Endothelium-dependent and-independent relaxation induced by resveratrol in rat superior mesenteric arteries

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Abstract. Resveratrol (Res) is a specific agonist of sirtuin 1, and has many cardioprotective effects. Although Res is able to relax various vascular beds, its pharmacological properties in rat superior mesenteric arteries and the underlying mechanism are not well clarified. The aim of present study was to investigate the vasorelaxant effects of Res on rat superior mesenteric arteries and the mechanisms involved. The isometric tension of rat superior mesenteric arterial rings was recorded *in vitro* using myography. It was found that Res concentration-dependently relaxed endothelium-intact superior mesenteric artery rings pre-contracted by phenylephrine hydrochloride (E_{max}, 97.66±0.79%; pD_2, 4.30±0.14) or KCl (E_{max}, 101.3±0.6%; pD_2, 4.12±0.03). The vasorelaxant effect of Res on the superior mesenteric artery rings was partially endothelium-dependent. N^G-nitro-L-arginine methyl ester (100 μM) significantly inhibited the Res-induced vasorelaxant effect. However, 1H-[1,2,4]oxadiazolo[4,3-a] quinoxalin-1-one (10 μM) and indomethacin (5 μM) each had no effect on the Res-induced vasorelaxation. In artery rings without endothelium, the vasorelaxation induced by Res was attenuated by 4-aminopyridine (100 μM) and glibenclamide (10 μM). However, barium chloride dehydrate (10 μM) and tetraethylammonium chloride (1 mM) did not affect the vasorelaxation induced by Res. Moreover, Res also inhibited the contraction induced by an increase in external calcium concentration in Ca^{2+}-free medium plus KCl (60 mM). These results suggest that Res induces relaxation in superior mesenteric arterial rings through an endothelium-dependent pathway, involving nitric oxide release, and also through an endothelium-independent pathway, with opening of voltage-dependent K^+ channels and ATP-sensitive K^+ channels and blockade of extracellular Ca^{2+} influx.

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Abbreviations: PE, phenylephrine hydrochloride; ACh, acetylcholine chloride; 4-AP, 4-aminopyridine; BaCl2, barium chloride dehydrate; eNOS, endothelial nitric oxide synthase; Gli, glibenclamide; Indo, indomethacin; L-NAME, NG-nitro-L-arginine methyl ester; NO, nitric oxide; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a] quinoxalin-1-one; ROCC, receptor-operated calcium channel; Res, resveratrol; TEA, tetraethylammonium chloride; VDCC, voltage-dependent calcium channel; vsMCs, vascular smooth muscle cells

Key words: resveratrol, vasorelaxant effects, nitric oxide, K^+ channel, Ca^{2+} influx, superior mesenteric artery

Introduction

Resveratrol (Res) is a natural polyphenolic compound present in grapes and red wine. It is a specific agonist of sirtuin 1 (Sirt1), and has many cardiovascular protective effects, such as anti-inflammatory, anti-oxidative and anti-proliferative effects (1,2).

Previous studies have shown that Res is able to relax vascular beds of various types, including conductance arteries, such as the uterine artery (3), aorta (4-6), abdominal aorta (7) and thoracic aorta (8), and resistance arteries, such as the internal mammary artery (9), mesenteric artery (3,10,11) and coronary artery (12). The vasorelaxant effects of Res on conductance arteries and the underlying mechanism have been well clarified. However, the vasodilatation and vasodilatory mechanisms in small resistance arteries are associated with cardiovascular events (13). Although Res possesses the pharmacological property of vasodilatation in resistance arteries, several pathways involved in the mechanism of vasodilatation are unclear.

Therefore, the present study was designed to explore the mechanism by which Res induces vasodilatation in rat superior mesenteric arteries. This should further reveal the underlying mechanisms involved in the vasorelaxant effect of Res on resistance arteries, and provide a theoretical basis for the development of cardiovascular drugs.

Materials and methods

Reagents. Phenylephrine hydrochloride (PE), acetylcholine chloride (ACh), N^G-nitro-L-arginine methyl ester (L-NAME), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), indomethacin (Indo), 4-aminopyridine (4-AP), barium chloride dehydrate ($BaCl_2$), glibenclamide (Gli), tetraethylammonium chloride (TEA) and Triton X-100 were obtained from Sigma-Aldrich (St. Louis, MO, USA). Res was obtained from the College of Life Science, Northwest University (Xi'an, China). ODQ, TEA, Gli, 4-AP, and Res were dissolved in dimethylsulfoxide. All other compounds were dissolved in distilled water.

Artery preparation and testing. Thirty male Sprague-Dawley rats (8 weeks old; body weight, 300-350 g), which were obtained from the Animal Center of Xi'an Jiaotong University (Xi'an, China), were euthanized with CO_2. The superior mesenteric artery was gently removed and freed from adhering tissue under a dissecting microscope. The animal experiments in this study were approved by the Laboratory Animal Administration Committee of Xi'an Medical University (Xi'an, China) and performed according to the Guidelines for Animal Experimentation of Xi'an Medical University and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Triton X-100 is a non-ionic detergent. It directly dissolves the lipid bilayer in endothelial cell membranes to cause destruction of endothelial cell surfaces. In this study, the endothelium was denuded by perfusion of the vessel for 10 sec with X-100 (0.1%, v/v) followed by another 10 sec with a physiological buffer solution (PSS; NaCl 119 mM, KCl 4.6 mM, $NaHCO_3$ 15 mM, NaH_2PO_4 1.2 mM, $MgCl_2$ 1.2 mM, $CaCl_2$ 1.5 mM and glucose 5.5 mM). The vessels were then cut into 1-3-mm long cylindrical segments.

The segments, with and without endothelium, were immersed in individual temperature-controlled (37˚C) myograph baths (Organ Bath Model 700MO; J.P. Trading, Aarhus, Denmark) containing PSS (5 ml). The solution was continuously aerated with gas comprising 5% CO_2 and 95% O_2, resulting in a pH of 7.4. The arterial segments were mounted for continuous recording of isometric tension using LabChart 7 Pro software (ADInstruments, Hastings, UK). A resting tone of 2 mN was applied to each segment, and the segments were allowed to stabilize at this tension for at ≥1.5 h prior to exposure to K^+-rich (60 mM) buffer solution with the same composition as the standard solution, with the exception that NaCl was replaced by an equimolar concentration of KCl (KPSS). The potassium-induced contraction was used as a reference for contractile capacity, and the segments were used only if potassium elicited reproducible responses >1.0 mN. Following equilibration, PE (10 μM) or KPSS (containing 60 mM K^+) was added to the bath. When a sustained tension was obtained, Res ($5x10^{-7}$-$5x10^{-4}$ M) was added cumulatively to the baths and concentration-response curves to Res were constructed. After the experiment, the bath was washed with PSS three times. PE (10 μM) or KPSS was added to the bath again following equilibration. The difference in contractile capacity between before and after the experiment was used as a reference for the toxicity of Res.

With regard to the endothelium, the completeness of endothelium denudation was tested with ACh (10 μM) following pre-contraction with KPSS. No relaxation in response to ACh in the denuded preparation indicated an effective functional removal of the endothelium. Endothelium-intact rings that produced <30% relaxation in response to ACh were discarded (14).

In vitro pharmacology. To evaluate the effects of Res on the contraction induced by PE or KCl, superior mesenteric artery rings were pre-contracted with PE (10 μM) or KCl (60 mM), and once a plateau was attained, concentration-response curves were obtained by adding cumulative doses of Res to the bath.

To identify the endothelial mediator (s) associated with the vasodilatory effect of Res, an endothelial nitric oxide synthase (eNOS) inhibitor [L-NAME (100 μM)], a guanylate cyclase inhibitor [ODQ (10 μM)] and a cyclooxygenase inhibitor [Indo (5 μM)] were used. The endothelium-intact artery rings were pre-incubated with each of these inhibitors for 20 min before KCl (60 mM) was added to the bath, and then Res was added cumulatively.

In order to demonstrate the role of K^+ channels in Res-induced relaxation, artery rings without endothelium were pre-incubated with the K^+ channel blockers 4-AP (100 μM), $BaCl_2$ (10 μM), Gli (10 μM) and TEA (1 mM), independently, for 20 min before KCl (60 mM) was added, and then Res was added cumulatively.

To clarify whether the relaxation induced by Res was associated with intracellular Ca^{2+} release, experiments were carried out in Ca^{2+}-free PSS (100 μM). Rings without endothelium were washed with Ca^{2+}-free PSS. Following incubation with or without Res (500 μM) for 20 min, PE (10 μM) was added to stimulate the release of intracellular Ca^{2+} and the contraction was recorded (15).

Finally, to determine whether the inhibition of extracellular Ca^{2+} influx was involved in the relaxation induced by Res, experiments were carried out in Ca^{2+}-free PSS (100 μM). Artery rings without endothelium were washed with Ca^{2+}-free PSS containing ethylene glycol tetraacetic acid (EGTA; 100 μM) and then rinsed with Ca^{2+}-free PSS (without EGTA) containing KCl (60 mM K^+). Following incubation with or without Res (500 μM) for 20 min, $CaCl_2$ (2 mM) was added to contract the artery rings (15).

Statistical analysis. Data are expressed as mean ± standard error of the mean. The effects of Res are expressed as percentage of relaxation from the pre-contraction. The negative logarithm of the dilator concentration that caused 50% of the maximum response (pD_2) and the maximum relaxation (Emax%) were calculated. Statistical analysis was performed with unpaired Student's *t*-test. $P<0.05$ was considered to indicate a statistically significant result. The analysis was performed using SPSS software, version 16.0 (SPSS Inc., Chicago, IL, USA).

Results

Effect of Res on rat superior mesenteric artery pre-constricted by PE or KCl. Res (0.5-500 μM) concentration-dependently relaxed the endothelium-intact superior mesenteric artery rings pre-contracted by PE (E_{max}, 97.66±0.79%; pD_2, 4.30±0.14) or

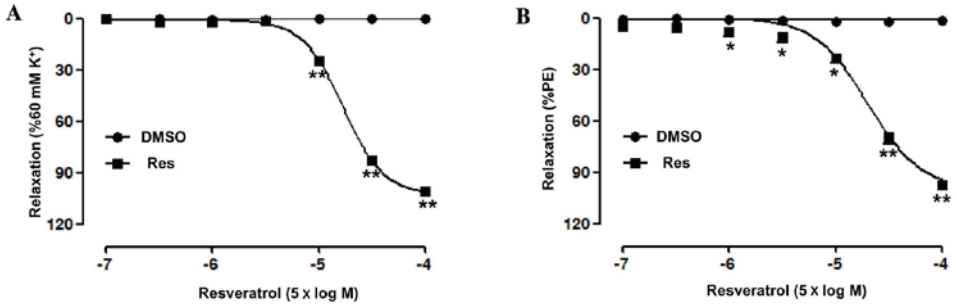

Figure 1. Vasodilatation effects of resveratrol on endothelium-intact superior mesenteric arterial rings pre-contracted with (A) KCl (60 mM) or (B) PE (10 μM). Data are presented as mean ± standard error of the mean (n=6-8). *P<0.05 and **P<0.01 vs. DMSO. PE, phenylephrine hydrochloride; DMSO, dimethylsulfoxide.

KCl (E_{max}, 101.3±0.6%; pD_2, 4.12±0.03) (Fig. 1). In addition, there was no significant change in the contractile capacity of the superior mesenteric artery induced by PE or KPSS between before and after the experiment, suggesting that Res has no toxicity.

Role of the endothelium in Res-induce relaxation of rat superior mesenteric artery pre-constricted by KCl. The vasorelaxant effect of Res on endothelium-intact superior mesenteric artery rings pre-contracted by PE (10 μM) was significantly stronger than that on artery rings without endothelium, with an E_{max} of 97.69±0.82 vs. 89.72±0.1.89% for the artery rings without endothelium group, and a pD_2 of 4.31±0.14 vs. 3.86±0.04 for the artery rings without endothelium group. Moreover, the vasorelaxation induced by Res in endothelium-intact artery rings pre-contracted by KCl (60 mM) also was significantly stronger than that in artery rings without endothelium, with an E_{max} of 100.94±0.59 vs. 95.63±0.63% for the artery rings without endothelium group and a pD_2 of 4.13±0.03 vs. 4.09±0.01 for the artery rings without endothelium group (P<0.05; Fig. 2).

The endothelial mediator(s) associated with the vasodilatory effect of Res were investigated by pre-incubation with the eNOS inhibitor L-NAME, guanylate cyclase inhibitor ODQ and cyclooxygenase inhibitor Indo, independently, prior to treatment with KCl or Res. The results showed that L-NAME significantly inhibited the relaxation induced by Res in the artery rings with endothelium, with an E_{max} of 89.93±0.17 vs. 100.96±0.76% in the control group, and a pD_2 of 3.91±0.03 vs. 4.15±0.02 in the control group (P<0.05; Fig. 3A). However, ODQ and Indo each did not significantly affect the relaxation induced by Res in the artery rings with endothelium (Fig. 3B and C).

Role of K^+ channels in the Res-induced relaxation of rat superior mesenteric artery pre-constricted by KCl. Artery rings without endothelium were pre-incubated with the K^+ channel blockers 4-AP, $BaCl_2$, Gli and TEA, independently, prior to treatment with KCl and Res in order to investigate the role of K^+ channels in the Res-induced relaxation. The results showed that 4-AP significantly reduced the relaxation induced by Res in the artery rings without endothelium, with an E_{max} of 90.15±1.6 vs. 96.38±0.44% in the control group and pD_2 of 3.94±0.03 vs. 4.1±0.02 in the control group (P<0.05; Fig. 4A). However, $BaCl_2$ did not significantly affect the relaxation induced by Res in the artery rings without endothelium (Fig. 4B). In addition, Gli also significantly reduced the relaxation induced by Res in the artery rings without endothelium, with an E_{max} of 89.75±1.24 vs. 95.82±0.49% in the control group and pD_2 of 3.86±0.05 vs. 4.07±0.02 in the control group (P<0.05; Fig. 4C), whilst TEA, similar to $BaCl_2$, did not significantly affect the relaxation induced by Res in the artery rings without endothelium (Fig. 4D).

Effect of Res on calcium release by the sarcoplasmic reticulum in rat superior mesenteric artery pre-constricted by PE. Experiments were carried out in Ca^{2+}-free buffer to clarify whether the relaxation induced by Res was associated with intracellular Ca^{2+} release. The results showed that PE induced a transient contraction due to the release of intracellular Ca^{2+} in the Ca^{2+}-free solution, with an E_{max} of 6.35±1.5%. Res attenuated the contraction induced by PE, with an E_{max} of 3.85±0.95%; however, the difference was not significant (Fig. 5A).

Effect of Res on extracellular Ca^{2+}-induced contraction in rat superior mesenteric artery pre-constricted by KCl. Experiments were carried out in Ca^{2+}-free buffer to determine whether the inhibition of extracellular Ca^{2+} influx is involved in the Res-induced relaxation of artery rings without endothelium. The results showed that Res significantly attenuated $CaCl_2$-induced contraction in the Ca^{2+}-free PSS containing KCl (60 mM K^+), with an E_{max} of 3.6±0.31 vs. 101.4±1.79% in the control group (P<0.01; Fig. 5B). This suggests that Ca^{2+} influx was inhibited by Res in the superior mesenteric artery.

Discussion

The present study found that Res concentration-dependently relaxed superior mesenteric artery rings with or without endothelium that had been pre-contracted using PE or KCl. This suggests that Res induced vasorelaxation via endothelium-dependent and-independent pathways. Moreover, the vasorelaxation induced by Res was inhibited by L-NAME, and not affected by ODQ or Indo in artery rings with endothelium. In addition, the vasorelaxation induced by Res was inhibited by 4-AP and Gli, and not affected by $BaCl_2$ and TEA in artery rings without endothelium. Finally, it was also found that the vasorelaxation induced by Res was mediated through blockade of Ca^{2+} influx from extracellular medium.

Vascular endothelium, occupying a location between circulating blood and vascular smooth muscle, is considered to be

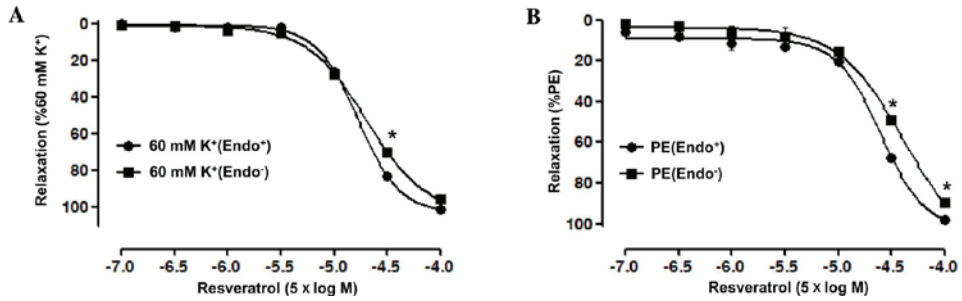

Figure 2. Vasodilatation effects of resveratrol on endothelium-intact and endothelium-denuded superior mesenteric arterial rings pre-contracted with (A) KCl (60 mM) or (B) PE (10 μM). Data are presented as mean ± standard error of the mean (n=6-8). *P<0.05 for Endo+ vs. Endo-. Endo+, artery ring with endothelium; Endo-, artery ring without endothelium. PE, phenylephrine hydrochloride.

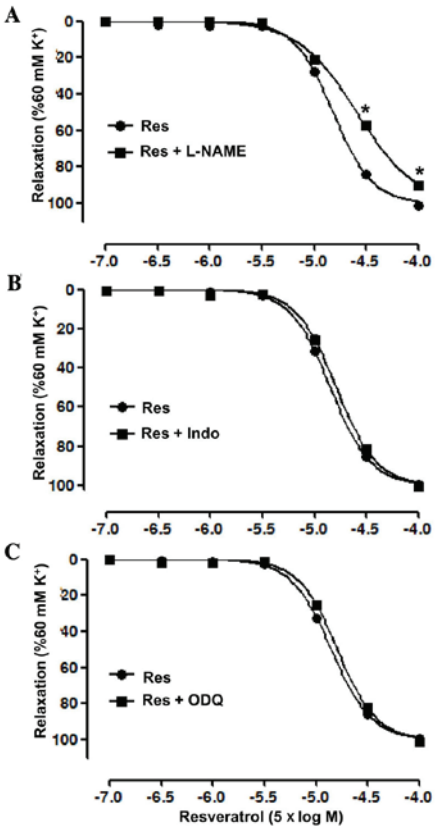

Figure 3. Vasodilatation effects of resveratrol (Res) on endothelium-intact superior mesenteric arterial rings pre-contracted with KCl (60 mM) in the presence of (A) the endothelial nitric oxide synthase inhibitor (L-NAME, 100 μM), (B) the cyclooxygenase inhibitor (Indo, 5 μM) and (C) the guanylate cyclase inhibitor (ODQ, 10 μM). Data are presented as mean ± standard error of the mean (n=6-8). *P<0.05 vs. Res. L-NAME, N^G-nitro-L-arginine methyl ester; Indo, indomethacin; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one.

important in the regulation of vascular tone. The vasorelaxation is mediated by relaxing substances synthesized in and released by the endothelium (16). In the present study, the relaxant effect induced by Res was attenuated in the superior mesenteric artery rings without endothelium, suggesting that Res relaxed the artery through an endothelium-dependent pathway. The data showed that L-NAME (an eNOS inhibitor) significantly reduced the vasorelaxation induced by Res. However, Indo (a cyclooxygenase inhibitor) and ODQ (a guanylate cyclase inhibitor) did not affect the action of Res. This suggests that nitric oxide (NO) is involved in the relaxation of Res in the superior mesenteric artery with endothelium, whereas the cGMP pathway and prostanoids are not associated with this effect. This finding is consistent with previous studies; in the abdominal aorta, thoracic aorta and coronary artery, the efficacy of Res has been found to be closely associated with the NO system in endothelial cells (4,7,8,12).

In the present study, it was found that Res also induced a relaxant effect in superior mesenteric artery without endothelium, suggesting that Res has a direct effect on vascular smooth muscle cells (VSMCs). The opening of K^+ channels in vsMCs causes membrane potential hyperpolarization, decreases Ca^{2+} entry through voltage-operated Ca^{2+} channels, and induces vasorelaxation (17,18). Several types of K^+ channels have been identified in vascular smooth muscle. The most abundant types include large conductance Ca^{2+}-activated K^+ channels, voltage sensitive K^+ channels, ATP-sensitive K^+ channels and inward rectifying potassium channels (19). In order to detect the contribution of different types of K^+ channels to the endothelium-independent relaxation induced by Res in superior mesenteric artery rings, agents that are known to possess K^+ channel-blocking activity, namely 4-AP (a voltage-dependent K^+ channel blocker), $BaCl_2$ (an inward rectifying potassium channel blocker), Gli (an ATP-sensitive K^+ channel blocker) and TEA (a Ca^{2+}-activated K^+ channel blocker) (20,21) were used.

Previous studies have found that Res relaxes many types of vascular beds without endothelium through the activation of different types of K^+ channels. The voltage-dependent K^+ channel plays an important role in the vasodilatation induced by Res in the aorta (6) and internal mammary artery (9), whereas, the voltage-dependent K^+ channel is involved in the vasodilatation induced by Res in the thoracic aorta (6). In addition, Res has been shown to induce relaxation of the abdominal aorta through activation of ATP-sensitive K^+ channels and Ca^{2+}-activated K^+ channels (7). However, Gojkovic-Bukarica *et al* found that K^+ channel-independent mechanisms are involved in its vasorelaxant effect in mesenteric arteries (10). In the present study, both 4-AP and Gli significantly inhibited the relaxant effect of Res, indicating that voltage-dependent K^+ channels and ATP-sensitive K^+ channels are involved in the relaxation of the superior mesenteric artery induced by Res. However, neither $BaCl_2$ nor TEA affected the concentration-response curves of Res, suggesting that inward rectifying K^+ channels and Ca^{2+}-activated K^+ channels are not involved in the Res-induced relaxation.

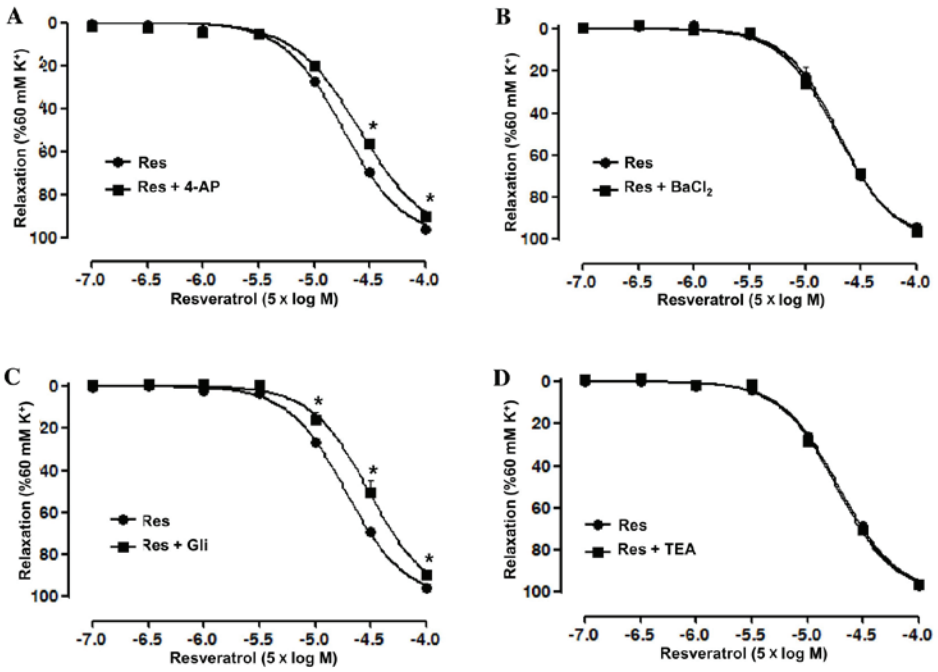

Figure 4. Vasodilatation effects of resveratrol (Res) on endothelium-denuded superior mesenteric arterial rings pre-contracted with KCl (60 mM) in the presence of the K^+ channel blockers (A) 4-aminopyridine (4-AP; 100 μM), (B) barium chloride dehydrate ($BaCl_2$, 10 μM), (C) glibenclamide (Gli; 10 μM) and (D) tetraethylammonium chloride (TEA; 1 mM). Data are presented as mean ± standard error of the mean (n=6-8). *P<0.05.

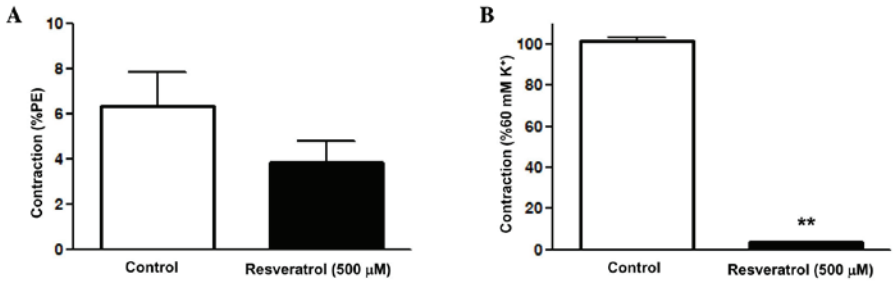

Figure 5. Inhibitory effect of resveratrol on (A) intracellular Ca^{2+} release induced by PE (10 μM) and (B) extracellular Ca^{2+} influx induced by KCl (60 mM) in Ca^{2+}-free solution in endothelium-denuded superior mesenteric arterial rings. Data are presented as mean ± standard error of the mean (n=6-8). **P<0.01 vs. control. PE, phenylephrine hydrochloride.

Accumulation of intracellular calcium is involved in vascular smooth muscle contraction. Moreover, both extracellular Ca^{2+} influx, through voltage-dependent calcium channels (VDCCs) or receptor-operated calcium channel (ROCCs), and intracellular Ca^{2+} release result in an increase of the intracellular calcium level (22). Contractions of vsMCs induced by KCl rely almost exclusively on Ca^{2+} influx induced by the activation of voltage-sensitive channels (23), whereas contractions induced by PE are mediated by an increase in Ca^{2+} influx through both receptor-operated channels (24) and voltage-sensitive channels (25). The results of the present study show that Res is able to inhibit the contractile effects induced by PE or KCl on the superior mesenteric artery without endothelium. This suggests that Res may exert effects on both VDCCs and ROCCs. The release of intracellular stored Ca^{2+} is mainly regulated by the inositol trisphosphate (IP3) receptor system and the ryanodine receptor system (26). Contractions induced by PE in Ca^{2+}-free medium occur due to intracellular Ca^{2+} release through Ca^{2+} channels in the sarcoplasmic reticulum activated by IP3 (27). Previous studies have shown that Res attenuates extracellular calcium influx and intracellular calcium release, which results in vasodilatation in the abdominal aorta (7) or thoracic aorta (8). However, Res has Ca^{2+} antagonistic properties and inhibits extracellular Ca^{2+} influx through VDCCs in coronary arteries (12). In the present study, it was found that Res significantly inhibited $CaCl_2$-induced contraction in the superior mesenteric artery rings without endothelium in Ca^{2+}-free PSS containing KCl (60 mM), indicating that Res exhibits Ca^{2+} entry blocking activity. However, Res did not inhibit the contraction triggered by PE in Ca^{2+}-free PSS, suggesting that Res does not inhibit Ca^{2+} mobilization from intracellular stores. In the superior mesenteric artery, it appears that Res induced vasorelaxation via the inhibition of extracellular calcium in vsMCs.

In conclusion, the results of the present study suggest that Res-induced relaxation of the rat superior mesenteric artery occurs via an endothelium-dependent pathway involving NO release, as well as an endothelium-independent pathway, with

opening of voltage-dependent K^+ channels and ATP-sensitive K^+ channels, and blockade of extracellular Ca^{2+} influx.

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