible mechanisms. Pulmonary vessels of normal Sprague–Dawley rats were isolated and pre-contracted using norepinephrine. Then, betaine was cumulatively added in differing concentrations (0.02-0.14 mg/mL), and the tension curve was observed and recorded. Changes in the tension of the pulmonary artery rings with an intact endothelium and a dissected endothelium were recorded. The interactions among betaine and NG-nitro-L-arginine methyl ester, indomethacin, 4-aminopyridine, barium chloride, and glibenclamide were evaluated. The experimental results show that betaine can relax the pulmonary artery rings pre-contracted by norepinephrine. Furthermore, pre-incubation with NG-nitro-L-arginine methyl ester and indomethacin did not inhibit betaine vasodilation, demonstrating that vasodilation by betaine is endothelium-dependent. Additionally, pretreatment of pulmonary artery rings with 4-aminopyridine and glibenclamide had no effect on betaine. However, pretreatment of pulmonary artery rings with barium chloride attenuated the effects of betaine. In conclusion, the vasodilatory effects of betaine on pulmonary artery rings is associated with inward rectifier potassium channels.

### 1. Introduction

Pulmonary arterial hypertension (PAH) is a clinical syndrome characterized by pulmonary arterial remodeling and excessive pulmonary vasoconstriction, threatening the health, and ultimately, life of patients (Hosokawa et al. 2013; Kearney et al. 2021). PAH, often regarded as a cancer of the cardiovascular system, is the third most common cardiovascular condition after coronary heart disease and hypertension, and is associated with high morbidity and mortality (Tuder et al. 2013; Gaine et al. 2021). Excessive pulmonary artery contraction has been closely related to the pathogenesis of PAH (Pruthi et al. 2014; Wang et al. 2020; Bernardo et al. 2020). Therefore, drugs that can dilate the pulmonary artery could be used for the treatment of PAH.

In the past few decades, traditional Chinese medicine has shown several advantages such as varied sources, less toxicity and side effects, cost efficacy, and ease of access. *Lycium barbarum* is known as “red gold” in Northwestern China. It is a valuable resource in Ningxia Chinese herbal medicines and a health food in many countries (Bingül et al. 2016; Miwa et al. 2011). Chinese Wolfberry has many properties such as anti-oxidant, anti-aging, neuroprotective, and anti-Alzheimer's disease (Kim et al. 2014; Ilić et al. 2020; Wu et al. 2015). Betaine is an important alkaloid isolated from *Lycium barbarum* (Fig. 1), primarily found in berries, leaves, and stems of Goji Berry, and has various pharmacological properties such as anti-inflammatory, anti-fibrotic, and anti-oxidant (Bingül et al. 2016; Wu et al. 2020). Betaine cannot only lead to an improvement in PAH, but can also relax the vascular rings of the thoracic aorta in isolated rats. However, whether betaine can dilate the pulmonary artery rings is not clear.

In the present study, we investigated the vasodilatory effects of betaine on isolated pulmonary artery rings of rats and its related

mechanisms. Further studies on the protective effect of betaine on PAH by the ion-channel mechanism could provide experimental and theoretical support for the treatment of PAH by betaine.

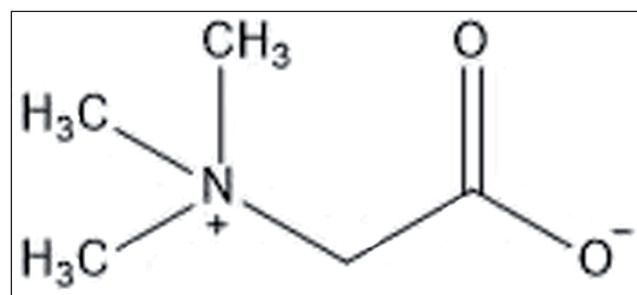


Fig. 1: Chemical structure of betaine.

### 2. Investigations and results

#### 2.1. Vasodilatory effects of cumulative concentrations of betaine on normal isolated rat pulmonary artery rings

Cumulative concentrations of betaine had a vasodilatory effect on normal isolated pulmonary artery rings in rats (Fig. 2; n = 6). When the betaine concentration was greater than 30 mM, significant vasodilation was seen. When the concentration of betaine was 60 mM, the vascular rings showed a maximum relaxation of  $43.75 \pm 2.19\%$  ( $P < 0.01$ ; n = 6). The above results indicate that the vasodilatory effect of betaine is concentration-dependent.

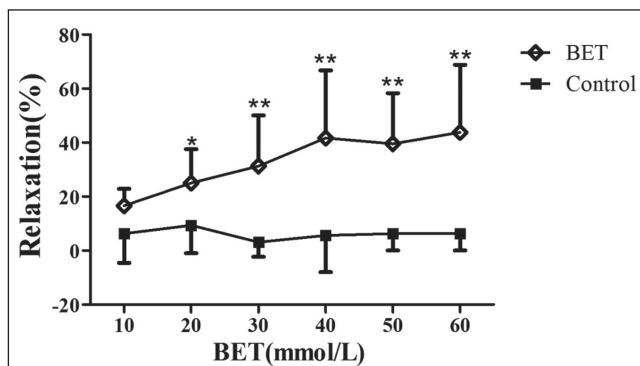


Fig. 2: Influences of different concentrations of betaine on untreated rat pulmonary artery rings (n = 6 per group). Data are expressed as the mean  $\pm$  SEM. BET: betaine.

### 2.2. Vasodilatory effects of cumulative concentrations of betaine on pre-contracted isolated rat pulmonary artery rings using NE

Betaine showed a concentration-dependent vasodilatory effect on NE pre-contracted isolated rat pulmonary artery rings. Significantly greater vasodilation was observed when the concentration of betaine was greater than 20 mM in the experimental group than that in the normal group. At a concentration of 60 mM, the vascular annulus showed a maximal vasodilation of  $55.20 \pm 1.23\%$  ( $P < 0.01$ ; Fig. 3; n = 6).

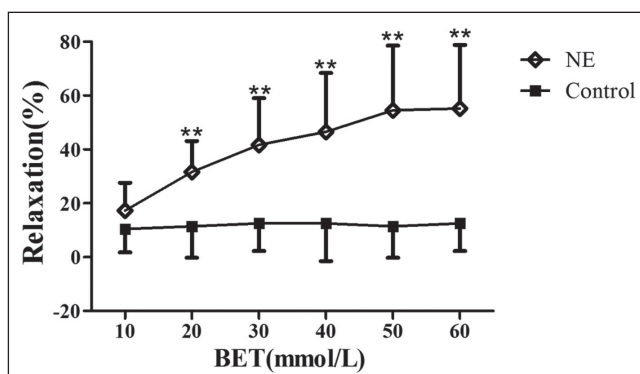


Fig. 3: Respective influences of betaine on NE pre-contracted rat pulmonary artery rings. The value of tension of betaine on NE pre-contracted rat pulmonary artery rings (n = 6 per group). Data are expressed as the mean  $\pm$  SEM. BET: betaine.

### 2.3. Effects of L-NAME and INDO on vasorelaxation due to betaine in isolated rat pulmonary artery rings

No change was evident in vasodilation due to betaine in the rat pulmonary artery rings after L-NAME pretreatment (Fig. 4; n = 6). After incubation with the cyclooxygenase (COX) inhibitor, indomethacin for 20 min, the vasodilation due to betaine remained unchanged in the NE pre-contracted pulmonary vascular rings (Fig. 4; n = 6).

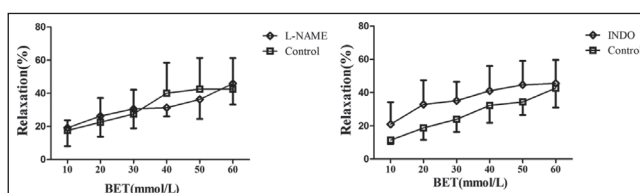


Fig. 4: Respective effects of (A) L-NAME and (B) indomethacin on vasorelaxation action of betaine in rat pulmonary artery rings (n = 6 per group). Data are expressed as the mean  $\pm$  SEM. BET: betaine.

### 2.4. Effect of potassium-channel blockers on vasorelaxation due to betaine in rat pulmonary artery rings

Both voltage-operated potassium-channel blocker 4-AP and KATP-channel blocker glibenclamide had no effect on the vasodilation due to betaine (Fig. 5A and B). However, pretreatment with the Kir-channel inhibitor BaCl<sub>2</sub> significantly antagonized the vasodilatation due to betaine ( $P < 0.05$ ; Fig. 5C).

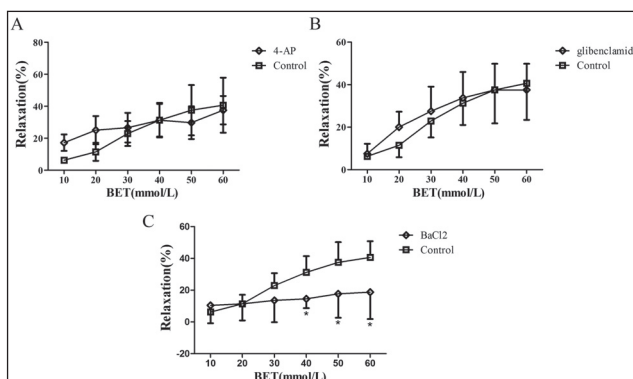


Fig. 5: Respective effects of (A) 4-AP, (B) glibenclamide and (C) BaCl<sub>2</sub> on vasorelaxation action of betaine in rat pulmonary artery rings (n = 6 per group). Data are expressed as the mean  $\pm$  SEM. BET: betaine.

## 3. Discussion

The Chinese herb *Lycium barbarum* is a genuine medicinal material from Ningxia province in China and is the label of Ningxia. The fruit of *L. barbarum* has been used for preventing and treating various diseases (Wu et al. 2015). Betaine is the main component isolated from *L. barbarum*. Pulmonary vasoconstriction is one of the pathological mechanisms of PAH (Bernardo et al. 2020). The vasodilatory effect of betaine on the pulmonary artery and its mechanism are key to the treatment of PAH. However, no study has analyzed the vasodilatory effects of betaine. To the best of our knowledge, this is the first study to demonstrate the vasodilation caused by betaine in isolated rat pulmonary artery rings. Regulation of vasoconstriction and vasodilation is important in the treatment of several cardiovascular diseases (Cosa and Costa 2016; Velayati et al. 2016). Therefore, this study aimed to investigate the vasodilatory effects of betaine on pulmonary artery rings. We used NE to construct a pre-contracted model of pulmonary artery rings (Zheng et al. 2008). NE is a strong  $\alpha$ -receptor agonist, which can cause small arteries and venules to contract through the agonistic action on  $\alpha$ -receptors (Tsai et al. 2008). Pulmonary vessels are primarily innervated by the vagus and sympathetic nerves, and both adrenergic and cholinergic nerve fibers are present in the walls of the smooth muscle cells in the pulmonary arteries. When the vascular tone is normal, sympathetic excitation or endogenous norepinephrine can cause pulmonary artery contraction (Stringham and Shah 2010). The results of this study indicated that betaine can relax NE pre-contracted pulmonary artery rings in rats. Vascular endothelial cells are a thin layer of flat cells, involved in the regulation of inflammatory response, smooth muscle formation, platelet function, coagulation function, and vascular tone (Fleming 2010). They also secrete nutrients and vasoactive substances, and produce a variety of vasodilators including Endothelium-dependent relaxing factor EDRF, prostacyclin, and nitric oxide (NO), which form vasoconstrictor substances that act on vascular smooth muscle cells (Tan et al. 2017). The nitric oxide synthase (NOS) inhibitor L-NAME can prevent vasorelaxation by inhibiting NO (Tan et al. 2017; Qu et al. 2015). Prostaglandins (PGs) are critical in vasodilation. COX and prostacyclin synthetase promote the conversion of arachidonic acid to PG. Vascular relaxation occurs after PG binds to the  $\alpha$ -receptor (Salahdeen et al. 2016). Indomethacin (INDO), a COX inhibitor, has been shown to inhibit prostacyclin-induced vasodilation (Selles et al. 2002). The results of this study indicate that betaine causes concentration-dependent

relaxation of NE pre-contracted pulmonary artery rings. However, this vasodilatory action of betaine is not attenuated by removal of the endothelium. In this study, even after treatment with two endothelium-dependent vasodilators, including L-NAME and INDO, betaine caused vasodilatation of blood vessels. The experimental results showed that these two blockers did not change the effect of betaine on vascular ring tension. Therefore, it can be deduced that the vasodilatory mechanism of betaine is endothelium-dependent. There are several ion channels on the cell membrane of the vascular smooth muscles (Selles et al. 2002; Milkau et al. 2010). Under normal physiological conditions, the opening and closing of the channels can effectively regulate the concentration of sodium, potassium, and calcium ions inside and outside the cell, and play an important role in ensuring the sensitivity of tissues and organs and the smooth performance of various physiological functions (Oloyo et al. 2011). When the potassium channel is inhibited, the cell membrane is depolarized, thereby opening the voltage-dependent calcium channel, and extracellular calcium influx causes excitatory-contraction coupling leading to contraction of blood vessels (Arsyad and Dobson 2016). Conversely, when the potassium channel is open, vascular smooth muscle tension is reduced and vasodilation occurs (Arsyad and Dobson 2016; Testai et al. 2016). Therefore, the potassium channel plays an important role in the regulation of vasomotor activity. Our experiment investigated the role of potassium channels in betaine-induced pulmonary artery ring vasodilation in rats. The most studied potassium channels are voltage-dependent potassium channels, Kir channels, and KATP channels (Oloyo et al. 2011; Bonaventura et al. 2011). In this experiment, we analyzed the effects of voltage-operated potassium channel blocker (4-AP), Kir channel blocker ( $\text{BaCl}_2$ ), and KATP channel blocker (glibenclamide) on the vasodilation caused by betaine.

The results of this experiment showed that pretreatment of pulmonary artery rings with 4-AP and glibenclamide had no effect on the vasodilatation caused by betaine. However, pretreatment of pulmonary artery rings with  $\text{BaCl}_2$  attenuated the effects of betaine. Taken together, these results indicate that activation of Kv (voltage-dependent potassium channel) and KATP channels is not involved in the vasodilatory effect of betaine on pulmonary artery rings. However, the Kir channel affects the vasodilation caused by betaine in pulmonary artery rings in rats.

In conclusion, the results of this study demonstrate for the first time that betaine causes endothelium-dependent vasodilation in the pulmonary vasculature of rats. This vasodilation is associated with open Kir channels. This experiment proves that betaine can help in the treatment of cardiovascular diseases, Especially it provides novel insights into PAH, and further long-term prospective studies should analyze the related mechanisms in the future.

## 4. Experimental

### 4.1. Animals and reagents

Sprague–Dawley rats (220–320 g) were purchased by the experimental animal center of Ningxia Medical University. All experimental rats were reviewed and approved by the Animal Experimental Committee of the Ningxia Medical University (Certificate no. SYXK Ningxia 2015–0001). Experimental rats were raised in a temperature-controlled room (12-h light–dark cycles) with free access to food and water. Rats were acclimated to the environment for 1 week and then tested.

### 4.2. Chemicals and drugs

Betaine was purchased from Shanghai YuanYe Biotechnology Co., Ltd (Shanghai, China), with a purity by high-performance liquid chromatography of 98%. Norepinephrine bitartrate (NE) was purchased from Grand Pharmaceutical China Co., Ltd (Wuhan, China). NG-nitro-L-arginine methyl ester (L-NAME) and glibenclamide were purchased from Ron Reagent Co., Ltd (Shanghai, China). Barium chloride ( $\text{BaCl}_2$ ) was purchased from Sigma-Aldrich (St. Louis, MO, USA). 4-Aminopyridine (4-AP) was purchased from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan). Indomethacin (INDO) was purchased from Shanghai Aladdin Biochemical Technology Co., Ltd (Shanghai, China). Acetylcholine (ACh) was purchased from Beijing Suo Laibao Technology Co., Ltd (Beijing, China).

Potassium chloride (KCl) was supplied by Mingsheng Pharmaceutical Group Co., Ltd (Zhejiang, China). Sodium hydrogen carbonate ( $\text{NaHCO}_3$ ), glucose, calcium chloride hexahydrate ( $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ), anhydrous magnesium sulfate ( $\text{MgSO}_4$ ), and sodium chloride (NaCl) were supplied by Tianjin Damao Chemical Reagent Factory (Tianjin, China).

Isolated pulmonary artery rings were preserved in Krebs salt solution (6.92 g NaCl; 0.35 g KCl; 2.21 g  $\text{NaHCO}_3$ ; 0.28 g  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ; 0.16 g  $\text{KH}_2\text{PO}_4$ ; 0.29 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ; 0.29 g glucose; pH 7.4, temperature 37 °C) with a mixed standard gas, i.e. 95%  $\text{O}_2$  + 5%  $\text{CO}_2$ .

### 4.3. Preparation of isolated rat pulmonary artery rings

Male Sprague–Dawley rats (n=6), intraperitoneally injected with 20% urethane (100 mg/kg), were tied to the operating table in the supine position. After the rats were completely anesthetized, the pulmonary arteries were removed and immediately placed in Krebs salt solution maintained at 37 °C. Thereafter, the isolated rat pulmonary artery was carefully retrieved, cleaned, and then cut into transverse 3-mm rings. More than two vasoactive pulmonary arteriole rings from the same rat were obtained. The isolated rat pulmonary artery rings were mounted in a 10-mL organ bath of a wire myograph (PowerLab System, ADInstruments) that was continuously ventilated with mixed normal oxygen gas (95%  $\text{O}_2$  + 5%  $\text{CO}_2$ ). The isolated rat pulmonary artery rings were stretched at a resting tension of 1 g, and the Krebs salt solution was changed every 15 min before the experiment and equilibrated for 60 min. The isolated rat pulmonary artery rings were challenged with 60-mM KCl to check the functional integrity of the rings. Next, the endothelial integrity was determined by verifying the response to ACh (1  $\mu\text{M}$ ) in vessels pre-contracted using NE (1  $\mu\text{M}$ ) for 15 min. If the ACh-induced relaxation was greater than 85%, the endothelium was considered intact.

### 4.4. Vasodilatory effects of cumulative concentrations of betaine on isolated rat pulmonary artery rings

Endothelial and fully functional respectively isolated rat pulmonary artery rings that had not been pretreated were challenged by cumulative concentrations of betaine (0.02–0.14 mg/mL). Divided into two groups, a control group and a drug group. At the same time, the rings were contracted steadily using NE (1  $\mu\text{M}$ ), the same volume of vehicle as that of betaine was added to rings in the control group.

### 4.5. Effects of L-NAME and INDO on vasorelaxation due to betaine in isolated rat pulmonary artery rings

The isolated rat pulmonary artery rings in the experimental and control groups were exposed to L-NAME (0.1 mM) and INDO ( $1 \times 10^3$  mM) for 20 min, followed by exposure to NE to pre-contrast the rings. After stable vasoconstriction was achieved, betaine (0.02–0.14 mg/mL) was added cumulatively.

### 4.6. Effect of potassium-channel blockers on vasorelaxation due to betaine in isolated rat pulmonary artery rings

To explore the effect of potassium-channel blockers on the vasodilatory effects of betaine, the rat pulmonary artery rings were incubated with 5-mM voltage-operated potassium channel blocker, 4-AP; 1-mM inward-rectifier potassium-channel (Kir) blocker,  $\text{BaCl}_2$ ; and 0.01-mM ATP-sensitive potassium (KATP) channel blocker, glibenclamide; for 20 min after precontraction using NE (1  $\mu\text{M}$ ). After stable vasoconstriction was achieved, betaine (0.02–0.14 mg/mL) was added cumulatively.

### 4.7. Statistical analysis

Data processing analysis was performed using SPSS 24.0 statistical software. Data are presented as mean  $\pm$  standard error of the mean (mean  $\pm$  SEM). The independent sample t-test was used to compare the two samples. One-way analysis of variance was used to compare the mean of various parameters. The Kruskal-Wallis H test was used when multiple sets of mean did not show normality. For all statistical tests,  $P < 0.05$  was considered statistically significant.

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Conflict of Interest: The authors declare no conflict of interest.

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