Physiol. Res. 58: 521-527, 2009

Sulfur Dioxide Relaxes Rat Aorta by Endothelium-Dependent and -Independent Mechanisms

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Received November 24, 2007
Accepted June 23, 2008
On-line July 25, 2008

Summary

This study aimed to investigate the vasoactivity of sulfur dioxide (SO_2), a novel gas identified from vascular tissue, in rat thoracic aorta. 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, SO_2 derivatives, and various drugs as pharmacological interventions. In endothelium-intact aortic rings constricted by 1 μM phenylephrine (PE), SO_2 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 SO_2 derivatives, but not that to relatively high doses (≥ 2 mM). In endothelium-denuded rings, SO_2 derivatives attenuated vasoconstriction induced by high K^+ (60 mM) or $CaCl_2$ (0.01-10 mM). The relaxation to SO_2 derivatives in PE-constricted rings without endothelium was significantly inhibited by blockers of ATP-sensitive K^+ (K_{ATP}) and Ca^{2+}-activated K^+ (K_{Ca}) channels, but not by those of voltage-dependent K^+ channels, Na^+-K^+-ATPase or Na^+-Ca^{2+} exchanger. SO_2 relaxed vessel tone via endothelium-dependent mechanisms associated with NOS activation, and via endothelium-independent mechanisms dependent on the inhibition of voltage-gated Ca^{2+} channels, and the opening of K_{ATP} and K_{Ca} channels.

Key words

Sulfur dioxide • Vasorelaxation • Ion channel • Endothelium • Aorta • Rat

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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 (H_2S) (Bhatia 2005). Sulfur dioxide (SO_2) is a novel gas first detected in porcine coronary artery by Balazy *et al.* (2003). A possible precursor of SO_2 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 β-sulfinylpyruvate, which decomposes spontaneously to pyruvate and SO_2 (Griffith 1983). Another pathway that can produce SO_2 is the oxidation of H_2S by NADPH oxidase (Mitsuhashi *et al.* 2005).

Little is known about the vasoactivity of SO_2. A few studies addressed the systemic impact of SO_2 inhalation under the concern that SO_2 is a common air pollutant. Meng *et al.* (2003) reported that both SO_2 inhalation and intraperitoneal injection of SO_2

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derivatives decreased blood pressure in rats, implying possible vasorelaxant activity of SO_2. Besides, ACh was shown to stimulate the formation of SO_2 (Balazy *et al.* 2003), raising a possibility that SO_2 is involved in the profound vasodilation response to ACh. However, the direct vasoactivity of SO_2 has never been described.

SO_2 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 SO_2 derivatives, a mixture of sulfite and bisulfite (Na_2SO_3/$NaHSO_3$) 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 SO_2 was revealed by denuding the endothelium and applying a NOS inhibitor N^G-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 Ca^{2+} channels, Na^+-K^+ pump and Na^+-Ca^{2+} 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 % O_2 – 5 % CO_2. The modified K-H solution contained (in mM): NaCl 118, KCl 4.6, $MgSO_4$ 1.2, KH_2PO_4 1.2, glucose 11.1, $NaHCO_3$ 27.2, EGTA 0.03, $CaCl_2$ 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 SO_2 derivatives (Na_2SO_3 and $NaHSO_3$, 3:1 M/M) were added cumulatively into the bathing solution once steady contraction was obtained. The equivalent SO_2 concentration ranged from 0.5 to 8 mM. We have examined the pH of the Krebs solution that it is about 7.40 under normal conditions. After we add SO_2 derivates at the lower concentrations (0.5~2 mM), the pH does not change. It will decrease to 7.37 after SO_2 derivates added at the higher concentrations (4~8 mM) and can return to 7.40 in 3-4 min. 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 SO_2 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 (N^G-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 SO_2 derivatives, aortic rings without endothelium were constricted with high K^+ (60 mM, to abolish the effect of K^+ channel activation) (Sang *et al.* 2003), and SO_2 derivatives were applied cumulatively. To further identify the types of K^+ channels associated with SO_2, a K_{ATP} blocker glibenclamide (3 μM), a K_{Ca} blocker tetraethylammonium chloride (TEA, 5 mM) or a K_V 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 SO_2 derivatives was obtained per ring in the presence of each inhibitor.

The ability of SO_2 to modulate Ca^{2+} influx *via* VGCCs was studied using $CaCl_2$-constricted rings without endothelium, as described previously (Chan *et al.* 2005). For this set of experiments, two consecutive

concentration-dependent constrictions to $CaCl_2$ were obtained in the absence and in the presence of SO_2 derivatives (0.5-8 mM, 5-min incubation). For constructing $CaCl_2$ concentration-response curve, arterial rings were rinsed three times in Ca^{2+}-free solution containing 30 mM Na_2-EGTA and then incubated in Ca^{2+}-free, 60 mM K^+ solution before the cumulative addition of $CaCl_2$ (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 SO_2 derivatives (0.5-8 mM). Na^+-K^+-ATPase inhibitor ouabain (100 mM) or Na^+-Ca^{2+} 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 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 ± S.E.M. SO_2-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. $P<0.05$ value was considered significant.

Results

SO_2 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 SO_2 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 SO_2 derivatives (0.5-8 mM) in both endothelium-intact and -denuded rings. Figure 1A shows representative recordings. Lower concentrations of SO_2 derivatives (0.5 and 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 SO_2 derivatives (2, 4 and 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 SO_2 derivatives at lower doses, while the endothelium-independent relaxation is predominantly displayed at higher doses.

In agreement with the phenomenon, a non-selective NOS inhibitor L-NAME (100 μM) abolished the relaxation of endothelium-intact rings induced by lower doses of SO_2 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 SO_2 derivatives.

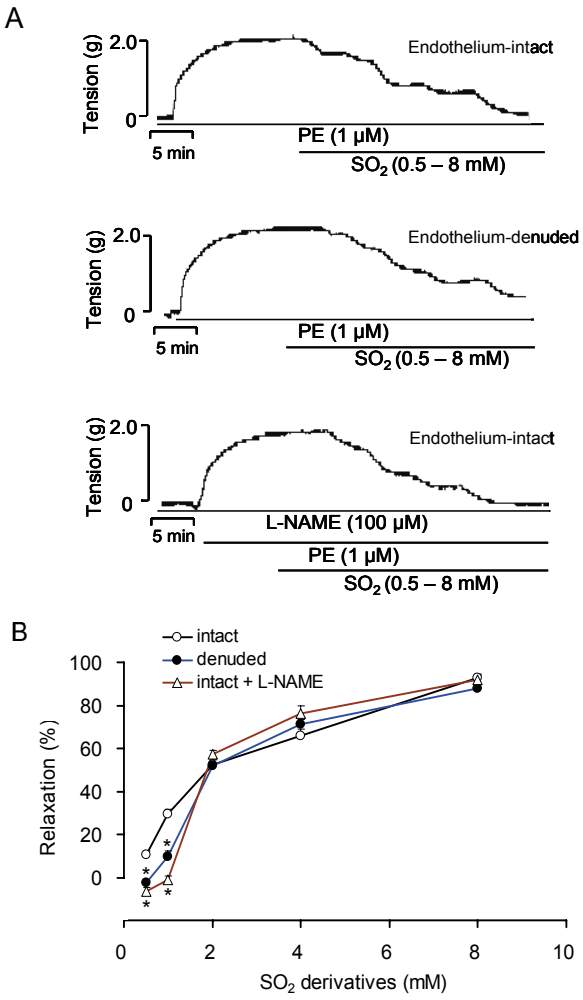

Fig. 1. Relaxation to SO_2 derivatives (0.5-8 mM) in rat thoracic aortic rings pre-constricted by 1 μM phenylephrine (PE). (**A**) Representative traces of vessel tension in response to PE and cumulative SO_2 derivatives; (**B**) Relaxation curves in (○) endothelium-intact rings (n = 18), (●) endothelium-denuded rings (n = 18), and (△) endothelium-intact rings treated with 100 μM of a non-specific NOS inhibitor L-NAME (n = 8). Results are mean ± S.E.M. *$P<0.01$ vs. endothelium-intact rings.

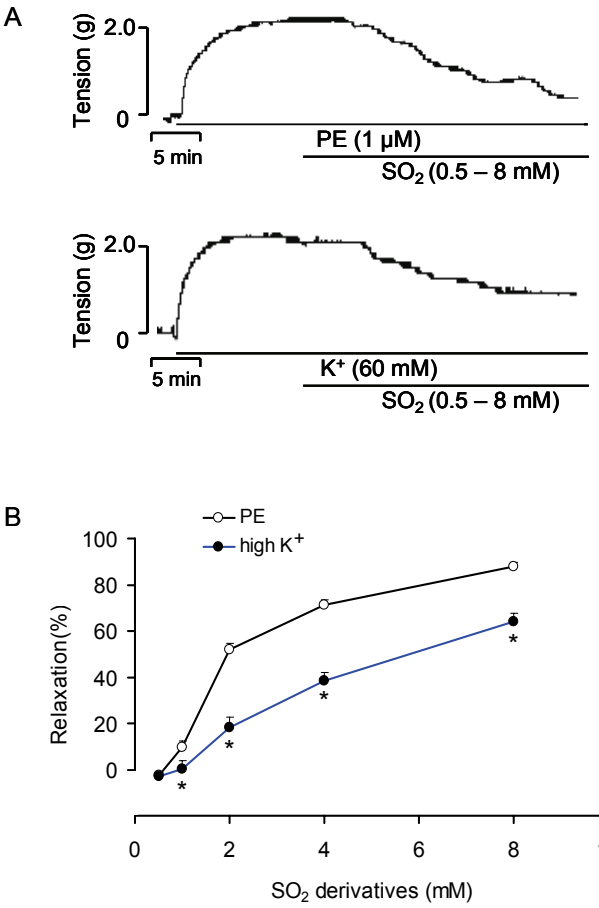

Fig. 2. Relaxation to SO_2 derivatives (0.5-8 mM) in endothelium-denuded aortic rings preconstricted by 1 μM phenylephrine (PE, n = 18) or 60 mM K^+ (high K^+, n = 8). (**A**) Representative traces of vessel tension; (**B**) Relaxation curves in rings pre-constricted by 1 μM phenylephrine (PE, ○) or 60 mM K^+ (high K^+,●). Results are mean ± S.E.M. * P<0.01 vs. PE.

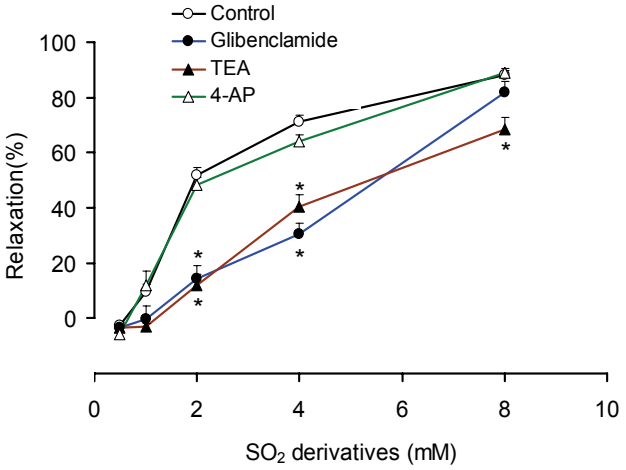

Fig. 3. Relaxation to SO_2 derivatives (0.5-8 mM) in endothelium-denuded aortic rings preconstricted by 1 μM PE. Glibenclamide (3 μM, n = 10), TEA (5 mM, n = 15) or 4-AP (100 μM, n = 12) were incubated for 30 min before the addition of PE. Results are mean ± S.E.M. *P<0.01 vs. control.

Ion channels involved in endothelium-independent relaxation induced by SO_2 derivatives

Involvement of K^+ channels

In endothelium-denuded aortic rings constricted with 1 μM PE, SO_2 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 SO_2 derivatives was significantly inhibited (maximum 63.88±3.78 %, P<0.01, Fig. 2B), 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 SO_2 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 SO_2 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 SO_2-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 Figure 3, glibenclamide significantly reduced the relaxation caused by 4 mM and 2 mM SO_2 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 SO_2 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 SO_2 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 SO_2-induced relaxation is associated with the opening of K_{ATP} and K_{Ca} channels in smooth muscle cells, but not K_V channels.

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

In Ca^{2+}-free, 60 mM K^+ solution, cumulative $CaCl_2$ (0.01-10 mM) induced progressive constriction of aortic rings without endothelium. The maximal tension was approximately 2.5 g. SO_2 derivatives (0.5-8 mM) reduced $CaCl_2$-induced constriction in a dose-dependent manner with progressive suppression of the maximal constriction (n ≥ 6 for each group, Fig. 4). In control

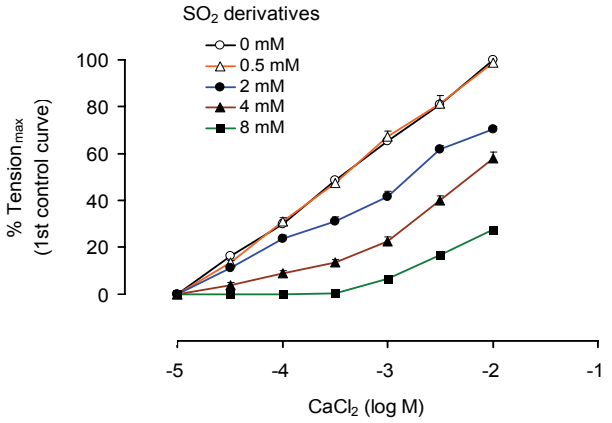

Fig. 4. $CaCl_2$-induced contraction in Ca^{2+}-free, 60 mM K^+ solution in the absence and presence of SO_2 derivatives (0.5-8 mM) in aortic rings without endothelium. Results are mean ± S.E.M. of eight experiments.

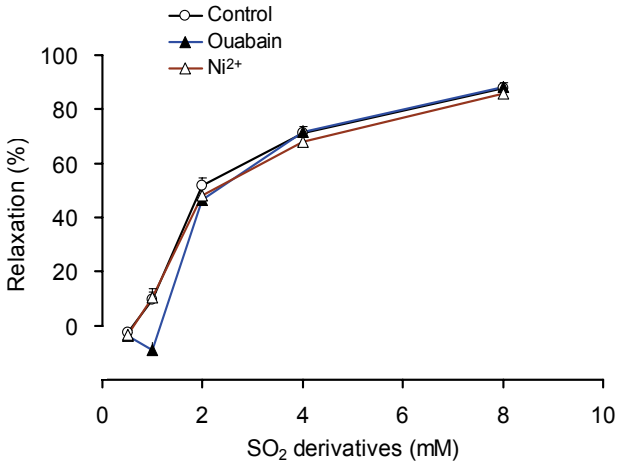

Fig. 5. Relaxation to SO_2 derivatives (0.5-8 mM) in endothelium-denuded aortic rings pre-constricted by 1 μM phenylephrine (PE). Rings were incubated with 100 mM ouabain (n = 14) or 30 μM $NiCl_2$ (n = 13) for 30 min before the addition of PE. Results are mean ± S.E.M.

experiments, 1 μM nifedipine abolished vasoconstriction to $CaCl_2$ (data not shown).

No involvement of Na^+-K^+ pump and Na^+-Ca^{2+} exchanger

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 SO_2-induced vasodilation is associated with the stimulation of Na^+-K^+ pump or forward Na^+-Ca^{2+} exchanger, the present study used a Na^+-K^+-ATPase inhibitor ouabain (100 mM) and a putative Na^+-Ca^{2+} exchanger inhibitor Ni^{2+} (30 μM). Neither ouabain nor Ni^{2+} affected the vasodilation resulting from SO_2 derivatives in PE-constricted aortic rings without endothelium (Fig. 5). It is suggested that Na^+-K^+ pump and Na^+-Ca^{2+} exchanger are not involved in the vasorelaxant response to SO_2 derivatives.

Discussion

The vasoactivity of SO_2 has never been described before, although it was found in vascular tissue (Balazy *et al.* 2003). The present study first revealed the direct vasorelaxant activity of SO_2. In constricted rat aortic rings under isometric recording, we found that the derivatives of SO_2 hydration, sulfite and hydrogen sulfite (Shapiro 1977), reduced vessel tension in a dose-dependent manner. The vasorelaxation resulting from low doses of SO_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_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_2. Previous report showed that the endothelium-independent relaxation by SO_2 derivates was not mediated by NO (Meng and Zhang 2007). 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_2 derivatives, using pharmacological interventions. It is well known 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.* (2003) proposed SO_2 as a candidate for the unidentified endothelium-derived hyperpolarizing factor (EDHF), based on the facts that of 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 and Zhang 2007, Meng *et al*. 2007). 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. Multiple types of K^+ channels have been identified in vascular smooth muscle cells, among which K_{ATP}, K_{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 to attenuate 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 revealed 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 contributed to the endothelium-dependent relaxation, and the endothelium-independent relaxation was associated with the activation of K_{ATP} and K_{Ca}, and the inactivation of VGCCs. In addition, K_V, Na^+-K^+-ATPase and Na^+-Ca^{2+} exchanger were not suggested to be involved in the vasorelaxant response to SO_2 derivatives.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

This study was supported by grants from the National Basic Research Program of China (No. 2006CB503807) and the National Natural Science Foundation of China (No. 30572190, No. 30600763 and No. 30670760).

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