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Vasodilatory effects of nitric oxide, hydrogen sulfide and sulfur dioxide in rats: Time-dependent interaction study

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ABSTRACT

The vasodilator response of nitric oxide (NO), hydrogen sulfide (H_2S) and sulfur dioxide (SO_2) were studied to determine the significance of the actions and interactions of these gasotransmitters for controlling aortic tone in rats. The isometric tension of five separate sets of experiments was recorded. Sodium nitroprusside (SNP; NO donor), sodium disulphide (Na_2S; H_2S donor), SO_2 derivatives and their paired combinations were added to phenylephrine (PE)-induced contraction during the peak value. Then maximal relaxation rate was calculated four times at 5 min intervals. Tetraethylammonium (TEA) and Glibenclamide (GLIB) were applied for investigating the molecular mechanism of the gasses. While, in a separate set of experiments, we used either L-Arginine (L-Arg), L-Cysteine (L-Cyst) or L-nitroarginine methyl ester (L-NAME) before applying gasotransmitters. Highest and prolonged relaxation rate were recorded when SNP was combined with SO_2. The combination of Na_2S and SO_2-induced vasorelaxation was blocked by TEA and GLIB pretreatments. L-Cyst decreased relaxation compared to SNP and vice versa to SO_2 induced vasorelaxation. L-Arg markedly attenuated relaxation responses of Na_2S and SO_2 derivatives. Also, L-NAME delayed relaxation compared to Na_2S and SO_2. These results suggest that exogenous paired combinations of H_2S, NO and SO_2 will enhance and elongate the rate of aortic relaxation. Meanwhile, preincubation of aortic rings with precursors attenuate the dilatory effects of exogenous studied gases.

Keywords: Gasotransmitters, Nitric Oxide; Hydrogen Sulfide; Sulfur Dioxide; Vasotion.

Introduction

The last few years have seen a great interest in the biology of endogenously active gases; taken together, these gases compose what has come to be known as a family of "gasotransmitters" (1). These gases include NO, carbon monoxide, H_2S and SO_2 (2) that are membrane permeable and activator of different molecular targets (3).

The discovery of NO as the first gasotransmitter enhanced the researchers in the field of physiology and pharmacology (4). Within the vasculature, NO is produced endogenously in the endothelial cells from

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L-Arg by a family of nitric oxide synthases (NOS) enzymes (5). Functionally, NO regulates vascular tone through opening of multiple potassium (K^+) channels via the activation of soluble guanylyl cyclase enzyme and generation of cyclic GMP (6). Along with NO and CO, H_2S is recognized as the third 'gasotransmitter,' which plays multiple physiological and pathophysiological functions; such as long term synaptic potentiation, vasorelaxation, pro- and anti-inflammatory conditions, cardiac inotropic regulation and cardioprotection (7). H_2S is synthesized via desulfhydration of L-Cys, mainly by two pyridoxal-5-phosphate-dependent enzymes: cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) in the brain and peripheral tissues (8). Furthermore, major physiological actions of H_2S include relaxation of different arteries via activation of different K^+ channels mainly ATP-dependent K^+ (K_{ATP}) channels (9).

The first detection of SO_2 in the porcine coronary artery encouraged some researchers to study the physiological and pathophysiological role of SO_2 (10). Then, endogenous SO_2 generation in vascular tissues and its blood pressure lowering (11) and vasodilator effects were later discovered (12). SO_2 is produced endogenously from intracellular H_2S or normal metabolism of L-Cys (13). At high concentration, SO_2 could relax vascular smooth muscles (VSMCs) directly via modulation of calcium (Ca^{2+}) channels and K_{ATP} channels (14). While at basal and low concentrations, vasorelaxation was endothelium-dependent and related to the opening of the Ca^{2+}-activated K^+ channel (K_{Ca}) (15).

However, gasotransmitters in vascular tissue might not exist independently; they might have interactions (16), which are complicated and currently unclear (17). Although some researchers have shown that H_2S either positively increases NO production and function (18); or may interact to produce a new molecule (possibly nitrosothiol) (19), or a network of cascading chemical reactions that generate radical intermediates, anionic and uncharged solutes each with a distinct bioactivity (20). On the other hand, they may exert negative and opposite effects (21). Although, whether and how NO and H_2S affect SO_2 function is still unclear. The previous studies demonstrate that there are still many challenges for future gasotransmitter research to tackle, including clarifying the interactions among these gases; understanding the significance of the cellular and molecular gasotransmitter signaling networks. Therefore, this study was designed to explore the possible synergistic effects of these gases and their precursors on the relaxation of rat aortic rings at different time intervals and to understand further about the molecular mechanism of gasotransmitters.

Material and methods

Experimental animals

The experimental procedures were carried out according to the Guide for the Care and Use of Laboratory Animals and approved by the Animal Research Committee of Salahaddin University-Erbil. Adult male Wistar rats (*Rattus norvegicus*) weighing about 200–300 g were used for this study. The animals were kept under standard laboratory conditions.

Tissue preparation

After anaesthetizing the rats with ketamine (40 mg/kg) and xylazine (10 mg/kg) intraperitonealy, the chest cavity was opened, after removal of excess tissue and fat, thoracic aorta was isolated and transferred to beaker containing Krebs solution (composition in mM: NaCl-136.9, KCl-5.4, Glucose-5.5, $NaHCO_3$-23.8, $MgCl_2$-1, $CaCl_2$-1.5, and EDTA-0.003), equilibrated with 95% O_2 and 5% CO_2. The beaker was placed in the water bath at 37˚C.

The procedure of Furchgott and Zawadzki (22) with some modifications was followed to study the vascular reactivity in the isolated aorta. Briefly, two stainless steel wires were carefully inserted into the lumen of the aortic rings. One wire was anchored to the hook at the base of an organ bath (Model 166051, Radnoti, Monrovia Ca, USA) and another wire was connected to force transducer (MLT0201/RAD 5 gm to 25 gm, AD Instruments, Sydney, Australia) coupled to the transbridge amplifier (ML 224, Quad Bridge Amp, AD Instruments). Data was acquired with PowerLab Data Acquisition System (ML 870, Power Lab, AD Instruments) using the LabChart software 7 for measurement of isometric tension. The degree of contraction and relaxation were indicated by the

tension development in the recording system and expressed in gram (g).

Aortic relaxation studies

Rings were allowed to equilibrate for 60 min at a resting tension of 2 g with changes of buffer every 15 min. When the isometric tension had stabilized, the effects of relaxants were taken about the difference of the tension induced by phenylephrine (PE; 1 µM) and the original tension. This difference was set at 100%.

Aortic rings precontracted with PE were first relaxed by cumulative addition of increasing concentrations of either SNP (1 nM-10 µM), Na_2S (1-6 mM) or SO_2 derivatives "1:3 M/M $NaHSO_3$ and Na_2SO_3" (3-8 mM) (23). Based on these preliminary experiments, relative half-inhibitory concentration (IC_{50}) of SNP (2.3 µM), Na_2S (2.4 mM) SO_2 derivatives (6 mM) had retested for the ability to relax precontracted rings in four separate sets of experiments. First, when the PE-induced contraction reached the peak value, SNP (2.3 µM), Na_2S (2.4 mM) SO_2 derivatives (6 mM) were added and left for 20 min, and the maximal relaxation rate (%) were calculated four times at 5 min intervals. In the second set, every two vasodilators were added to the organ bath simultaneously. Then, to test the role of K_{Ca} and K_{ATP} channels in the development of relaxation induced by every two vasodilators, the aortic rings were preincubated for 20 min with either tetraethylammonium (TEA; 1 mM) or glibenclamide (GLIB; 10 µM), blockers of both K^+ channels, respectively. While in the third set of experiments we used either L-Arg (3 mM; precursor of NO) or L-Cys (3 mM; precursor of both H_2S and SO_2) were applied to the organ bath 1 hr before the addition of vasorelaxant gases. Finally, to remove the effect of NOS, aortic rings were preincubated with L-NAME (0.3 mM), an antagonist of NOS for 10 min before applying gasotransmitters.

Statistical Analysis

All values represented as mean±SE. The statistical analysis was performed using two-way analysis of variance (ANOVA) followed by Bonferroni *post hoc* test. P-value less than 0.05 ($P<0.05$) were considered as statistically significant. All the graph, calculation and statistical analyses were performed using GraphPad Prism software 6.0 (GraphPad Software, San Diego, California, USA).

Results

Comparison between SNP, Na_2S and SO_2 Derivatives Induced Aortic Relaxation

To investigate which of the used gasotransmitters have high aortic relaxant effects and remains at all time-intervals over the course of 20 min comparisons were made at each 5 min intervals. SNP (2.3 µM, *n*=6) caused significant time-dependent relaxation of the rat aorta compared to SO_2 derivatives (6 mM) treated arteries at all time-intervals over the course of 20 min. Furthermore, Na_2S (2.4 mM, *n*=5) caused significant time-dependent relaxation of the rat aorta compared to SO_2 derivatives (6 mM, *n*=4) at 10 and 15 min time-intervals. On the contrary, SNP did not show any significant differences on the rat aorta compared to Na_2S at all time-intervals over the course of 20 min (Fig. 1A, Table 1). LabChart traces from representative experiments on the relaxant effect of SNP, Na_2S and SO_2 derivatives on PE-precontracted aortic rings are shown in Figure 1B.

Table 1. Time-dependent and combination effects of gasotransmitters on vascular contractility in rat aortic rings

Na_2S+SO_2	$SNP+SO_2$	$SNP+Na_2S$	SO_2	Na_2S	SNP	
59.14±6.44	68.32±6.09	64.94±14.16	9.33±2.17	26.54±5.21	47.8±10.49	**5 min**
87.68±6.46	107.45±4.21	98.25±8.64	43.11±5.95	69.31±6.51	85.1±8.03	**10 min**
83.56±9.18	110.46±5.2	102.26±8.29	43.11±5.95	77.41±6.12	87.29±7.99	**15 min**
78.65±11.61	110.72±6.15	99.42±7.99	56.56±3.533	73.14±5.62	87.46±8.6	**20 min**

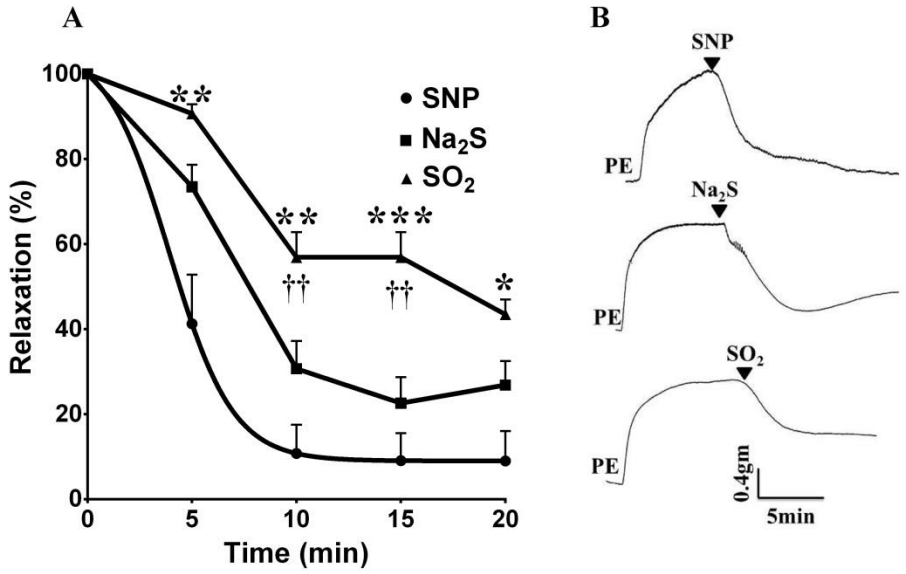

Figure 1. Time-dependent tension modulation by SNP (2.3 µM;●), Na_2S (2.4 mM;■) and SO_2 derivatives (6 mM;▲) on the tone of the PE (1 µM)-precontracted rat thoracic aorta. (A) Represent comparison between SNP and Na_2S induced time-dependent aortic relaxation. (B) SNP significantly potentiated aortic relaxation more than SO_2 derivatives (P<0.01, 5 and 20 min; *** P<0.001, 10 to 15 min). (C) Aortic relaxation significantly enhanced by Na_2S in comparison to SO_2 derivatives (††P<0.01, 10 to 15 min). All data are expressed as (%) of relaxation of PE-induced aortic tone and are represented as the mean±SE. (D, E and F) Represent typical traces showing time-dependent vasorelaxant effects of SNP, Na_2S and SO_2 derivatives in isolated rat aortic rings precontracted with PE, respectively. SNP, Sodium nitroprusside; Na_2S, Sodium disulfide; SO_2, Sulfur dioxide; PE, phenylephrine.**

Interaction effects of SNP, Na_2S and SO_2 derivatives on aortic tone

An additional series of experiments were undertaken to test the ability of the combination of these gases on the relaxation of rat aortic rings. LabChart traces from representative experiments on the combined effect of SNP, Na_2S and SO_2 derivatives on PE-precontracted aortic rings are shown in Figure 2D, E and F, respectively.

The highest degree of relaxation during this study was observed when SNP combined with SO_2 derivatives (n=4) at 20 min of relaxation. However, the combination of SNP with SO_2 derivatives slightly increased maximal relaxation at all time-intervals over the course of 20 min in comparison to the relaxation induced by SNP (Fig. 2A, Table 2). On the other hand, a combination of Na_2S with SNP (n=8) significantly enhanced maximal response as compared to individual Na_2S at all time intervals over the course of 20 min (Fig. 2B, Table 2). Furthermore, the combination of SO_2 derivatives with SNP increased vascular tone at all time-intervals over the course of 20 min compared to the relaxation induced by SO_2 derivatives. These effects were remained significant for 15 min when SO_2 derivatives combined with Na_2S (n=4) (Fig. 2C, Table 2).

Table 2. Involvement of K_{Ca} and K_{ATP} channels in the mechanism of time-dependent combination effects of gasotransmitters vascular contractility in rat aortic rings

	TEA			GLIB		
	SNP+Na_2S	SNP+SO_2	Na_2S+SO_2	SNP+Na_2S	SNP+SO_2	Na_2S+SO_2
5 min	76.48±13.51	60.85±8.33	-12.8±7.65	86.14±6.92	85.14±31.42	-26.8±11.44
10 min	97.16±9.2	90.05±12.38	6.28±14.66	107.91±9.93	127.36±41.32	-23.7±28.47
15 min	105.08±9.52	107.61±19.6	32.84±12.08	113.3±15.27	129.07±43.85	-32.4±34.58
20 min	95.92±11.94	118.85±22.12	54.56±14.99	110.84±14.31	133.21±46.37	-14.8±27.44

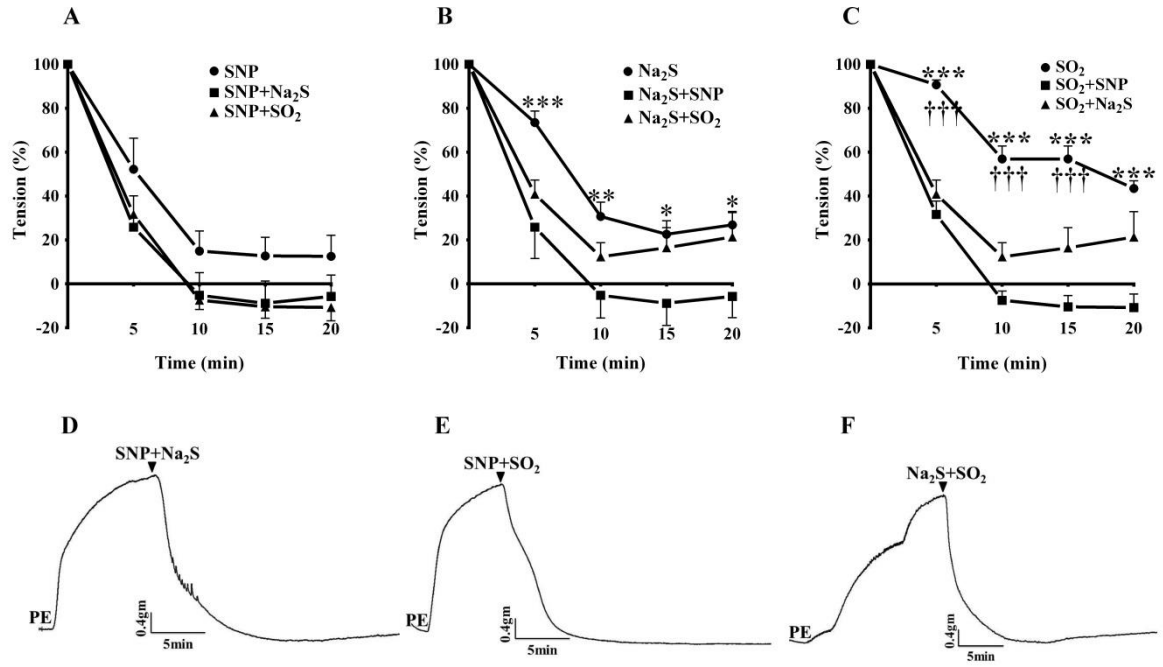

Figure 2. Combination effects of SNP, Na_2S and SO_2 derivatives on time-dependent tension modulation induced by PE in rat thoracic aortic rings. (A) Show SNP (●) induced time-dependent relaxation in the presence of Na_2S (■) or SO_2 derivatives (▲), compared to the relaxation induced by SNP. (B) Show significant differences between Na_2S (●) induced time-dependent relaxation in the presence of SNP (■) (* P<0.001, 5 min, ** P<0.01, 10 min; * P<0.05, 15 and 20 min), compared to the relaxation induced by Na_2S. (C) Show significant differences between SO_2 derivatives (●) induced time-dependent relaxation in the presence of SNP (■) (*** P<0.001, from 5 to 20 min) or Na_2S (▲) (†††P<0.001, from 5 to 15 min), compared to the relaxation induced by SO_2 derivatives. All data are expressed as (%) of relaxation of PE-induced aortic tone and are represented as the mean±SE. (D, E and F) Represent typical traces showing time-dependent vasorelaxant combination effects of SNP, Na_2S and SO_2 derivatives in isolated rat aortic rings precontracted with PE, respectively. SNP, Sodium nitroprusside; Na_2S, Sodium disulfide; SO_2, Sulfur dioxide; PE, phenylephrine.**

Effect of TEA or GLIB on aortic relaxation induced by combination activity of vasodilators

To identify the role of K_{Ca} and K_{ATP} channels on the time-dependent change of relaxation responses to combination effects of SNP, Na_2S and SO_2 derivatives aortic rings were incubated with either TEA or GLIB for 20 min before to the application of vasodilators. LabChart traces from representative experiments on the effect of TEA or GLIB are shown in Figure 3D, E and F, respectively.

In the presence of the GLIB (10 μM, *n*=5) the net vasorelaxation effect of Na_2S with SO_2 derivatives was significantly reduced at all time-points, while blocking of K_{Ca} channels with TEA (1 mM, *n*=6) significantly reduced vasorelaxation only at 5 and 10 min (Fig. 3C, Table 2). Neither the TEA (*n*=4) nor GLIB (*n*=5) had any significant effect on the time-dependent vascular responses to SNP with Na_2S (Fig. 3A, Table 2). Similarly, pre-treating arteries with the TEA (*n*=7) or GLIB (*n*=7) had no effect on the time-dependent vascular response to SNP with SO_2 derivatives (Fig. 3B, Table 2).

Table 3. The role of L-Arg and L-Cys in the time-dependent vasorelaxant effects of gasotransmitters on vascular contractility in rat aortic rings

	L-Arg			**L-Cys**		
	SNP	Na_2S	SO_2	SNP	SNP	SNP
5 min	60.7±6.28	-23.2±16.26	108.56±2.61	58.32±2.86	58.32±2.86	58.32±2.86
10 min	85.46±3.64	-0.86±18.22	121.47±9.52	69.92±4.25	69.92±4.25	69.92±4.25
15 min	86.14±4.71	17.67±13.33	126.91±12.32	54.72±6.18	54.72±6.18	54.72±6.18
20 min	85. 7±6.24	15.18±14.41	126.53±13.44	41.23±7.96	41.23±7.96	41.23±7.96

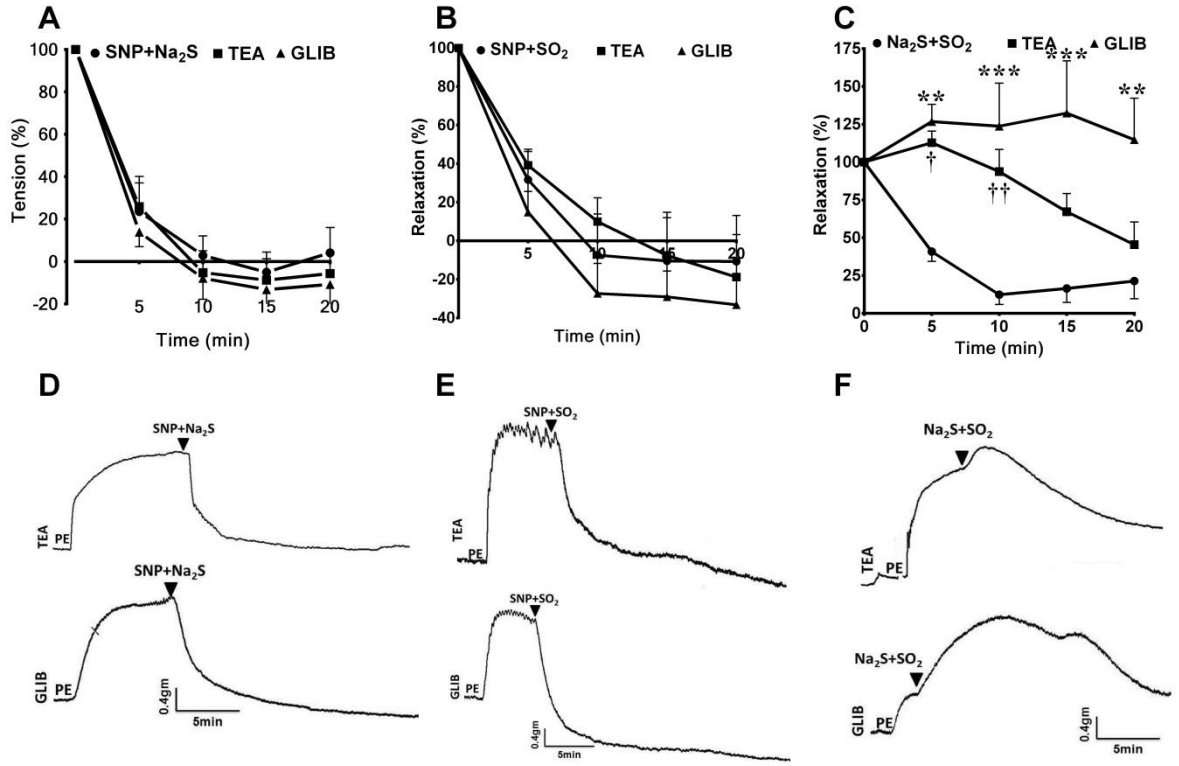

Figure 3. Time-dependent change of relaxation responses to combination effects of SNP, Na_2S and SO_2 derivatives in rat thoracic aortic rings preincubated with either TEA (1 mM) or GLIB (10 µM). (A and B) Preincubation of aortic rings with either TEA (■) or GLIB (▲) had no significant effect on SNP+Na_2S- or SNP+SO_2-induced time-dependent vasorelaxation. (C) Na_2S+SO_2-induced vasorelaxation significantly inhibited by either TEA (■) pretreatment (P<0.01, 5 and 20 min, *** P<0.001, 10 and 15 min) or GLIB (▲) (†P<0.05, 5 min and ††P<0.01, 5 min). While, (D, E and F) represents typical traces showing the effects of TEA or GLIB preincubation on time-dependent aortic relaxation responses to combination of every two vasodilators precontracted with PE. All data are expressed as (%) of relaxation of PE-induced aortic tone and are represented as the mean±SE. SNP, Sodium nitroprusside; Na_2S, Sodium disulfide; SO_2, Sulfur dioxide; PE, phenylephrine; TEA, tetraethylammonium; GLIB, Glibenclamide.**

Effect of L-Arg or L-Cys on aortic relaxation induced by SNP, Na_2S and SO_2 derivatives

In this set of experiment, we tested whether endogenous sources of these gases (NO from L-Arg; H_2S and SO_2 from L-Cys) were able to modulate vasorelaxation induced by exogenously applied NO and H_2S donors and SO_2 derivatives. Typical traces from representative experiments of L-Arg and L-Cys preincubated aortic rings on time-dependent relaxation to SNP, Na_2S and SO_2 derivatives are shown in Figure 4 D, E and F, respectively.

Preincubated thoracic aortic rings with L-Cys (3 mM, *n*=6) caused a significant decrease of time-dependent relaxation compared to SNP-induced relaxation at 15 and 20 min. By contrast, L-Arg (3 mM, *n*=4) did not have any significant effect on the rat aorta compared to time-dependent relaxation induced by SNP (Fig. 4A, Table 3).

At the same time, preincubation of aortic rings with L-Arg (3 mM, *n*=5) caused a marked time-dependent attenuation of the vasorelaxation responses to Na_2S at all time-intervals over the course of 20 min. Pretreatment of aortic rings with L-Cyst (5 mM, *n*=6) had no effects on Na_2S-induced vasorelaxation (Fig. 4B, Table 3).

Vasorelaxation mediated by SO_2 derivatives was stimulated in the presence of L-Cys (*n*=4), their potencies were significantly increased at 10 and 15 min of relaxation. On the other hand, pretreatment of aortic rings with L-Arg (*n*=6) markedly suppressed SO_2-induced relaxation at 10 to 20 min of relaxation (Fig. 4C, Table 3).

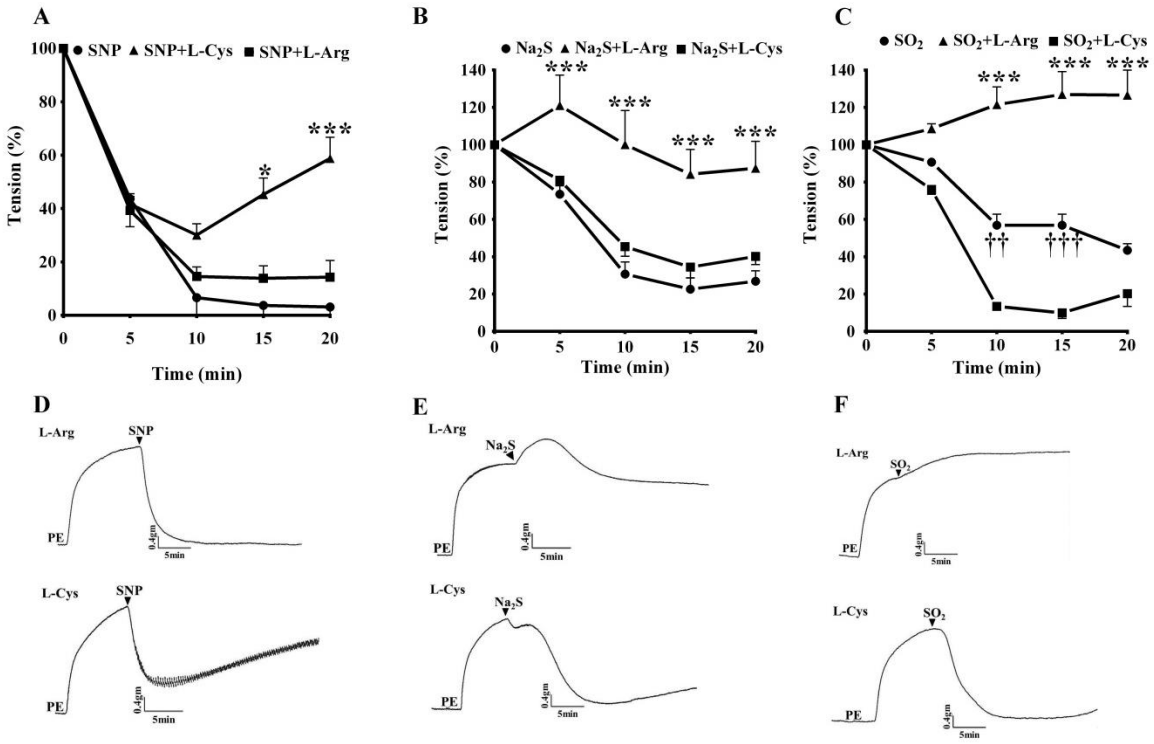

Figure 4. The effects of L-Arg (3 mM;■) and L-Cys (3 mM;▲) preincubation on rat aortic time-dependent relaxation to SNP, Na_2S and SO_2 derivatives (●) precontracted with PE. (A) L-Cyst pretreatment inhibited time-dependent relaxation induced by SNP (*P<0.05, 15 min; *** P<0.001, 20 min), when preincubation of L-Arg did not change SNP-induced relaxation. (B) Preincubation of aortic rings with L-Arg reduced time-dependent relaxation induced by Na_2S (* P<0.01, 5 to 20 min), while L-Cys failed to change in SNP-induced relaxation. (C) L-Cyst pretreatment was enhanced relaxation responses to SO_2 derivatives (*** P<0.001, from 10 to 15 min). Meanwhile, preincubation of aortic rings with L-Arg inhibited time-dependent relaxation induced by SO_2 derivatives (††P<0.05, 10 min; †††P<0.001, 15 min). (D, E and F) Represent typical traces showing the effects of L-Arg and L-Cys preincubation on rat aortic time-dependent relaxation to SNP, Na_2S and SO_2 derivatives precontracted with PE, respectively. All data are expressed as (%) of relaxation of PE-induced aortic tone and are represented as the mean±SE. SNP, Sodium nitroprusside; Na_2S, Sodium disulfide; SO_2, Sulfur dioxide; PE, phenylephrine.**

Effect of L-NAME on aortic relaxation induced by SNP, Na_2S and SO_2 derivatives

Since L-NAME is known to inhibit endothelial NOS production, the actions of L-NAME (10 µM) were tested on the ability of SNP, Na_2S and SO_2 derivatives to depress PE-induced vascular tone. Preincubation of aortic rings with L-NAME (*n*=4) caused a significant decrease of time-dependent relaxation compared to Na_2S-induced relaxation at 10 and 15 min of relaxation (Fig. 5B). However, in the presence of L-NAME (*n*=4), relaxation produced by SO_2 was significantly enhanced at 15 and 20 min of relaxation, (Fig. 5C). Moreover, incubation of aortic rings with L-NAME (*n*=5) did not affect significantly the extent of the SNP-induced relaxation at all time-courses of the study (Fig. 5A, Table 4). LabChart traces from representative experiments of L-NAME preincubated aortic rings on time-dependent relaxation to SNP, Na_2S and SO_2 derivatives are shown in Figure 5D, E and F.

Table 4. Effect of L-NAME in the time-dependent vasorelaxant effects of gasotransmitters on vascular contractility in rat aortic rings

	L-NAME		
	SNP	Na_2S	SO_2
5 min	75.7±1.8	22.2±7.5	1.4±2.4
10 min	102.9±2.2	40.8±10.2	62.4±8.7
15 min	105±2.6	54.7±6.7	95.2±3.4
20 min	105.4±2.8	57.7±5.3	98.0±2.5

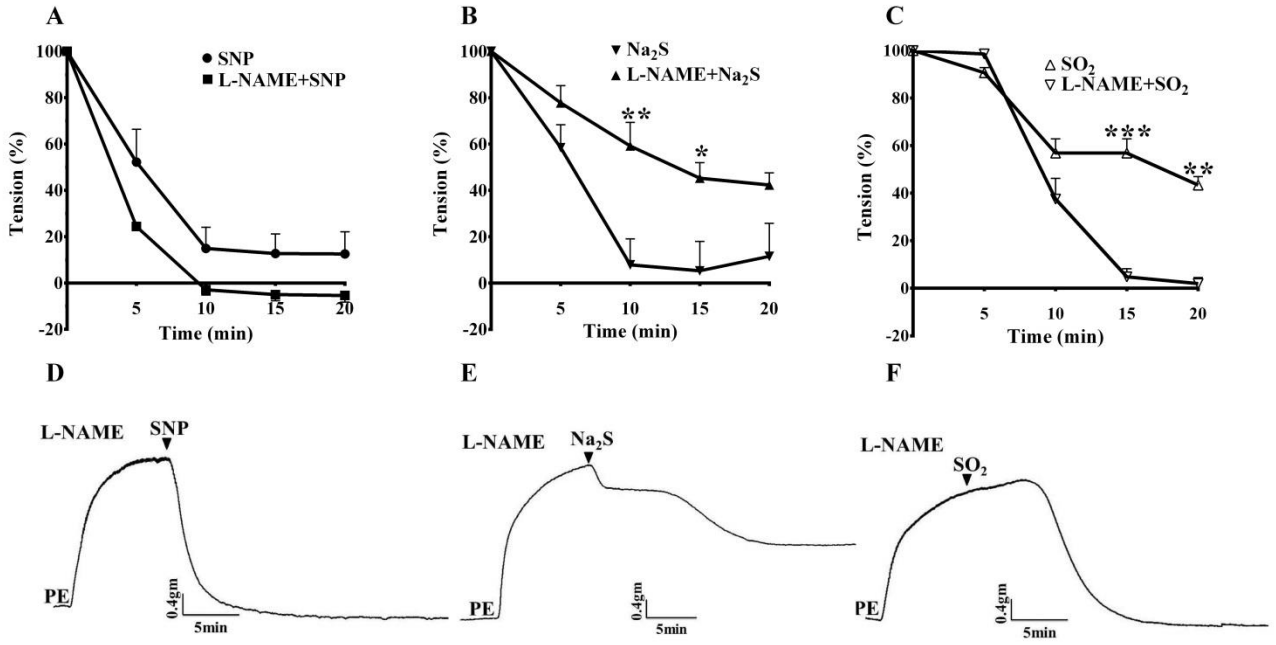

Figure 5. Time-dependent change of relaxation responses to SNP (●), Na_2S (▼) and SO_2 derivatives (△) in rat thoracic aortic rings preincubated with L-NAME (0.3mM), precontracted with PE (1 µM). (A) L-NAME (■) had no significant effect on SNP-induced time-dependent aortic relaxation. (B) Na_2S induced relaxation significantly inhibited by L-NAME (▲) pretreatment (P<0.01, 10 min, * P<0.05, 15 min). (C) In contrast, L-NAME (▽) enhanced inhibition of contraction induced by SO_2 derivatives (*** P<0.001, 15 min, ** P<0.01, 20 min). While, D, E and F represent typical traces showing the effects of L-NAME preincubation on time-dependent aortic relaxation responses to SNP, Na_2S and SO_2 derivatives precontracted with PE, respectively. All data are expressed as (%) of relaxation of PE-induced aortic tone and are represented as the mean±SE. SNP, Sodium nitroprusside; Na_2S, Sodium disulfide; SO_2, Sulfur dioxide; PE, phenylephrine; L-NAME, L-nitroarginine methylester.**

Discussion

The first important finding of this study is that exogenous application of NO has a greater effect of aortic relaxation compared to the other gases, and the power of relaxation will remain for a long time. The differences in relaxation rates and time prolonged by these gases are due to the types of blood vessels and differences in their molecular mechanism pathways. Furthermore, the rates of endogenous NO to H_2S production and clearance vary with time. Therefore, the sum of the biological effects of NO and H_2S stability will change in any system with time (1). NO plays an important vasorelaxant role in conduit artery, but H_2S may be more critical in controlling the relaxation of peripheral resistance arteries (3). The half-life of NO in blood ranges from 0.05 to 1.8 milliseconds (24). During this period, NO relaxes different blood vessels via the activation of guanylyl cyclase, elevation in cGMP levels, and opening of K_{Ca} channels (25) in many vessels, including rat and rabbit aorta (26). While actions of H_2S are complex, involve multiple pathways and dependent on the vascular bed, species, and experimental condition (27). The half-life of H_2S is ranging from seconds to minutes, but it relaxes blood vessels via direct stimulation of K_{ATP} channels and membrane hyperpolarization. It is markedly less potent than NO (25). Although SO_2 activates guanylyl cyclase to generate cGMP which elicits relaxation of VSMCs via cGMP-dependent protein kinases (28), the weakest relaxation response was induced by SO_2. In this regard, it is reasonable for us to suggest that NO is a more powerful among all tested gases and its effect remains for a long time.

Equally important to the first finding, the results of this study further advance our understanding of the interaction between NO and H_2S. In the present study, we show that the relaxation responses depend in the way of interaction between these gases. When both gas donors are added together, they prolong the aortic relaxation. This synergistic action may be due to the production of HSNO and HNO as a result of chemical reaction between H_2S and nitrite (29), which releases

NO and polysulfides and relax VSMCs through soluble guanylyl cyclase activation (30). In contrast, preincubation of aortic rings with L-Cys inhibits the tonic activity induced by NO donors. The suitable explanation for this observation is that the raised concentration of L-Cys within the endothelial cells converts a substantial portion of the $NO^{\cdot}$ Intracellularly to the polar, hydrophilic nitroso-L-Cysteine, which, unlike $NO^{\cdot}$, does not as easily pass through cell membranes, and trapped within the endothelial cells (31, 32). At the same time, pre-incubation of aortic rings with L-Arg caused a marked decrease in Na_2S-induced relaxation. Yong and his team (4) found that co-application of L-Arg and H_2S donor enhanced myocyte contractility via the increase in the amplitudes of intracellular Ca^{2+} transients. Also, the previous study showed that the H_2S-induced aortic relaxation can be decreased by removal of the endothelium, interruption of NO synthase or blocking of K_{Ca} channels (33). These observations are in agreement with our findings, in which a significant decline of H_2S-mediated relaxation was observed when aortic rings were preincubated with L-NAME. These data suggest that exogenous NO and H_2S interact enhance aortic relaxation, otherwise using one of the gas precursors inhibits relaxation produced by the second exogenous gas.

In the present study, our results indicate the highest degree of relaxation when SNP applied with SO_2 derivatives. NO, and SO_2 exert a synergistic effect on aortic relaxation (34), which might be mainly related to the activation of the cGMP pathway as both gases have similar cellular signaling mechanisms (35). The results also indicate that L-NAME can enhance the relaxation induced by SO_2 derivatives. The vasodilatory effects of SO_2 derivatives were independent of NO and could not be changed by the L-NAME preincubation (24) only at low concentrations (36). On the other hand, preincubation of aortic rings with L-Arg sharply reduced SO_2-induced aortic relaxation; it implies that endogenous NO inhibited SO_2-mediated vasodilation. Moreover, these data suggest that the synergistic activity of NO and SO_2 depend on the way interaction between these gases.

It is well known that the mechanism underlying the vasorelaxant activity of NO and SO_2 are related to the activation of K_{Ca} channels, while H_2S maintains vascular tone via the activation of K_{ATP} channels. However, most of the previous information on the biological interaction of these gases had been focused on the biochemical interactions, regulation of enzymatic expression and synergistic action of NO with H_2S. A few studies have examined the possible physiological combination of NO, H_2S and SO_2 in the modulation of K_{Ca} and K_{ATP} channels in the aorta. Thus, we hypothesised that K^+ channels partially mediate the vasorelaxant action of tested gases. Therefore, the current results point to the significant requirement of K_{Ca} and K_{ATP} channels to the vasorelaxant effect of both sulfur-containing gasotransmitters, while unable to modulate the synergistic activity of NO with H_2S and SO_2.

In this study, after preincubation of the aortic rings with either L-Cys or coapplication with H_2S, the vasodilator effects induced by SO_2 derivatives were strongly enhanced. This result was consistent with a previous study which reported that SO_2 can be either produced from L-Cys via a series of enzymatic conversion or via intracellular oxidation of H_2S (34). Furthermore, SO_2 could upregulate H_2S concentration and its generating enzymes in pulmonary hypertension (37), ischemia/reperfusion-induced myocardial injury (38) and in atherosclerotic rats (39). These results imply that SO_2 and H_2S have synergistic vasodilator activity.

In conclusion, the observations of this study can be summarized as the following: (1) NO is the most powerful among tested gases; (2) synergistic activity of NO, H_2S and SO_2 depend on the way of interaction between these gases; (3) exogenous gases interact to enhance aortic relaxation, otherwise using one of the gas precursors inhibits relaxation produced as a result of the second exogenous gas; and (4) the combination of SNP and SO_2 derivatives strongly enhances aortic relaxation. These findings provide insights into the combination effects of NO, H_2S and SO_2-mediated vasodilation and valuable information regarding the therapeutic antihypertensive application of these gases.

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REFERENCES

1. Li, L., Hsu, A. and Moore, P.K. (2009) Actions and interactions of nitric oxide, carbon monoxide and hydrogen sulphide in the cardiovascular system and in inflammation– a tale of three gases! *Pharmacol. Ther.*, **123**, 386-400.
2. Liu, D., Huang, Y., Bu, D., Liu, A.D., Holmberg, L., Jia, Y., Tang, C., Du, J. and Jin, H. (2014) Sulfur dioxide inhibits vascular smooth muscle cell proliferation via suppressing the Erk/MAP kinase pathway mediated by cAMP/PKA signaling. *Cell Death Dis.*, **22**, 229.
3. Wang, R. (2012) Shared signaling pathways among gasotransmitters. *PNAS*, **109**, 8801-8802.
4. Yong, Q.C., Cheong, J.L., Hua, F., Deng, L.W., Khoo, Y.M., Lee, H.S., Perry, A., Wood, M., Whiteman, M. and Bian, J.S. (2011) Regulation of heart function by endogenous gaseous mediators-crosstalk between nitric oxide and hydrogen sulfide. *Antioxid. Redox Signal*, **14**, 2081-2091.
5. Moncada, S., Higgs, A. and Furchgott, R. (1997) International Union of Pharmacology Nomenclature in Nitric Oxide Research. *Pharmacol. Rev.*, **49**, 137-142.
6. Koh, S.D., Campbell, J.D., Carl, A. and Sanders, K.M. (1995) Nitric oxide activates multiple potassium channels in canine colonic smooth muscle. *J. Physiol.*, **489**, 735-743.
7. Guo, C., Liang, F., Shah Masood, W. and Yan, X. (2014) Hydrogen sulfide protected gastric epithelial cell from ischemia/reperfusion injury by Keap1 s-sulfhydration, MAPK dependent anti-apoptosis and NF-kappaB dependent anti-inflammation pathway. *Eur. J. Pharmacol.*, **725**, 70-78.
8. Koenitzer, J.R., Isbell, T.S., Patel, H.D., Benavides, G.A., Dickinson, D.A., Patel, R.P., Darley-Usmar, V.M., Lancaster, J.R., Doeller, J.E. and Kraus, D.W. (2007) Hydrogen sulfide mediates vasoactivity in an O_2-dependent manner. *Am. J. Physiol. Heart Circ. Physiol.*, **292**, 19.
9. Zhao, W., Zhang, J., Lu, Y. and Wang, R. (2001) The vasorelaxant effect of H_2S as a novel endogenous gaseous K(ATP) channel opener. *Embo. J.*, **20**, 6008-6016.
10. Balazy, M., Abu-Yousef, I.A., Harpp, D.N. and Park, J. (2003) Identification of carbonyl sulfide and sulfur dioxide in porcine coronary artery by gas chromatography/mass spectrometry, possible relevance to EDHF. *Biochem. Biophys. Res. Commun.*, **311**, 728-734.
11. Meng, Z., Geng, H., Bai, J. and Yan, G. (2003) Blood pressure of rats lowered by sulfur dioxide and its derivatives. *Inhal. Toxicol.*, **15**, 951-959.
12. Du, S.X., Jin, H.F., Bu, D.F., Zhao, X., Geng, B., Tang, C.S. and Du, J.B. (2008) Endogenously generated sulfur dioxide and its vasorelaxant effect in rats. *Acta. Pharmacol. Sin.*, **29**, 923-930.
13. Wang, X.B., Jin, H.F., Tang, C.S. and Du, J.B. (2010) Significance of endogenous sulphur-containing gases in the cardiovascular system. *Clin. Exp. Pharmacol. Physiol.*, **37**, 745-752.
14. Du, S.X., Zhang, C.Y., Jin, H.F., Du, J.B. and Tang, C.S. (2006) Vasorelaxant effect of sulfur dioxide derivatives on isolated aortic rings of rats and its mechanisms. *Beijing Da Xue Xue Bao*, **38**, 581-585.
15. Zhang, Q. and Meng, Z. (2009) The vasodilator mechanism of sulfur dioxide on isolated aortic rings of rats: Involvement of the K^+ and Ca^{2+} channels. *Eur. J. Pharmacol.*, **602**, 117-123.
16. Jin, H.F., Du, J.B., Li, X.H., Wang, Y.F., Liang, Y.F. and Tang, C.S. (2006) Interaction between hydrogen sulfide/cystathionine gamma-lyase and carbon monoxide/heme oxygenase pathways in aortic smooth muscle cells. *Acta. Pharmacol. Sin.*, **27**, 1561-1566.
17. Kolluru, G.K., Shen, X. and Kevil, C.G. (2013) A tale of two gases: NO and H_2S, foes or friends for life? *Redox Biol.*, **1**, 313-318.

18. Kida, M., Sugiyama, T., Yoshimoto, T. and Ogawa, Y. (2013) Hydrogen sulfide increases nitric oxide production with calcium-dependent activation of endothelial nitric oxide synthase in endothelial cells. *Eur. J. Pharm. Sci.*, **48**, 211-215.
19. Ali, M.Y., Ping, C.Y., Mok, Y.Y., Ling, L., Whiteman, M., Bhatia, M. and Moore, P.K. (2006) Regulation of vascular nitric oxide in vitro and in vivo; a new role for endogenous hydrogen sulphide? *Br. J. Pharmacol.*, **149**, 625-634.
20. Cortese-Krott, M.M., Kuhnle, G.G., Dyson, A., Fernandez, B.O., Grman, M., DuMond, J.F., Barrow, M.P., McLeod, G., Nakagawa, H., Ondrias, K. *et al.* (2015) Key bioactive reaction products of the NO/H_2S interaction are S/N-hybrid species, polysulfides, and nitroxyl. *PNAS*, **112**, E4651-4660.
21. Geng, B., Cui, Y., Zhao, J., Yu, F., Zhu, Y., Xu, G., Zhang, Z., Tang, C. and Du, J. (2007) Hydrogen sulfide downregulates the aortic L-arginine/nitric oxide pathway in rats. *Am. J. Physiol. Regul. Integr. Comp. Physiol.*, **293**, 18.
22. Furchgott, R.F. and Zawadzki, J.V. (1980) The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*, **288**, 373-376.
23. Meng, Z., Li, Y. and Li, J. (2007) Vasodilatation of sulfur dioxide derivatives and signal transduction. *Arch. Biochem. Biophys.*, **467**, 291-296.
24. Rassaf, T., Preik, M., Kleinbongard, P., Lauer, T., Heiss, C., Strauer, B.E., Feelisch, M. and Kelm, M. (2002) Evidence for in vivo transport of bioactive nitric oxide in human plasma. *J. Clin. Invest.*, **109**, 1241-1248.
25. Tang, G., Wu, L., Liang, W. and Wang, R. (2005) Direct stimulation of K(ATP) channels by exogenous and endogenous hydrogen sulfide in vascular smooth muscle cells. *Mol. Pharmacol.*, **68**, 1757-1764.
26. Si, J.Q., Zhao, H., Yang, Y., Jiang, Z.G. and Nuttall, A.L. (2002) Nitric oxide induces hyperpolarization by opening ATP-sensitive K(+) channels in guinea pig spiral modiolar artery. *Hear. Res.*, **171**, 167-176.
27. Coletta, C., Papapetropoulos, A., Erdelyi, K., Olah, G., Modis, K., Panopoulos, P., Asimakopoulou, A., Gero, D., Sharina, I., Martin, E. *et al.* (2012) Hydrogen sulfide and nitric oxide are mutually dependent in the regulation of angiogenesis and endothelium-dependent vasorelaxation. *PNAS*, **109**, 9161-9166.
28. Li, X., Bazer, F.W., Gao, H., Jobgen, W., Johnson, A., Li, P., McKnight, J.R., Satterfield, M.C., Spencer, T.E. and Wu, G. (2009) Amino acids and gaseous signaling. *Amino Acids*, **37**, 65–78.
29. Filipovic, M.R., Miljkovic, J., Nauser, T., Royzen, M., Klos, K., Shubina, T., Koppenol, W.H., Lippard, S.J. and Ivanovic-Burmazovic, I. (2012) Chemical characterization of the smallest S-nitrosothiol, HSNO; cellular cross-talk of H_2S and S-nitrosothiols. *J. Amer. Chem. Soc.*, **134**, 12016-12027.
30. Cortese-Krott, M.M., Fernandez, B.O., Santos, J.L., Mergia, E., Grman, M., Nagy, P., Kelm, M., Butler, A. and Feelisch, M. (2014) Nitrosopersulfide (SSNO(-)) accounts for sustained NO bioactivity of S-nitrosothiols following reaction with sulfide. *Redox Biol.*, **2**, 234-244.
31. Ellis, A., Li, C. and M., R. (2000) Differential actions of L-cysteine on responses to nitric oxide, nitroxyl anions and EDRF in the rat aorta. *Br. J. Pharmacol.* **129**, 315–322.
32. Wanstall, J.C., Jeffery, T.K., Gambino, A., Lovren, F. and Triggle, C.R. (2001) Vascular smooth muscle relaxation mediated by nitric oxide donors: a comparison with acetylcholine, nitric oxide andnitroxyl ion. *Br. J. Pharmacol.*, **134**, 463-472.
33. Zhao, W. and Wang, R. (2002) H(2)S-induced vasorelaxation and underlying cellular and molecular mechanisms. *Am. J. Physiol. Heart. Circ. Physiol.*, **283**, H474-480.
34. Wang, X.B., Jin, H.F., Tang, C.S. and Du, J.B. (2011) The biological effect of endogenous sulfur dioxide in the cardiovascular system. *Eur. J. Pharm.*, **670**, 1-6.
35. Li, J., Li, R. and Meng, Z. (2010) Sulfur dioxide upregulates the aortic nitric oxide pathway in rats. *Eur. J. Pharmacol.*, **645**, 143-150.
36. Wang, Y.K., Ren, A.J., Yang, X.Q., Wang, L.G., Rong, W.F., Tang, C.S., Yuan, W.J. and Lin, L. (2009) Sulfur dioxide relaxes rat aorta by endothelium-dependent and -independent mechanisms. *Physiol. Res.*, **58**, 521-527.

37. Luo, L., Liu, D., Tang, C., Du, J., Liu, A.D., Holmberg, L. and Jin, H. (2013) Sulfur dioxide upregulates the inhibited endogenous hydrogen sulfide pathway in rats with pulmonary hypertension induced by high pulmonary blood flow. *Biochem. Biophys. Res. Commun.*, **433**, 519-525.
38. Jin, H., Wang, Y., Wang, X., Sun, Y., Tang, C. and Du, J. (2013) Sulfur dioxide preconditioning increases antioxidative capacity in rat with myocardial ischemia reperfusion (I/R) injury. *Nitric Oxide Biol. Chem.*, **32**, 56–61.
39. Li, W., Tang, C., Jin, H. and Du, J. (2011) Regulatory effects of sulfur dioxide on the development of atherosclerotic lesions and vascular hydrogen sulfide in atherosclerotic rats. *Atherosclerosis*, **215**, 323-330.