Sulfur dioxide relaxes rat aorta by endothelium-dependent and
-independent mechanisms

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Short title: Sulfur dioxide relaxes rat aorta
Summary

AIM: This study aimed to investigate the vasoactivity of sulfur dioxide (SO₂), a novel gas identified from vascular tissue, in rat thoracic aorta. METHODS: The thoracic aorta was isolated, cut into rings, and mounted in organ-bath chambers. After equilibrium, the rings were gradually stretched to a resting tension. Isometric tension was recorded under the treatments with vasoconstrictors, SO₂ derivatives, and various drugs as pharmacological interventions. RESULTS: In endothelium-intact aortic rings constricted by 1 µM phenylephrine (PE), SO₂ derivatives (0.5 – 8 mM) caused a dose-dependent relaxation. Endothelium removal and a NOS inhibitor L-NAME reduced the relaxation to low doses of SO₂ derivatives, but not that to relatively high doses (≥ 2 mM). In endothelium-denuded rings, SO₂ derivatives attenuated vasoconstriction induced by high K⁺ (60 mM) or CaCl₂ (0.01 – 10 mM). The relaxation to SO₂ derivatives in PE-constricted rings without endothelium was significantly inhibited by blockers for ATP-sensitive K⁺ (K_{ATP}) and Ca²⁺-activated K⁺ (K_{Ca}) channels, but not by those for voltage-dependent K⁺ channels, Na⁺-K⁺-ATPase or Na⁺-Ca²⁺ exchanger. CONCLUSIONS: SO₂ relaxed vessel tone via endothelium-dependent mechanisms associated with NOS activation, and endothelium-independent mechanisms dependent on the inhibition of voltage-gated Ca²⁺ channels, and the opening of K_{ATP} and K_{Ca} channels.

Key words: Sulfur dioxide; Vasorelaxation; Ion channel; Endothelium; Aorta; Rat
Introduction

The vascular endothelium has been established as an abundant source of vasoactive substances including gases, such as nitric oxide (NO), carbon oxide (CO) and sulfureted hydrogen (H₂S) (BHATIA 2005). Sulfur dioxide (SO₂) is a novel gas first detected in porcine coronary artery by Balazy et al (BALAZY et al. 2003). A possible precursor of SO₂ could be the intracellular thiol such as cysteine that can be oxidized to cysteinesulfinic acid by cysteine dioxygenase. Cysteinesulfinate can further be transformed by glutamate-oxaloacetate transaminase to generate b-sulfinylpyruvate, which decomposes spontaneously to pyruvate and SO₂ (GRIFFITH 1983). Another pathway that can produce SO₂ is the oxidation of H₂S by NADPH oxidase (MITSUHASHI et al. 2005).

Little is known about the vasoactivity of SO₂. A few studies addressed the systemic impact of SO₂ inhalation under the concern that SO₂ is a common air pollutant. Meng et al. reported that SO₂ inhalation and intraperitoneal injection of SO₂ derivatives both decreased blood pressure in rats (MENG et al. 2003), implying possible vasorelaxant activity of SO₂. Besides, ACh was shown to stimulate the formation of SO₂ (BALAZY et al. 2003), raising a possibility that SO₂ is involved in the profound vasodilation in response to ACh. However, the direct vasoactivity of SO₂ has never been described.

SO₂ is quite soluble in water. Upon solution, it hydrates rapidly to form sulfurous acid, which dissociates in turn to form sulfite and bisulfite ions (3:1 M/M, in neutral fluid) (SHAPIRO 1977). The present study observed that SO₂ derivatives, a mixture of sulfite and bisulfite (Na₂SO₃/NaHSO₃) in a molar ratio of 3:1, elicited potent vasorelaxation in isolated rat aortic rings constricted by phenylephrine (PE) through both endothelium-dependent and -independent mechanisms. The
endothelium-dependent relaxation induced by SO₂ was revealed by denuding the endothelium and applying a NOS inhibitor N⁶-nitro-L-arginine methyl ester (L-NAME), and the endothelium-independent mechanism was further investigated by using a variety of ion channel interventions such as antagonist of K⁺ channels, voltage-gated Ca²⁺ channels, Na⁺-K⁺ pump and Na⁺-Ca²⁺ exchange in endothelium-denuded aortic rings.

**Materials and methods**

**Preparation of rat aortic rings**

This study was performed in accordance with the Guide for Care and Use of Laboratory Animals published by the U.S. National Academy Press in 1996 and the Guidelines for Animal Experiments of the Second Military Medical University, China.

Male Sprague-Dawley rats (250 – 350 g) were anesthetized by intraperitoneal injection of 1 g/kg urethane with 750 U heparin. The thoracic aorta was quickly removed, cleaned of all connective and fat tissue, and cut into rings of 3 mm in length. The aortic rings were then mounted in organ-bath chambers containing modified Krebs-Henseleit (K-H) solution (pH 7.4) at 37 °C, continuously bubbled with 95% O₂ - 5% CO₂. The modified K-H solution contained (in mM): NaCl 118, KCl 4.6, MgSO₄ 1.2, KH₂PO₄ 1.2, glucose 11.1, NaHCO₃ 27.2, EGTA 0.03, CaCl₂ 1.8. After 30-min equilibrium, the rings were gradually stretched to a resting tension of 2 g over 40 min. Isometric tension was recorded with force displacement transducers coupled to a computerized recording system (Nanjing MedEase Science and Technology Co. LTD, Nanjing, China).
Protocols

After two challenges with 60 mM KCl, aortic rings were constricted by 1 µM phenylephrine (PE) and subsequently challenged with 1 µM acetylcholine (ACh) to confirm the integrity or removal of the endothelium. Then they were washed in K-H solution to restore tension to baseline level and allowed to stabilize for 60 – 90 min. The rings were constricted submaximally by 1 µM phenylephrine (PE) again, and SO₂ derivatives (Na₂SO₃ and NaHSO₃, 3:1 M/M) were added cumulatively into the bathing solution once steady contraction was obtained. The equivalent SO₂ concentration ranged from 0.5 to 8 mM. We have examined the pH of the Kreb’s solution that in the normal condition it is about 7.40. After we add SO₂ derivates at the lower concentrations (0.5~2mM), the pH doesn’t change. It will decrease to 7.37 after SO₂ derivates added at the higher concentrations (4~8mM) and can return to 7.40 in 3 or 4 minutes. Relaxation was calculated as a percentage of the maximal tension induced by PE (ENGLER et al. 2000).

The role of endothelium/nitric oxide (NO) in vasorelaxant responses to SO₂ derivatives was first examined. For this set of experiments, the endothelium was removed mechanically by gently rubbing the luminal surface of aortic rings with a wire, and the functional removal was confirmed by the lack of relaxation in response to ACh as aforementioned. To examine the involvement of NO, endothelium-intact rings were exposed for 30 min to a non-specific NOS inhibitor L-NAME (N⁶-nitro-L-arginine methyl ester, 100 µM) before the addition of PE.

To study the participation of K⁺ channels in endothelium-independent relaxation induced by SO₂ derivatives, aortic rings without endothelium were constricted with high K⁺ (60 mM, to annul the effect of K⁺ channel activation) (SANG et al. 2003), and SO₂ derivatives were applied cumulatively. To further
identify the types of K⁺ channels associated with SO₂, a K<sub>ATP</sub> blocker glibenclamide (3 µM), a K<sub>Ca</sub> blocker tetraethylammonium chloride (TEA, 5 mM) or a K<sub>V</sub> blocker 4-aminopyridine (4-AP, 100 µM) were applied to endothelium-denuded rings 30 min prior to the addition of PE. Only one concentration-response curve to SO₂ derivatives was obtained per ring in the presence of each inhibitor.

The ability of SO₂ to modulate Ca²⁺ influx via VGCCs was studied on constrictions to CaCl₂ in rings without endothelium, using a protocol described previously (CHAN et al. 2005). For this set of experiments, two consecutive concentration-dependent constrictions to CaCl₂ were obtained in control and in the presence of SO₂ derivatives (0.5 – 8 mM, 5-min incubation). For constructing CaCl₂ concentration-response curve, arterial rings were rinsed three times in Ca²⁺-free solution containing 30 mM Na₂-EGTA and then incubated in Ca²⁺-free, 60 mM K⁺ solution before the cumulative addition of CaCl₂ (0.01 – 10 mM). The effect of 1 µm nifedipine was tested as control.

In the final set of experiments, the rings were constricted with 1 µM PE and relaxed with cumulative SO₂ derivatives (0.5 – 8 mM). A Na⁺-K⁺-ATPase inhibitor ouabain (100 mM) or a Na⁺-Ca²⁺ exchanger inhibitor nickel chloride (30 µM) was applied 30 min prior to the addition of PE.

**Drugs**

All the drugs were purchased from Sigma-Aldrich (St Louis, MO, USA). They were all dissolved in K-H solution except for glibenclamide, which was dissolved in dimethyl sulfoxide first and diluted with K-H solution before use. The final content of dimethyl sulfoxide was 0.2% (v/v), which did not affect vessel tension.
Statistical Analyses

All data are presented as means ± standard errors of means (SEM). SO₂-induced relaxation is expressed as the percentage change from the PE-contracted levels of tension. One-way repeated-measures ANOVA was used for comparisons of concentration-response curves. One-way ANOVA was used for comparisons at each drug concentration. A P value of less than 0.05 was considered significant.

Results

1. SO₂ derivatives relaxed aortic rings by endothelium-dependent and -independent mechanisms

The thoracic aortic rings were equilibrated for 60 - 90 min at the optimal preload of 2 g, and then cumulative concentrations of SO₂ derivatives (0.5, 1, 2, 4, 8 mM) were administered. No relaxation or constriction was observed (data not shown).

The vasoconstriction induced by 1 µM PE was progressively relaxed by cumulative SO₂ derivatives (0.5 – 8 mM) in both endothelium-intact and -denuded rings. Fig. 1A shows representative recordings. Lower concentrations of SO₂ derivatives (0.5, 1 mM) caused less relaxation in endothelium-denuded rings than that in endothelium-intact rings (P < 0.01. Fig. 1B). However, relatively higher concentrations of SO₂ derivatives (2, 4, 8 mM) relaxed the rings with and without endothelium to the same extent (Fig. 1B). These data suggest that both endothelium-dependent and -independent relaxations are induced by SO₂ derivatives at lower doses, while the endothelium-independent relaxation is
predominantly displayed at higher doses.

In consistent with the phenomenon observed in endothelium removal versus integrity, a non-selective NOS inhibitor L-NAME (100 µM) abolished the relaxation of endothelium-intact rings induced by lower doses of SO₂ derivatives (0.5 and 1 mM), but failed to affect that by higher doses (Fig. 1). It is suggested that NOS pathway is involved in the endothelium-dependent relaxation induced by SO₂ derivatives.

2 Ion channels involved in endothelium-independent relaxation induced by SO₂ derivatives

2.1 Involvement of K⁺ channels

In endothelium-denuded aortic rings constricted with 1 µM PE, SO₂ derivatives caused a dose-dependent relaxation with the maximum of 87.99 ± 1.82 % at 8 mM (Fig. 2A). In the rings constricted with high K⁺ (60 mM), however, the relaxation to SO₂ derivatives was significantly inhibited (maximum 63.88 ± 3.78 %, \( P < 0.01 \), Fig. 2A), though the contraction induced by high K⁺ was comparable to that by PE (data not shown). On the one hand, the lessened vasorelaxant activity of SO₂ derivatives suggests that the opening of K⁺ channels contributes to the relaxation, since high K⁺ is presumed to block all K⁺ channels (SANG et al. 2003). On the other hand, the persistent existence of vasorelaxation in response to SO₂ derivatives under high-K⁺ conditions suggests that K⁺ channel-independent mechanisms also contribute to the relaxation.

To further identify the types of K⁺ channels involved in SO₂-induced relaxation, aortic rings without endothelium were treated with glibenclamide (3 µM), TEA (5
mM) and 4-AP (100 µM) to block K_ATP, K_Ca and K_V, respectively. The vasoconstriction induced by PE remained unchanged by the presence of glibenclamide, TEA or 4-AP. As shown in Fig. 2B, glibenclamide significantly reduced the relaxation caused by 4 mM and 2 mM SO2 derivatives from 71.06 ± 2.42 % to 30.54 ± 4.05 % (P < 0.01), and from 51.77 ± 2.77 % to 14.44 ± 4.63 % (P < 0.01), respectively. Similarly, TEA significantly reduced the relaxation resulting from 4 mM and 2 mM SO2 derivatives to 40.36 ± 4.22 % (P < 0.01) and 11.97 ± 4.14 % (P < 0.01), respectively. Besides, the relaxant response to 8 mM SO2 derivatives was also significantly reduced by TEA (68.62 ± 4.27 % vs. 87.99 ± 1.82 %, P < 0.01). However, 4-AP did not affect the relaxation (n = 12, P > 0.05 vs. control). These data indicate that SO2-induced relaxation is associated with the opening of K_ATP and K_Ca channels in smooth muscle cells, but not K_V channels.

2.2 Involvement of voltage-gated Ca^{2+} channels

In Ca^{2+}-free, 60 mM K+ solution, cumulative CaCl2 (0.01 – 10 mM) induced progressive constriction of aortic rings without endothelium. The maximal tension was approximately 2.5 g. SO2 derivatives (0.5 – 8 mM) reduced CaCl2-induced constriction in a dose-dependent manner with progressive suppression of the maximal constriction (n >= 6 for each group, Fig. 3). In control experiments, 1 µM nifedipine abolished vasoconstriction to CaCl2 (data not shown).

2.3 Noninvolvement of Na^{+}-K^{+} and Na^{+}-Ca^{2+} exchangers

Increased activity of Na^{+}-K^{+} pump results in a reduction in [Na+]i, which may stimulate the forward mode of Na^{+}-Ca^{2+} exchanger and facilitate muscle relaxation. To test whether SO2-induced vasodilation is associated with the stimulation of
Na\textsuperscript{+}-K\textsuperscript{+} pump or forward Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger, the present study used a Na\textsuperscript{+}-K\textsuperscript{+}-ATPase inhibitor ouabain (100 mM) and a putative Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger inhibitor Ni\textsuperscript{2+} (30 \mu M). Neither ouabain nor Ni\textsuperscript{2+} affected the vasodilation resulting from SO\textsubscript{2} derivatives in PE-constricted aortic rings without endothelium (Fig. 4). It is suggested that Na\textsuperscript{+}-K\textsuperscript{+} pump and Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger are not involved in the vasorelaxant response to SO\textsubscript{2} derivatives.

**Discussion**

The vasoactivity of SO\textsubscript{2} has never been described before though it was found in vascular tissue (BALAZY et al. 2003). The present study first revealed the direct vasorelaxant activity of SO\textsubscript{2}. In constricted rat aortic rings under isometric recording, we found that the derivatives of SO\textsubscript{2} hydration, sulfite and hydrogen sulfite (SHAPIRO 1977), reduced vessel tension in a dose-dependent manner. The vasorelaxation resulting from low doses of SO\textsubscript{2} derivatives (0.5 and 1 mM) was attenuated by endothelium removal and a non-specific NOS inhibitor L-NAME (Fig.1), indicating the involvement of endothelium-dependent mechanisms associated with NO. In contrast, the relaxation induced by high doses of SO\textsubscript{2} derivatives (2 - 8 mM) was not changed by endothelium removal or NOS inhibition (Fig.1), suggesting that the endothelium-independent relaxation was predominant in the presence of a relatively large amount of SO\textsubscript{2}. Previous report showed that the endothelium-independent relaxation of SO\textsubscript{2} derivates was not mediated by NO (MENG et al. 2007a). It is not in agreement with our results and need further research.

We further studied the ion channels involved in the potent endothelium-independent vasodilation induced by SO\textsubscript{2} derivatives, using
pharmacological interventions. It is well demonstrated that K\(^+\) plays a vital role in regulating muscle contractility and vascular tone (NELSON et al. 1995). A rise in K\(^+\) permeability normally hyperpolarizes cell membrane and thus inhibits Ca\(^{2+}\) influx through VGCCs, resulting in muscle relaxation. To test whether or not SO\(_2\) can increase K\(^+\) permeability, we observed the relaxant response to SO\(_2\) derivatives in endothelium-denuded rings challenged with high K\(^+\) (60 mM), a putative blocker for all K\(^+\) channels (SANG et al. 2003). The vessel tone caused by high K\(^+\) (60 mM) was comparable to that by PE (1 µM). However, the relaxant response to SO\(_2\) derivatives was smaller in rings receiving K\(^+\) than those receiving PE, suggesting that SO\(_2\) derivatives activated K\(^+\) channels. Balazy et al. proposed SO\(_2\) as a candidate for the unidentified endothelium-derived hyperpolarizing factor (EDHF), based on the facts that SO\(_2\) has a short-life comparable to EDHF and the formation of SO\(_2\) can be stimulated by ACh (BALAZY et al. 2003). Here we demonstrate that SO\(_2\) derivatives relax vascular smooth muscle at least partially through opening K\(^+\) channels, a well-characterized property of EDHF, supporting SO\(_2\) as a candidate for EDHF.

Previous report also studied the mechanisms of the endothelium-independent vasodilation induced by SO\(_2\) derivatives and the vasorelaxation was mediated in partly by the inhibition of Ca\(^{2+}\) channels and the signal transduction pathway of PGI\(_2\)-AC-cAMP-PKA (MENG et al. 2007a, MENG et al. 2007b). In the present study, we further investigated the mechanisms of vasodilation induced by SO\(_2\) by using a variety of ion channel interventions such as antagonist of K\(^+\) channels, Na\(^+\)-K\(^+\) pump and Na\(^+\)-Ca\(^{2+}\) exchange besides voltage-gated Ca\(^{2+}\) channels. Multi-types of K\(^+\) channels have been identified in vascular smooth muscle cells, among which K\(_{\text{ATP}}\), K\(_{\text{Ca}}\) and K\(_V\) are relatively predominant (FERRER et al. 1999). To
examine their involvement in SO$_2$-induced relaxation, glibenclamide, TEA and 4-AP were administered in concentrations tested previously (BOLOTINA et al. 1994, KITAGAWA et al. 1994, KITAZONO et al. 1995, MURPHY et al. 1995, RANDALL et al. 1991) to block $K_{ATP}$, $K_{Ca}$ and $K_{V}$, respectively. The relaxation of PE-constricted rings in response to SO$_2$ derivatives was significantly reduced by glibenclamide and TEA, but not by 4-AP. These findings suggest the involvement of $K_{ATP}$ and $K_{Ca}$ channels in the vasodilating effect of SO$_2$. It is understood that $K_{ATP}$ channels play a crucial role under the condition of ischemia and reperfusion, where they are activated to hyperpolarize the membrane and therefore attenuated injuries. The ability of SO$_2$ to activate $K_{ATP}$ channels indicates a potential protective role of SO$_2$ in ischemia-reperfusion.

SO$_2$ derivatives inhibited high K$^+$-induced vasoconstriction (Fig. 2A), indicating that SO$_2$ may act as a Ca$^{2+}$ channel inhibitor to cause vascular relaxation. In endothelium-denuded aortic rings, SO$_2$ derivatives (0.5 – 8 mM) reduced CaCl$_2$-induced constriction in a dose-dependent manner, suggesting that SO$_2$ derivatives inhibit Ca$^{2+}$ influx through VGCCs in smooth muscle cells. In contrast, the whole cell patch-clamp technique recorded an increased voltage-gated L-type Ca$^{2+}$ current under the treatment with SO$_2$ derivatives in isolated rat ventricular myocytes (NIE et al. 2006). It is indicated that SO$_2$ derivatives may exert different modulations on VGCCs in different cell types.

Sarcolemmal Na$^+$-Ca$^{2+}$ exchange plays a significant role in regulating [Ca$^{2+}$]$_i$ in smooth muscle cells and thus vessel tone (MOTLEY et al. 1993). The activity of the Na$^+$-Ca$^{2+}$ exchanger is coupled to [Na$^+$]$_i$, which is primarily regulated by membrane permeability to Na$^+$ ions and Na$^+$-K$^+$-ATPase activity. Decreased permeability to Na$^+$ or increased activity of Na$^+$-K$^+$ pump results in a reduction in [Na$^+$]$_i$, which in
turn stimulates the forward mode of Na\(^+\)-Ca\(^{2+}\) exchanger and facilitates vasodilation. Treatment with a Na\(^+\)-K\(^+\)-ATPase inhibitor ouabain or a Na\(^+\)-Ca\(^{2+}\) exchanger inhibitor Ni\(^{2+}\) failed to prevent SO\(_2\)-induced relaxation. This phenomenon suggests that the relaxation by SO\(_2\) is unlikely associated with the stimulation of Na\(^+\)-K\(^+\)-ATPase or forward Na\(^+\)-Ca\(^{2+}\) exchanger.

Altogether, the present study showed that SO\(_2\) derivatives dose-dependently dilated rat aortic rings via mechanisms that were both endothelium-dependent and independent. NOS activation was contributive to the endothelium-dependent relaxation, and the endothelium-independent relaxation was associated with the activation of K\(_{\text{ATP}}\) and K\(_{\text{Ca}}\) and the inactivation of VGCCs. In addition, K\(_{\text{V}}\), Na\(^+\)-K\(^+\)-ATPase and Na\(^+\)-Ca\(^{2+}\) exchanger were not suggested to be involved in the vasorelaxant response to SO\(_2\) derivatives.

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**References**


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**Fig 1.** Relaxation to SO$_2$ derivatives (0.5 - 8 mM) in rat thoracic aortic rings pre-constricted by 1 µM phenylephrine (PE). (A) Representative traces of vessel tension in response to PE and cumulative SO$_2$ derivatives; (B) Relaxation curves in (○) endothelium-intact rings (n = 18), (●) endothelium-denuded rings (n = 18), and (▲) endothelium-intact rings receiving 100 µM of a non-specific NOS inhibitor L-NAME (n = 8). Results are mean ± SEM. *$P$ < 0.01 vs. endothelium-intact rings.
**Fig 2.** Relaxation to SO$_2$ derivatives (0.5 - 8 mM) in endothelium-denuded aortic rings pre-constricted by 1 µM phenylephrine (PE, n = 18) or 60 mM K$^+$ (high K$^+$, n = 8). (A) Representative traces of vessel tension; (B) Relaxation curves in rings pre-constricted by 1 µM phenylephrine (PE, ○) or 60 mM K$^+$ (high K$^+$, ●). Results are mean ± SEM. * $P < 0.01$ vs. PE.
**Fig 3.** Relaxation to SO₂ derivatives (0.5 - 8 mM) in endothelium-denuded aortic rings pre-constricted by 1 µM PE. Glibenclamide (3 µM, n = 10), TEA (5 mM, n = 15) or 4-AP (100 µM, n = 12) were incubated for 30 min before the addition of PE. Results are mean ± SEM. *P < 0.01 vs. control.
**Fig 4.** CaCl$_2$-induced contraction in Ca$^{2+}$-free, 60 mM K$^+$ solution in the absence and presence of SO$_2$ derivatives (0.5 - 8 mM) in aortic rings without endothelium. Results are mean ± SEM of eight experiments.
**Fig 5.** Relaxation to SO$_2$ derivatives (0.5 - 8 mM) in endothelium-denuded aortic rings pre-constricted by 1 µM phenylephrine (PE). Rings were incubated with 100 mM ouabain (n = 14) or 30 µM NiCl$_2$ (n = 13) for 30 min before the addition of PE. Results are mean ± SEM.