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Involvement of RhoA/Rho-kinase in L-cysteine/H_2S pathway-induced inhibition of agonist-mediated corpus cavernosal smooth muscle contraction

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ABSTRACT

Rho-kinase activity is a key regulator in the maintenance of corporal vasoconstriction and penile detumescense. Also, importance of L-cysteine/H_2S pathway in erectile tissue has been shown; however it is currently unknown the role RhoA/Rho-kinase pathway in H_2S-induced inhibition in cavernosal tissue. We investigated the role of RhoA/Rho-kinase pathway in the inhibitory effect of L-cysteine and NaHS, as endogenous and exogenous H_2S, respectively, on phenylephrine-induced contractions of mouse cavernosal strips. Phenylephrine, α_1 receptor agonist, (10 nM-100 μM) induced a concentration-dependent contraction in CC. L-cysteine (endogenous H_2S substrate; 10 mM) and exogenous H_2S (NaHS; 1 mM) significantly inhibited the contractile response to phenylephrine ($P < 0.05$). Inhibition of CSE and CBS enzymes by PAG (10 mM) and AOAA (1 mM), respectively, significantly reversed the inhibitory effects of L-cysteine on phenylephrine-induced contraction ($P < 0.05$). Y-27632 (1 μM), a specific Rho-kinase inhibitor, significantly augmented the inhibitory effect of L-cysteine and NaHS on phenylephrine-induced contraction, and this inhibition was reversed by PAG and AOAA ($P < 0.05$). In addition, the formation of H_2S was increased by approximately 1.8 fold over basal values after incubation of tissue homogenates with L-cysteine. Y-27632 significantly increased both basal and L-cysteine-induced H_2S formation and this augmentation diminished by PAG and AOAA ($P < 0.05$). Furthermore, the pMYPT-1 expression was significantly decreased by L-cysteine, NaHS or Y-27632 alone. Also, pMYPT-1 expression was completely abolished by the L-cysteine/NaHS plus Y-27632 combination, and this inhibition was reversed by PAG and AOAA ($P < 0.05$). These results suggest that there is an interaction between Rho-kinase and H_2S pathways. Rho-kinase may be, at least in part, inhibits CSE/CBS enzymes in mouse corpus cavernosal tissue; however, it is not excluded the other kinases such as PKC and Zip-kinase.

1. Introduction

In recent studies, H_2S has been accepted as a gas neurotransmitter in mammalian like nitric oxide and carbon monoxide [1]. In common with other gas neurotransmitter, H_2S is a small molecule gas, endogenously synthesized through enzymes in mammalian tissues, passes through the cell membranes without binding to a specific receptor or carrier with short half-life after release [2,3]. H_2S is produced from L-cysteine endogenously by three different enzymes, cystathionine gamma lyase (CSE), cystathionine beta synthase (CBS) or 3-mercaptopurivate sulfurtransferase (3-MST) along with cysteine amino transferase in various tissues [4–6]. The synthesis of H_2S from L-cysteine is catalyzed particularly by pyridoxal-5′-phosphate-dependent enzymes CSE and CBS [7]. The relaxant effect of H_2S has been reported in several tissues such as vascular, corpus cavernosal and other smooth muscle [8–10]. Recent studies have reported the presence of L-cysteine/H_2S in erectile tissue and the contribution of this pathway in penile erectile function [10–15]. Penile erection is promoted by the tonus of corpus cavernosum smooth muscle, and the tone of cavernosal smooth muscle is regulated by the balance between contractile and relaxant factors. Smooth muscle contraction is primarily regulated by the phosphorylation levels of myosin light chains (MLC). The extent of MLC phosphorylation is determined by the ratio of MLC kinase (MLCK) to MLC phosphatase activities. Since, contractile signaling pathways that directly lead to inhibition of MLC can enhance contractile force independently from additional increases in $[Ca^{2+}]_I$, this mechanism is called "Ca^{2+} sensitization" [16,17]. Also, calcium sensitization by the RhoA/Rho-kinase pathway contributes to the contraction in smooth muscle. It is revealed that RhoA/Rho-kinase pathway plays an important role in the maintenance of the vasoconstrictive state of the

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cavernosal vasculature [18]. Also, the expression of RhoA and the involvement of RhoA/Rho-kinase signaling pathway in the noradrenergic contractile response have been shown in human [19] and mouse corpus cavernosum (CC) [20]. The few recent studies investigating the involvement of Rho-dependent pathway in H_2S-induced relaxation using rabbit, mouse and human colon [21], rat mesenteric artery [22] mouse gastric fundus [9] and rabbit gastric smooth muscle cells [23] have focused on MLC phosphatase (MLCP) activity. However, it is not extensively clarified the role RhoA/Rho-kinase pathway in H_2S-induced relaxations in cavernosal tissue. Most recently, we have shown that fasudil, a Rho-kinase inhibitor, decreased the relaxant response to exogenous H_2S in mouse corpus cavernosum [24]. To our knowledge, there is no information about the interaction of RhoA/Rho-kinase and L-cysteine/H_2S pathway on agonist-induced contraction in corpus cavernosal tissue. For this purpose, we investigated the role of RhoA/Rho-kinase pathway in the inhibitory effect of L-cysteine and NaHS, as endogenous and exogenous H_2S, respectively, on phenylephrine-induced contractions of mouse cavernosal strips.

2. Materials and methods

2.1. Experimental animals

Male Swiss albino mice weighing 20–25 g were obtained from Cukurova University Experimental Research and Application Center of Medical Science (DETAUM). Mice were located in Plexiglas cages and kept under environmentally conditions (12 h light/darkness cycles) and allowed free access to food and water. Protocols were approved by local Ethic Committee of the University of Cukurova. This investigation conforms to 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).

2.2. Tissue preparations

Male mice were killed by cervical dislocation. The penises were removed and placed in a Petri dish containing (composition in mM; NaCl 119, KCl 4.6, $CaCl_2$ 1.5, $MgCl_2$ 1.2, $NaHCO_3$ 15, $NaPO_4$ 1.2, glucose 11). The glans penis and urethra were excised and fibrous septum between two corpus cavernosum strips were cut and each corpus cavernosum (0.3 × 0.3 × 4 mm) was carefully dissected from the adherent tissues, keeping tunica albuginea intact. Cavernosal strips were mounted under 0.2 g tension in organ bath (5 ml) containing Krebs solution. The bath medium was maintained at 37 °C and gassed with 5% CO_2 and 95% O_2. The tissue strips were allowed to equilibrate for a period of 60 min during equilibration, the bath solution was replaced every 15 min. The responses were recorded with isotonic transducer (Ugo Basile, 7006) on a recorder (Ugo Basile Gemini, 7070).

2.3. Functional studies

Following the equilibration period of 60 min, isolated mouse cavernosal strips were pre-contracted with phenylephrine (α_1-receptor agonist; 10 μM) to evaluate contractility of tissue. The tissues were then washed out with Krebs solution and tissues were left re-equilibration for 30 min. After this period, cumulative phenylephrine (10 nM-100 μM) concentration-response curve was obtained. After the first series of cumulative contractile responses were obtained, the tissues were left equilibration for 30 min and the second series of cumulative response curve obtained with phenylephrine.

To investigate the effect of L-cysteine/H_2S pathway on contractile response induced by phenylephrine in mouse cavernosal strips, concentration-response curve to phenylephrine were studied in the presence of L-cysteine (endogenous H_2S substrate; 10 mM). In this set of experiments, after the contractile responses to phenylephrine were obtained cumulatively (10 nM-100 μM), the tissues were washed and incubated for 30 min. L-cysteine (10 mM) was added into the bath medium at the last 5 min of incubation period, and the second series of cumulative response curve obtained with phenylephrine. In addition, to confirm the H_2S-mediated inhibitory effect of L-cysteine in mouse cavernosal strips, contractile responses to phenylephrine were studied in the presence of H_2S donor NaHS (1 mM) in the same manner.

In the other sets of experiments, to investigate the contribution of endogenous H_2S production to the inhibitory effect of L-cysteine on the phenylephrine-induced contractile response, the effects of propargylglycine (PAG, 10 mM), a non-competitive cystathionine-gamma–lyase inhibitor and amino-oxyacetic acid (AOAA, 1 mM), a cystathionine-β-synthetase inhibitor, were studied. With this propose, after the cumulatively (10 nM-100 μM) contractile responses to phenylephrine were obtained the tissues were washed and incubated for 30 min with PAG (10 mM), AOAA (1 mM) alone. Then, L-cysteine (10 mM) was added into the bath medium and phenylephrine cumulative-response curves were obtained.

The contribution of RhoA/Rho-kinase pathway to the inhibitory effect of L-cysteine/H_2S pathway on the contractile responses induced by phenylephrine was investigated in the presence of specific Rho-kinase enzyme inhibitor (*R*)-(+)-*trans*-*N*-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide (Y-27632; 1 μM). In this set of experiments, after the contractile responses to phenylephrine were obtained cumulatively (10 nM-100 μM), tissues were washed and incubated with Y-27632 (1 μM) for 30 min. Then, the second series of cumulative response curves obtained with phenylephrine. In some experiments, L-cysteine or NaHS were added to bath medium at the last 5 min of incubation period with Y-27632 incubation and second series contractile responses to phenylephrine were obtained same manner. In addition, the contribution of RhoA/Rho-kinase to the inhibitory effect of L-cysteine was investigated in the presence of PAG and AOAA in cavernosal strips. With this purpose, after first series contractile responses to phenylephrine were obtained cumulatively (10 nM-100 μM), tissues were washed and incubated with PAG (10 mM) plus Y-27632 (1 μM) or AOAA (1 mM) plus Y-27632 (1 μM) for 30 min. L-cysteine (10 mM) was added to bath medium at the last 5 min incubation period and then, contractile responses to phenylephrine were obtained same manner.

2.4. Cavernosal endogenous H_2S synthesis

H_2S production in corpus cavernosal tissue samples was determined with a commercially available H_2S Colorimetric Assay kit (Elabscience Biotechnology Co., Ltd, Wuhan, China) through the reaction between H_2S and zinc acetate, N, N-Dimethyl-p-phenylenediamine and ammonium ferric sulfate. Protein concentration was determined by using bicinchoninic acid assay kit (Sigma Chemical Co, St. Luis, MO). Corpus cavernosal tissues were homogenized in extraction solution and centrifuged for 10 min at 4 °C at 10.000 × *g*, and the supernatant was collected. The supernatant solution was mixed an equal volume of Reagent 1 and 2. After centrifugation, the sediment was dissolved in Reagent 1, 3 and 4. The supernatant obtained after centrifugation was mixed Reagent 5. The absorbance of solutions was measured after 20 min at a wavelength of 665 nm H_2S concentrations in corpus cavernosal tissues were expressed as nmol/mg protein.

2.5. Measurement of phosphorylated MYPT1

We investigated the phosphorylation status at Thr696 of myosin phosphatase targeting subunit (MYPT), which is a substrate for the Rho-kinase, using a protocol described in detail below. Phosphorylated MYPT1 (pMYPT1) protein expression were determined by Western-blot analysis of cavernosal strips tissues. Tissues that had been frozen were homogenized in ice cold RIPA buffer system containing Halt protease inhibitor and total protein content determinant by Bradford method. Proteins from cavernosal tissues were obtained and boiled in the presence of Laemmli gel loading buffer containing SDS and β-

mercaptoethanol as reducing agent at pH 6.8 and kept at −20 °C until use. Proteins were separated in a 12% SDS-PAGE gel containing a 4% of stacking gel, under denaturing conditions at 100 V for 1 h and 45 min at room temperature. Proteins in the gel were then transferred to a PVDF membrane (Millipore) which was previously rehydrated in methanol and equilibrated with transfer buffer. Then a sandwich cassette was prepared according to the manufacturer's instructions (Bio-Rad) and proteins were electro blotted on to the PVDF membrane for 1 h and 15 min at 4 °C. After transfer, the membrane was briefly washed with Tris Buffered Saline (TBS) containing 1% Tween-20. Bovine serum albumin (BSA) at concentrations 5% was used in the wash buffer as blocking agent. Membranes were blocked for 1 h at room temperature with gentle and constant agitation and incubated with primary antibodies pMYPT1 (dilution 1:500, CST-5163S) and beta-actin (dilution 1:1000, CST-4967S) over night. The membranes were washed three times for 10 min each with TBS-T and incubated with a horseradish peroxidase-conjugated second antibody (dilution 1:5000, Santa Cruz Biotechnology) at room temperature for 1 h with constant agitation. After briefly drying, the membrane was incubated with 3 ml of HRP ECL substrate mixture (1.5 ml hydrogen peroxide and 1.5 ml enhancer) (Bio-rad) and incubated for 1 min at room temperature. The membranes were wrapped with stretch film and placed in Chemi Doc MP (Bio-rad) for 1–10 min. The bands were quantified using the Image J program. The protein expression was normalized to the β-actin content.

2.6. Drugs and solutions

The following drugs were used; amino-oxyacetic acid (*o*-carboxymethyl) hydroxylamine, dl-propargylglycine, L-cysteine, phenylephrine hydrochloride and sodium hydrosulphide hydrate (Sigma Chemical Co., St Louis, MO, U.S.A.), and Y-27632 dihydrochloride [(R)-(þ)-trans-N-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide] (Tocris Bioscience, Minneapolis, MN, USA). All drugs were dissolved in distilled water. NaHS was prepared as 1 Mm solution in H_2O and kept on ice.

2.7. Data analysis

The contractile responses to cumulative applied phenylephrine were expressed as a percentage of the maximal contractile response to first series of cumulative phenylephrine. The sensitivity to the agonist was expressed as pD2 (negative logarithm of the agonist concentration required for half-maximum response). All data are presented as mean ± S.E.M. Differences in results between tissues were tested by analysis of variance one-way ANOVA and unpaired *t*-test corrected for multiple comparisons (Bonferroni corrections). *P* values less than 0.05 were considered to be significant.

3. Results

3.1. Effects of CBS, CSE and Rho-kinase inhibition on H_2S generation in mouse CC

Mouse CC generated detectable amounts of H_2S. The formation of H_2S was increased by approximately 1.8 fold over basal values after incubation of tissue homogenates with L-cysteine (the CBS and CSE substrate) (Fig. 1). PAG (10 mM) and AOAA (1 mM), CSE and CBS inhibitor, respectively, significantly diminished the increase in H_2S production stimulated with L-cysteine. Thus, mouse CC is capable of synthesizing H_2S from L-cysteine. Rho-kinase inhibition with Y-27632 increased both basal and L-cysteine-induced H_2S formation in CC. PAG (10 mM) and AOAA (1 mM) significantly diminished the increase in H_2S production in the presence of Y-27632 (Fig. 1). To confirm the H_2S production, H_2S level was determined in the presence of NaHS. Also, H_2S generation increased in the presence of exogenous H_2S.

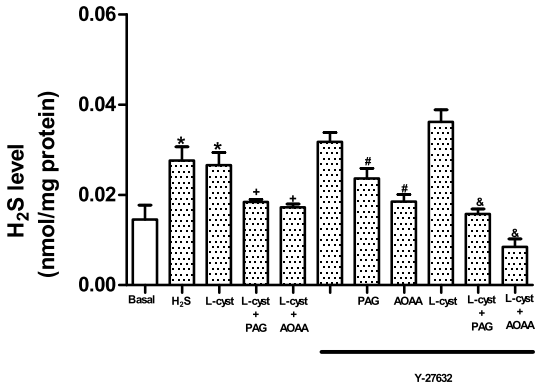

Fig. 1. The role of CBS, CSE and Rho-kinase inhibition on endogenous H_2S formation. The bar graph showing to endogenous H_2S production in the absence or presence of exogenous H_2S (NaHS; 1 mM), L-cysteine (L-cyst; 10 mM), L-cysteine (10 mM) plus PAG (10 mM), L-cysteine (10 mM) plus AOAA (1 mM), Y-27632 (1 μM), L-cysteine plus Y-27632 (1 μM), L-cysteine (10 mM) + PAG (10 mM) plus Y-27632 (1 μM) and L-cysteine (10 mM) + AOAA (1 mM) plus Y-27632 (1 μM) in mouse CC. All values are mean ± S.E.M. (n = 4). $^{*}P < 0.05$ significantly different from control; $^{+}P < 0.05$ significantly different from L-cysteine; $^{\#}P < 0.05$ significantly different from Y-276322; $^{\&}P < 0.05$ significantly different from L-cysteine plus Y-27632; one-way ANOVA and unpaired *t*-test followed by Bonferroni's comparison test.

3.2. Effect of L-cysteine/H_2S pathway on phenylephrine-induced contractions in mouse CC

Phenylephrine, a selective α_1 receptor agonist, applied at ascending concentrations (10 nM-100 μM) produced sustained contraction in concentration-dependent manner with a maximum response obtained at 100 μM in isolated mouse corpus cavernosum strips. Pre-treatment with L-cysteine (10 mM) significantly reduced contractile responses induced by phenylephrine ($P < 0.05$; Fig. 2A). Maximum contractile responses (E_{max}) to phenylephrine but not pD_2 values were significantly reduced by L-cysteine ($P < 0.05$; Table 1). In addition, to confirm the H_2S-mediated inhibitory effect of L-cysteine in mouse cavernosal strips, contractile responses induced by phenylephrine were studied in the presence of NaHS in response to exogenous H_2S. NaHS (1 mM) significantly reduced maximum contractile response (E_{max}) to phenylephrine ($P < 0.05$) and increased pD_2 values ($P < 0.05$; Table 1).

To clarify the inhibitory effect of endogenous H_2S on contractile responses to phenylephrine in mouse cavernosal strips, the inhibitory effect of L-cysteine on contractile responses induced by phenylephrine was studied in the presence of PAG (10 mM) and AOAA (1 mM), CSE and CBS inhibitor, respectively. PAG and AOAA significantly reversed the inhibitory effect of L-cysteine ($P < 0.05$; Fig. 2B and C). Maximum contractile responses (Emax) to phenylephrine but not pD_2 values were significantly increased by PAG and AOAA in the presence of L-cysteine (Table 1). In addition, cumulative concentration-response curves to phenylephrine were investigated in the presence of PAG (10 mM) and AOAA (1 mM) alone. The contractile responses to lower concentration of phenylephrine were significantly augmented in the presence of PAG and AOAA compared to control group ($P < 0.05$; Fig. 2B and C). Maximum contractile responses (E_{max}) to phenylephrine were not significantly altered by PAG and AOAA compared to control group (Table 1). pD_2 values were increased by AOAA ($P < 0.05$) but not PAG compared to control group (Table 1).

3.3. The contribution of Rho-kinase to the inhibitory effect of L-cysteine/H_2S pathway on phenylephrine-induced contractions in mouse CC

Pre-treatment with Y-27632 (1 μM), a Rho-kinase inhibitor, significantly reduced contractile responses induced by phenylephrine ($P < 0.05$; Fig. 3A). Maximum contractile responses (E_{max}) to

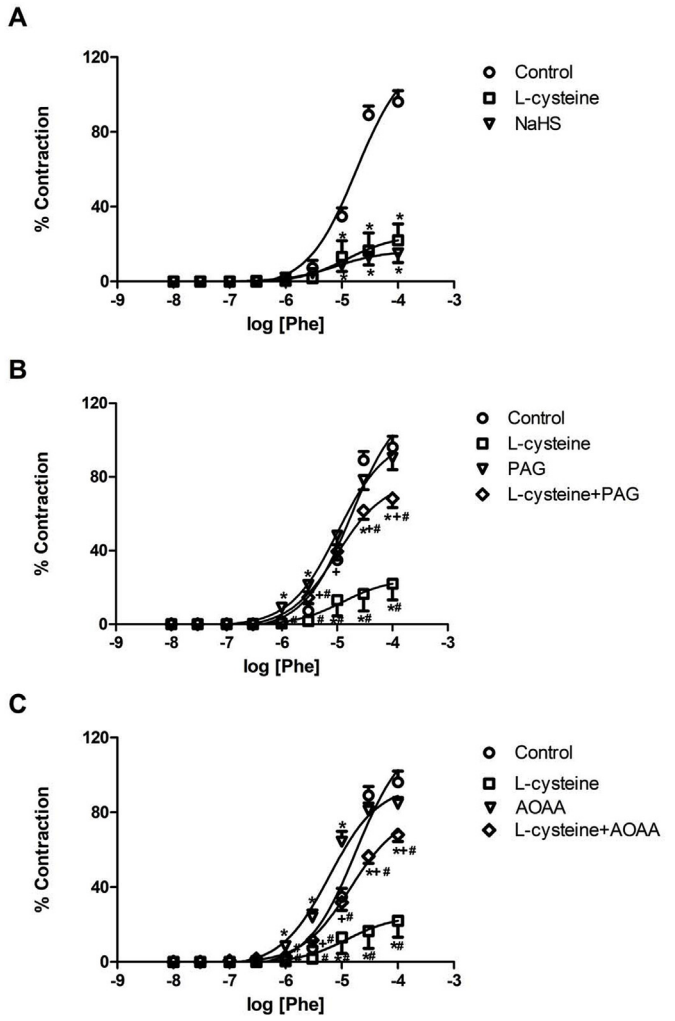

Fig. 2. The effect of L-cysteine/H_2S pathway on phenylephrine-induced contractions. The effects of L-cysteine (10 mM) and exogenous H_2S (NaHS; 1 mM) on concentration-response curve induced by cumulative phenylephrine (10 nM-100 μM) **(A),** and the effect of L-cysteine on phenylephrine-induced (10 nM-100 μM) contractile responses in the absence or presence of PAG (10 mM) **(B)** and AOAA (1 mM) **(C)** in mouse CC. All values are mean ± S.E.M. (n = 6). $^{*}P < 0.05$ significantly different from control; $^{+}P < 0.05$ significantly different from L-cysteine; one-way ANOVA and unpaired *t*-test followed by Bonferroni's comparison test.

Table 1
The effect of L-cysteine/H_2S pathway on the pD_2 and E_{max} values obtained from contractile responses to phenylephrine in mouse cavernosal strips.

	pD_2	E_{max} (%)
Control	4.74 ± 0.15	96.14 ± 5.86
L-cysteine	4.90 ± 0.14	22.05 ± 8.80*
NaHS	5.10 ± 0.03*	15.08 ± 4.89*
PAG	4.99 ± 0.04	90.35 ± 6.42
AOAA	5.22 ± 0.09*	84.69 ± 3.25
L-cysteine + PAG	5.01 ± 0.07	68.33 ± 5.02+
L-cysteine + AOAA	4.85 ± 0.05	68.10 ± 3.66+

Data represent mean ± SE for Emax and pD_2.
$^{*}P < 0.05$ significantly different from control, $^{+}P < 0.05$ significantly different from L-cysteine by analysis of variance one-way ANOVA and unpaired *t*-test corrected from multiple comparison's (Bonferroni corrections).

phenylephrine but not pD_2 values were significantly reduced in the presence of Y-27632 (Table 2). To clarify the contribution of Rho-kinase pathway to the inhibitory effect of endogenous and exogenous H_2S on phenylephrine-induced contractions in mouse cavernosal strips, we investigated the effect of L-cysteine (10 mM) and NaHS (1 mM) on concentration-response curve to phenylephrine (10 nM-100 μM) in the presence of Y-27632 (1 μM). Y-27632 almost abolished the contractile responses to phenylephrine in the presence of L-cysteine and NaHS ($P < 0.05$; Fig. 3A and B). Maximum contractile responses (E_{max}) to phenylephrine but not pD_2 values were significantly decreased in the presence of Y-27632 and L-cysteine or NaHS (Table 2). In addition, the reversal effect of PAG and AOAA on these inhibitory responses of L-cysteine and Y-27632 combination on contractile responses to phenylephrine was also investigated. The both of PAG and AOAA significantly reversed the inhibitory effect of L-cysteine plus Y-27632 combination on the contractile responses to phenylephrine ($P < 0.05$; Fig. 3C and D). Maximum contractile responses (E_{max}) to phenylephrine were significantly increased compared to L-cysteine plus Y-27632, and pD_2 values were significantly decreased (Table 2).

3.4. The effect of L-cysteine/H_2S pathway on phosphorylation of MYPT-1 at Thr696 in CC

To investigate the role of L-cysteine/H_2S pathway on calcium desensitization in cavernosal tissues, we next measured levels of MYPT-1 phosphorylation at the inhibitory site (Thr696) in the presence of L-cysteine and NaHS. Endogenous (L-cysteine; 10 mM) and exogenous H_2S (NaHS; 1 mM) caused significant decrease of phenylephrine-induced phosphorylation of MYPT-1 at Thr696 in cavernosal tissues (Fig. 4A). Similarly, Rho kinase inhibitor Y-27632 (1 μM) attenuated phosphorylated MYPT-1 level (Fig. 4B). The effects of L-cysteine/NaHS and Y-27632 (1 μM) combination on pMYPT-1 level were also investigated in cavernosal tissues. The pMYPT-1 expression was completely abolished by the incubation of L-cysteine and Y-27632 or NaHS and Y-27632 combination (Fig. 4B) at concentrations equivalent to those inhibiting contractions, and the inhibition of pMYPT-1 levels were reversed in the presence of PAG (10 mM) and AOAA (1 mM) in cavernosal strips exposed to L-cysteine (10 mM) + Y-27632 (1 μM) (Fig. 4B).

4. Discussion

In the present study, we investigated the role of RhoA/Rho-kinase in the inhibitory effect of L-cysteine/H_2S pathway on the agonist-induced contraction of corpus cavernosal smooth muscle. The main findings of this study are summarized as follows: 1) L-cysteine and the H_2S donor NaHS inhibited phenylephrine-induced contraction; 2) inhibition of CSE and CBS by PAG and AOAA, respectively, reversed the inhibitory effects of L-cysteine on contraction of phenylephrine; 3) inhibition of Rho-kinase by Y-27632 almost abolished phenylephrine-induced contraction in the presence of L-cysteine, and this inhibitory effect was reversed by PAG and AOAA; 4) Y-27632 increased both basal and L-cysteine-induced H_2S formation, and this augmentation diminished by PAG and AOAA; 5) the pMYPT-1 expression was completely abolished by the L-cysteine/NaHS plus Y-27632 combination, and the inhibition was reversed by PAG and AOAA, suggesting that there is an interaction between Rho-kinase and H_2S pathways in mouse corpus cavernosal tissue.

The role of H_2S in erectile function firstly was demonstrated by Srilatha et al. [25], suggesting a possible role for endogenous H_2S in erectile function through facilitation of nerve-mediated penile tumescence. Also, the production of endogenous H_2S and its relaxant effect has been shown in isolated rabbit [11] human [12], rat [12,13] and mice corpus cavernosum tissues [10,26]. Several mechanisms have been proposed to contribute to the relaxant effect of L-cysteine/H_2S on corpus cavernosal smooth muscle tone. In animal and human studies, the contribution of the endothelium [10], nitric oxide/soluble guanosine monophosphate (NO/cGMP) pathway [27–29] adenylyl cyclase/phosphodiesterase (PDE) [10,11,13], potassium (K+) channels

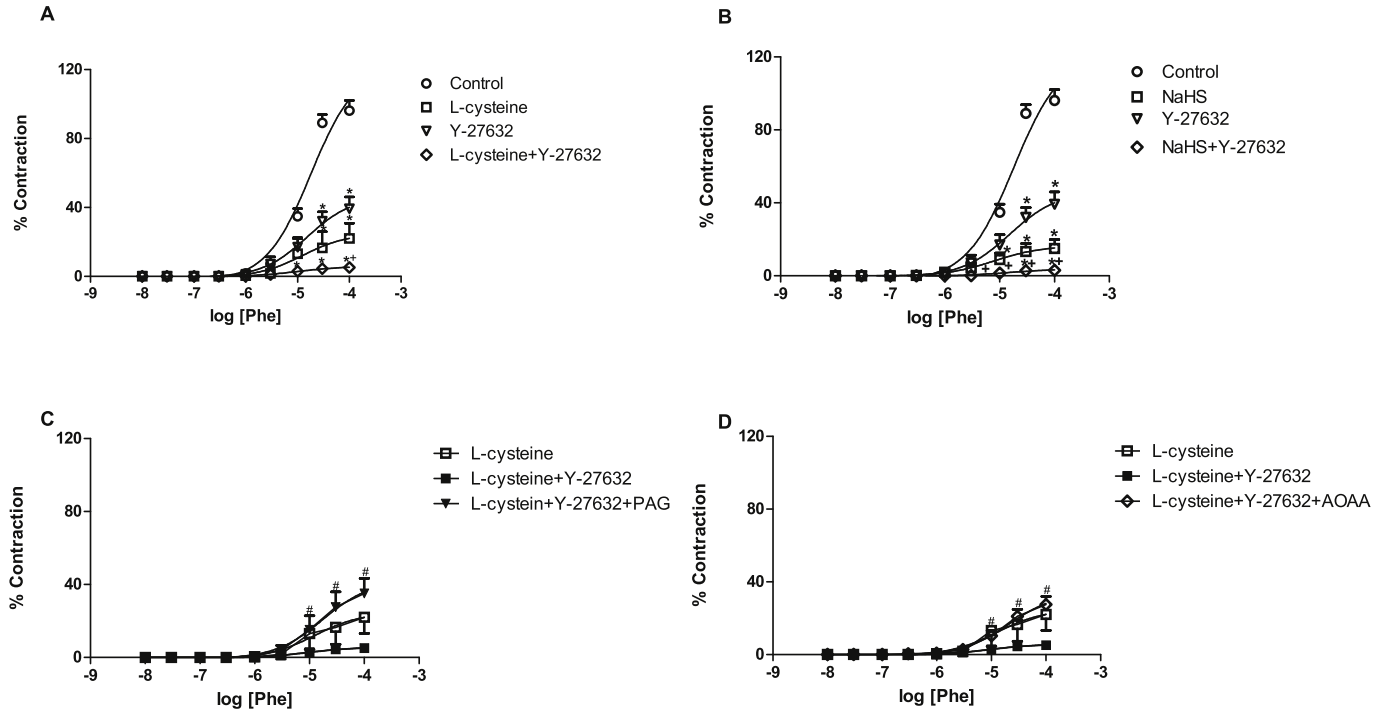

Fig. 3. The contribution of Rho-kinase to the inhibitory effect of L-cysteine/H2S pathway on phenylephrine-induced contractions. The effects of L-cysteine (10 mM) **(A)** and exogenous H_2S (NaHS; 1 mM) **(B)** on phenylephrine-induced (10 nM-100 μM) contractile responses in the absence or presence of Y-27632 (1 μM), and the effect of L-cysteine plus Y-27632 on phenylephrine-induced (10 nM-100 μM) contractile responses in the absence or presence of PAG (10 mM) **(C)** and AOAA (1 mM) **(D)** in mouse CC. $^{*}P < 0.05$ significantly different from control; $^{+}P < 0.05$ significantly different from L-cysteine or exogenous H_2S; $^{\#}P < 0.05$ significantly different from L-cysteine plus Y-276322; one-way ANOVA and unpaired *t*-test followed by Bonferroni's comparison test.

Table 2
The effect of L-cysteine/H_2S pathway on the pD_2 and E_{max} values of phenylephrine obtained from mouse cavernosal strips in the absence and presence of Y-27632.

	pD_2	E_{max} (%)
Control	4.74 ± 0.15	96.14 ± 5.86
L-cysteine	4.90 ± 0.14	22.05 ± 8.80*
NaHS	5.10 ± 0.03*	15.08 ± 4.89*
Y-27632	4.82 ± 0.05	39.27 ± 6.78*
NaHS + Y-27632	4.84 ± 0.14	3.07 ± 1.32$^{\infty}$
L-cysteine + Y-27632	5.00 ± 0.08	5.16 ± 1.19^{+}
L-cysteine + Y-27632 + PAG	4.76 ± 0.06$^{\#}$	35.12 ± 8.26$^{\#}$
L-cysteine + Y-27632 + AOAA	4.67 ± 0.06$^{\#}$	27.56 ± 4.39$^{\#}$

Data represent mean ± SE for E_{max} and pD_2.
$^{*}P < 0.05$ significantly different from control, $^{\infty}P < 0.05$ significantly different from NaHS, $^{+}P < 0.05$ significantly different from L-cysteine, $^{\#}P < 0.05$ significantly different from L-cysteine + Y-27632 by analysis of variance one-way ANOVA and unpaired *t*-test corrected from multiple comparison's (Bonferroni corrections).

[12,13,15], L-type calcium channels [10], Na + -K + -ATPase [10], muscarinic acetylcholine receptors [30], cyclooxygenase (COX)/arachidonic acid cascade [24] to the relaxant mechanism of L-cysteine/H_2S pathway in corpus cavernosum has been reported. However, H_2S-induced relaxations in some tissues were not completely abolished or failed to inhibit by inhibitors of channels and/or pathways mentioned above, and additional pathways such as Rho-kinase, PKC or Zip kinase might contribute to relaxation in response to H_2S in cavernosal smooth muscle. Since, the importance of Rho-kinase pathway in the regulation of corpus cavernosal tone and penile detumescence has been shown [20]. Also, Rho-kinase is expressed in human CC [19], and RhoA/Rho-kinase signaling pathway is involved in the noradrenergic contractile response in human cavernosal smooth muscle [31]. Recently, we reported that RhoA, ROCK-1 and ROCK-2 are expressed in mouse corpus cavernosum and RhoA/Rho-kinase pathway is involved to contractions induced by α_1 receptor agonist in this tissue [20]. Although the importance of Rho-kinase activity in the maintenance of corporal vasoconstriction and penile detumescense has been reported [32–34], so far there have not been attempts to directly correlate RhoA/Rho-kinase and H_2S in corpus cavernosum tissue. Our observation is important as it is the first reported investigating the involvement of RhoA/Rho-kinase in L-cysteine/H_2S pathway-induced inhibition of agonist-mediated corpus cavernosal smooth muscle contraction.

In the present study, we found that agonist-induced contraction was inhibited by L-cysteine, and this inhibition was reversed by PAG and AOAA, inhibitors of CSE and CBS, respectively. Also, we most recently showed the existence and localization of CBS and CSE in mouse CC tissues [30]. In the present study, the inhibitory effect of endogenous H_2S was confirmed with H_2S donor NaHS which inhibited the contractile response to agonist similar to L-cysteine. Our present data suggest that both endogenous H_2S produced from L-cysteine through CSE/CBS enzymes and exogenous H_2S inhibits agonist-induced contractions in mouse corpus cavernosal muscle strips. Consistent with the present results, recent studies in rabbit gastric [23] and colonic [21] smooth muscle strips and cells of rabbit, mouse and human have demonstrated that agonist-induced contractions was inhibited by L-cysteine and NaHS in a concentration-dependent fashion, and the inhibitory effect of L-cysteine on muscle contraction was blocked by inhibition of CSE with PAG. In order to evaluate the involvement of RhoA/Rho-kinase to the inhibitory effect of L-cysteine/H_2S pathway on the agonist-induced contraction of corpus cavernosal smooth muscle, we used Y-27632, a specific inhibitor of Rho-kinase. Y-27632 almost abolished the contractile responses to phenylephrine in the presence of L-cysteine and NaHS, and PAG or AOAA reversed the inhibitory effect. Also, these results consistent with our previous findings that fasudil, a Rho-kinase inhibitor, decreased the relaxant response to exogenous H_2S in mouse corpus cavernosum [24]. In the our present study, another evidence supporting the interplay between Rho-kinase and H_2S

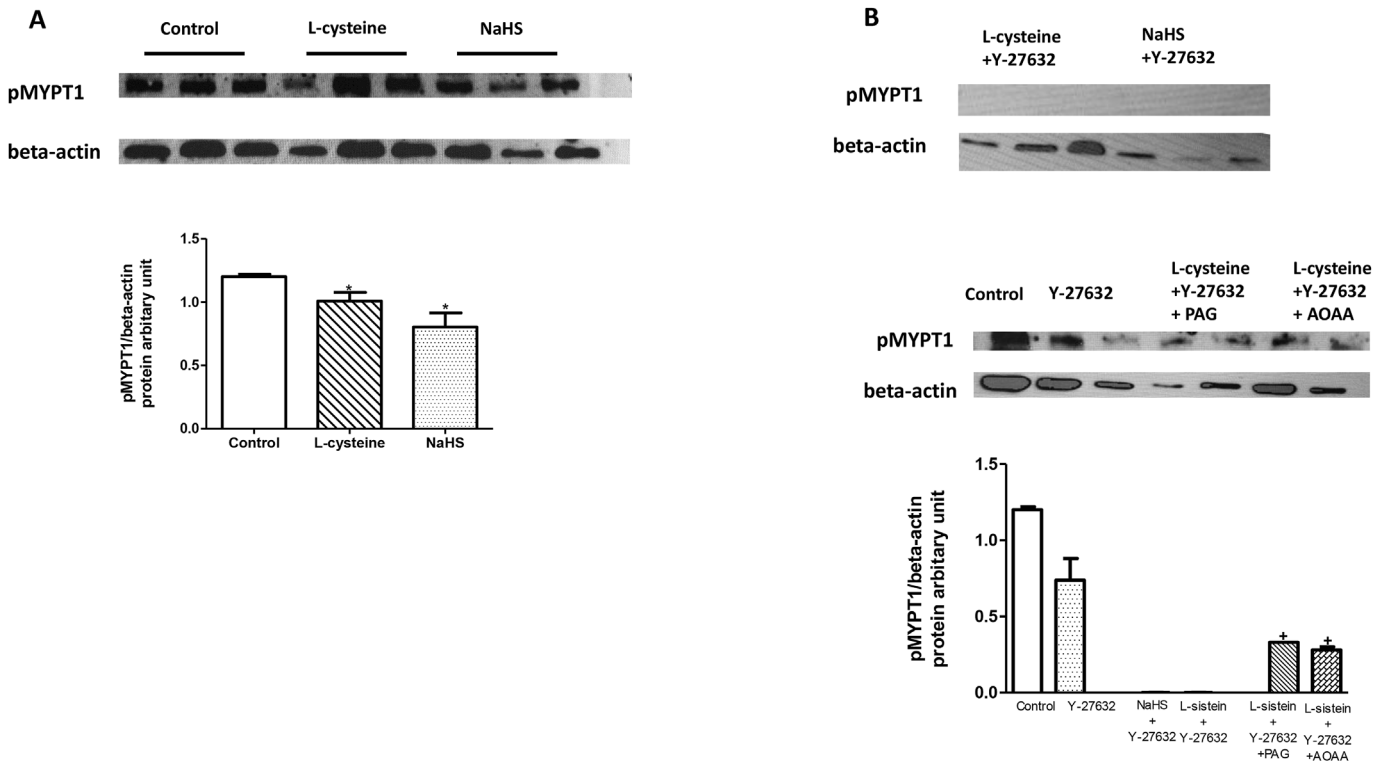

Fig. 4. The effect of L-cysteine/H_2S pathway on phosphorylated MYPT-1 expressions. Representative image of western blot analysis showing the effects of L-cysteine (10 mM), exogenous H_2S (NaHS; 1 mM) and Y-27632 (1 μM) **(A)**, on expression of phosphorylated MYPT-1 (pMYPT-1) at Thr696 and β-actin, and the effect of L-cysteine/NaHS combination on pMYPT-1 and β-actin expression in the absence or presence of PAG (10 mM) and AOAA (1 mM) **(B)** in mouse CC. The bar graphs showing the relative protein expression levels of pMYPT-1 versus β-actin in mouse CC. Values were normalized by the intensity of each band relative to the intensity of the loading control: All values are mean ± SEM (n = 3–4). $^{*}P < 0.05$ significantly different from control; $^{+}P < 0.05$ significantly different from L-cysteine + Y-27632 (unpaired *t*-test).

pathway that Rho-kinase inhibition with Y-27632 increased both basal and L-cysteine-induced H_2S formation, and PAG or AOAA significantly diminished the increase in H_2S production in the presence of Y-27632, suggesting that Rho-kinase may be inhibit the activity of CSE and CBS, H_2S-producing enzymes. It is well known that an important pathway controlling smooth muscle contraction is the RhoA/Rho-kinase pathway, acting by inhibition of MLCP through phosphorylation of the MYPT1 subunit and thus promoting calcium sensitization and contraction [35]. Previous studies have shown that activation of Rho kinase leads to inhibition of MLCP activity via phosphorylation of MYPT1 at Thr696 [36,37]. L-cysteine and NaHS decreased the basal level of MYPT1 phosphorylation maintained in CC tissues stimulated with phenylephrine. Also, MYPT1 phosphorylation level was completely abolished by the Rho kinase inhibitor Y-27632 in cavernosal tissue exposed to L-cysteine, and the inhibition of pMYPT-1 levels with L-cysteine were reversed by PAG and AOAA, suggesting endogenous generation of H_2S via activation of CSE/CBS by L-cysteine caused inhibition of contraction, at least in part, via inhibition of Rho-kinase mediated phosphorylation of MYPT1. However, the expression of pMYPT1 may be affected by other compensatory mechanisms such as PKC and Zip-kinase. Also, Nalli and co-workers recently reported that L-cysteine or NaHS caused inhibition of agonist-induced PKC activity and CPI-17 at Thr38 in rabbit and mouse gastric smooth muscle cells, suggesting the inhibitory effect of H_2S on agonist-induced contraction is mediated via inhibition of PKC-mediated phosphorylation of CPI-17 [21]. We cannot exclude the possibility that PKC and/or the other kinases may be involved to inhibitory effect of L-cysteine/H_2S pathway on agonist-induced smooth muscle contraction. Further studies are needed to explain the role of kinases on H_2S-induced inhibition. Our results is also correspondence with recent study in rabbit gastric smooth muscle cells [23] that L-cysteine and NaHS caused inhibition of carbachol-induced phosphorylation of MYPT1 at Thr^{696}, suggesting endogenous and exogenous H_2S caused inhibition of sustained contraction via inhibition of Rho-kinase-mediated phosphorylation of MYPT1. Also, Nalli and coworkers showed that L-csyteine and NaHS inhibited agonist-induced Rho kinase activity in a concentration manner in rabbit gastric fundus and colon [21,23]. Recently, it has been reported that H_2S inhibits muscle contraction via S-sulfhydration of RoA and inhibition of RhoA and Rho-kinase activity in colonic smooth muscle cells [21]. The underlying molecular targets of H_2S in the pathways cause to inhibition of RhoA/Rho-kinase signaling activity are not clear. It is needed to further studies to reveal precise molecular mechanism of interplay between RhoA/Rho-kinase and H_2S pathways in corpus cavernosal tissue. On the other hand, it has been observed that there was no difference in pMYPT1/MYPT1 in arteries exposed to NaHS in rat mesenteric small arteries [22]. Also, Dhaese and Lefebvre [9] reported that NaHS acts through activation of MLCP, without affecting the RhoA/Rho-kinase pathway in mouse gastric fundus. These differences may be explained by tissue differences, different experimental conditions or type of contractile drug.

In conclusion, our studies suggest that there is an interaction between Rho-kinase and H_2S pathways in regulation of phenylephrine-induced contraction, and Rho-kinase may be, at least in part, inhibits CSE/CBS enzymes in corpus cavernosal smooth muscle tissue. Also, we cannot exclude the possibility that pMYPYT1 may be affected by other compensatory mechanisms. It is needed further studies.

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