Pharmacological Reports 69 (2017) 610–615

Contents lists available at ScienceDirect

Pharmacological Reports

journal homepage: www.elsevier.com/locate/pharep

Original article

The role of arachidonic acid/cyclooxygenase cascade, phosphodiesterase IV and Rho-kinase in H_2S-induced relaxation in the mouse corpus cavernosum

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ARTICLE INFO

Article history:
Received 20 September 2016
Received in revised form 10 January 2017
Accepted 22 February 2017
Available online 24 February 2017

Keywords:
Arachidonic acid pathway
Corpus cavernosum
Hydrogen sulfide
Phosphodiesterase IV
Rho-kinase

ABSTRACT

Background: Penile corpus cavernosum is an extremely vascularized tissue and cavernosal smooth muscle tone is regulated by the balance between contractile and relaxant factor. We investigated the possible role of arachidonic acid/cyclooxygenase cascade, phosphodiesterase IV (PDEIV) and Rho-kinase in exogenous hydrogen sulfide (H_2S)-induced relaxation in mouse corpus cavernosum.
Methods: The relaxant response to H_2S (NaHS as exogenous H_2S; 1–1000 μM) were obtained in isolated mouse corpus cavernosum tissues which pre-contracted by phenylephrine (5 μM). The effects of 4-(4-octadecylphenyl)-4-oxobutenoic acid (OBAA; 10 μM), a selective phospholipase A_2 (PLA_2) inhibitor, indomethacin (1 μM), a non-selective cyclooxygenase (COX) inhibitor, baicalein (10 μM), a lipoxygenase (LOX) inhibitor, and proadifen (10 μM), cytochrome P450 inhibitor, on the relaxant responses to H_2S were investigated. Furthermore, the effects of theophylline (500 μM) and rolipram (1 μM), a non-selective and selective PDEIV inhibitor, and fasudil (3 μM), a specific Rho-kinase inhibitor, were studied on H_2S-induced relaxation.
Results: H_2S-induced relaxations were significantly reduced by OBAA, indomethacin and proadifen but not baicalein. Furthermore, theophylline, rolipram and fasudil reduced H_2S-induced relaxations.
Conclusion: These results suggest that PLA_2, COX, cytochrome P450, PDEIV and Rho-kinase pathway may involve in H_2S-induced relaxation in mouse corpus cavernosum tissues.

Introduction

Hydrogen sulfide (H_2S) not only exists as an environmental pollutant, but is also synthesized endogenously from L-cysteine in a reaction catalyzed by cystathionine beta synthase (CBS), cystathionine gama lyase (CSE) or 3-mercaptopurivate sulphurtranspherase (3-MST) enzymes in mammalian tissues [1–4]. The gas H_2S has biologic effects including vasoregulation as other gases carbon monoxide and nitric oxide (NO). Much interest in the role of H_2S in the body has focused on its role in the vascularity, particularly due to the similarities with NO [5]. The relaxant effect of H_2S has been reported in several tissues such as vascular, corpus cavernosal and other smooth muscle [6–11].

Penile erection is a complex neurovascular process of the corpus cavernosum tissue [12], and normal erectile function depends on relaxation of corporal smooth muscle [13]. Penile corpus cavernosum is an extremely vascularized tissue and the tone of cavernosal smooth muscle is regulated by contractile and relaxant factor [14]. The involvement of a functionally L-cysteine/H_2S pathway has been demonstrated in human penile erection [15]. Recently, it has been demostrated that H_2S endogenously synthesizes from L-cysteine by CSE endothelium-dependent in mouse cavernosum tissue, and exogenous H_2S may cause endothelium-independent relaxations via activation of L-type voltage-gated Ca^{2+} channels and K channels (K_{ATP} channel, K_V channels, K_{IR} channels) [11]. However, additional pathways may contribute to relaxation in response to H_2S, as inhibitors of channels and/or pathways mentioned above do not completely abolish or fail to inhibit H_2S-induced relaxation in corpus cavernosum and other some tissue. These additional relaxant pathways might include arachidonic acid/cyclooxygenase cascade, phosphodiesterase IV (PDEIV) or RhoA/Rho-kinase-regulated pathway in mouse corpus cavernosum. Since, di Villa Bianca et al. reported that hydrogen sulfide induces vascular effect *via*

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http://dx.doi.org/10.1016/j.pharep.2017.02.018
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arachidonic acid cascade in rat mesenteric arterial bed [15]. Recent study indicates that one of the biological actions of H_2S involves inhibition of PDEV in various smooth muscles [11,16]. Also, it has been suggested that endogenous and exogenous H_2S inhibits the Rho-kinase pathway and contraction in rabbit gastric smooth muscle [17]. In this study, to investigate contribution arachidonic acid/cyclooxygenase cascade to H_2S intracellular signaling, we studied the effects of 4-(4-octadecylphenyl)-4-oxobutenoic acid (OBAA) as a selective phospholipase A_2 (PLA_2) inhibitor, indomethacin as non-selective cyclooxygenase (COX) inhibitor, baicalein as lipoxygenase (LOX) inhibitor and proadifen as cytochrome P450 inhibitor, on relaxations to H_2S. Furthermore, we investigated the effects of theophylline or rolipram as a non-selective and selective PDEIV inhibitors, and fasudil, specific Rho-kinase inhibitor, on exogenous H_2S-induced relaxations.

Materials and methods

Animals

Male Swiss albino mice weighing 25–30 g were used throughout the study. Protocols were approved by local Ethic Committee. Mice were kept under environmentally conditions (12 h light/darkness cycles) and allowed free access to food and water. This investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (Bethesda, MA, USA; NIH Publication No. 85-23 revised 1996).

Tissue preparation

Mice were killed by cervical dislocation. The penises were removed and placed in a Petri dish containing (composition in mmol/L; 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 ($0.3 \times 0.3 \times 4$ mm) was carefully dissected from the adherent tissues, keeping the tunica albuginea intact. Cavernosal strips were mounted under 0.2 g tension in an organ bath (10 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, 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).

Experimental protocols

Following the equilibration period of 60 min, cavernosal strips were pre-contracted by phenylephrine (5 μM). After a steady state of contraction was obtained, the relaxant response to cumulatively H_2S (1–1000 μM) was studied. To carry out the relaxant mechanism of H_2S, we used NaHS as its exogenous source. After the first series of relaxant responses were obtained, the tissues were left equilibration for 30 min and the second series of responses were recorded in the same manner. At the end of the experimental protocol, papaverine (100 μM) was added to the organ bath to achieve maximal relaxation. Papaverine was applied after tissues washed out with Krebs and incubation with only Krebs solution for 30 min solution to remove inhibitor agents from bath medium. In the other sets of experiments, the possible roles of arachidonic acid/cyclooxygenase cascade, phosphodiesterase and Rho-kinase enzymes in the relaxant response to H_2S were investigated in the presence of enzyme inhibitors. With this propose, after the first series of relaxant responses to H_2S were obtained, the tissue was incubated in a medium containing OBAA (10 μM), indomethacin (1 μM), baicalein (10 μM), proadifen (10 μM), theophylline (500 μM), rolipram (1 μM) and fasudil (3 μM), PLA2, COX, LOX, cytochrome P450, PDEIV and Rho-kinase enzyme inhibitors, respectively. Fasudil, a RhoA/Rho-kinase inhibitor, had a small inhibiting effect on the PE-induced re-contraction. For this reason, the higher concentrations of PE than 5 μM was added to induce a similar absolute tone level to the first contraction.

Statistical analysis

The relaxant responses to H_2S were expressed as a percentage of the papaverine-induced (100 μM) relaxations at the end of experiment. All data are presented as means ± standard error of the mean (SEM), and n refers to the number of strips obtained from different animals for each experiment. The concentration-response curves for H_2S were obtained in parallel strips in the presence of inhibitors or vehicle (aqua, ethanol, NaOH or DMSO). Differences in results between tissues were tested by analysis of variance (ANOVA) and *t*-test corrected for multiple comparisons (Bonferroni corrections). *P* values less than 0.05 were considered significant. The sensitivity to the agonist was expressed as pD_2 (negative logarithm of the agonist concentration required for half-maximum response).

Drugs and solutions

Indomethacine, L-cysteine, NaHS, theophylline, papaverine, rolipram and phenylephrine were obtained from Sigma Chemical (St Louis, MO, USA). Baicalein (1761), fasudil (0541) and 4-(4-octadecylphenyl)-4-oxobutenoic acid (0606), were obtained from TOCRIS Bioscience (Minneapolis, MN, USA). Proadifen was obtained from Fluka (Gillingham, UK). Stock solution of theophylline was prepared in NaOH. Stock solutions of baicalein and 4-(4-octadecylphenyl)-4-oxobutenoic acid were prepared in DMSO. Stock solution of Rolipram was dissolved in ethanol and all other drugs were dissolved in distilled water. The vehicles had no effects on the relaxant responses to H_2S. NaHS as exogenous H_2S solution (1 M) was prepared in H_2O and kept on ice.

Results

Relaxations induced by exogenous H_2S

To study the relaxant response of corpus cavernosum to exogenous H_2S, the tissue was contracted with phenylephrine (5 μM) and after a steady state of contraction, H_2S was applied cumulatively at 1–1000 μM concentration. H_2S caused a concentration-dependent relaxation in mouse cavernosal strips pre-contracted with phenylephrine There was no significant difference between first and second relaxant responses to H_2S (data not shown).

The role of arachidonic acid/COX cascade pathway in H_2S-induced relaxation

To clarify contribution of PLA_2 to H_2S-induced relaxation in isolated mice cavernosal strip, we investigated the role of OBAA, a selective PLA_2 inhibitor, on relaxations induced by H_2S. OBAA (10 μM) significantly reduced the maximal relaxant response to H_2S from 112.7 ± 4% to 97.4 ± 4.5% ($p < 0.05$; n = 7; Fig. 1A). However, there was no significant difference between pD_2 values for control and OBAA, respectively, 3.68 ± 0.10 and 3.82 ± 0.13 ($p > 0.05$, n = 7). To evaluate whether other arachidonic acid metabolites were involved in H_2S-induced relaxant effect in mouse corpus cavernosum, non-selective COX inhibitor indomethacin, LOX inhibitor baicalein and cytochrome P450 inhibitor

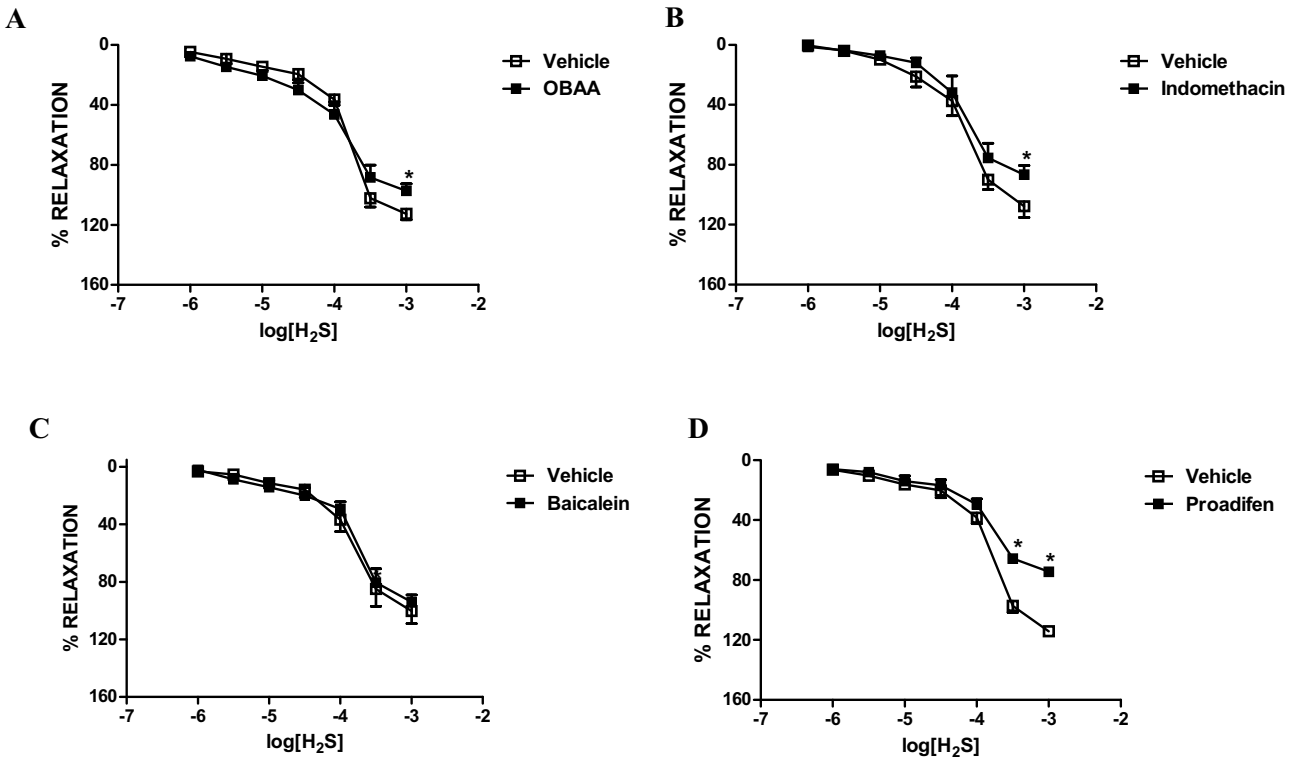

Fig. 1. The contribution of phospholipase A_2, cyclooxygenase, lipooxygenase and cytochrome P450 to exogenous H_2S-induced relaxations. The cumulative concentration-response curve showing the effect of OBAA (10 μM) **(A)**, indomethacin (1 μM) **(B)**, baicalein (10 μM) **(C)** and proadifen (10 μM) (**D**) on exogenous H_2S (1–1000 μM)-induced relaxations in mice cavernosal strips. All values are expressed as mean ± SEM ($n = 7$). *$p < 0.05$ significantly different from vehicle; one-way ANOVA and unpaired t test followed by Bonferroni's comparison test.

proadifen were studied on relaxant responses to H_2S. Indomethacin at 1 μM concentration caused a significant decrease in H_2S-induced maximal relaxation from 107.8 ± 7.5% to 86.7 ± 6.1% ($p < 0.05$; n = 7; Fig. 1B). There were no significant differences in the pD_2 values between the control group and indomethacin group, 3.70 ± 0.13 and 3.72 ± 0.15, respectively, ($p > 0.05$; n = 7). Also, the involvement of LOX pathway was evaluated by using baicalein (10 μM), a selective LOX inhibitor. Baicalein did not affect the H_2S-induced relaxations and there were no significant differences in the maximal relaxant response to H_2S between the control and baicalein group, 100.4 ± 8.7% and 94.0 ± 4.8% respectively ($p > 0.05$; n = 7; Fig. 1C). Also, there were no significant differences in the pD_2 values between the control group and baicalein group, 3.69 ± 0.15 and 3.65 ± 0.15, respectively ($p > 0.05$; n = 7). In addition, to evaluate the contribution of cytochrome P450 pathway to H_2S-induced relaxation in isolated mice cavernosal strip, we investigated the role of proadifen. Proadifen (10 μM) markedly reduced the maximal relaxant response to H_2S from 114.3 ± 2.5.0% to 74.6 ± 3.0% ($p < 0.05$; n = 7; Fig. 1D). There was no significant differences in pD_2 values between the control and proadifen group, 3.64 ± 0.09 and 3.7 ± 0.10, respectively ($p > 0.05$; n = 7).

The role of phosphodiesterase IV in H_2S-induced relaxation

In order to determine the possible role of phosphodiesterase IV (PDEIV) in exogenous H_2S-induced relaxation in mouse corpus cavernosum, theophylline, a non-selective PDEIV inhibitor, and rolipram, a selective PDEIV inhibitor, were studied. The maximal relaxant response to H_2S were significantly reduced in the presence of theophylline (500 μM) and rolipram (1 μM) from 108.1 ± 8.9% to 82.2 ± 6.4% ($p < 0.05$; n = 7) and 103.8 ± 2.3% to 84.1 ± 3.1% ($p < 0.05$; n = 7), respectively (Fig. 2A and B). However,

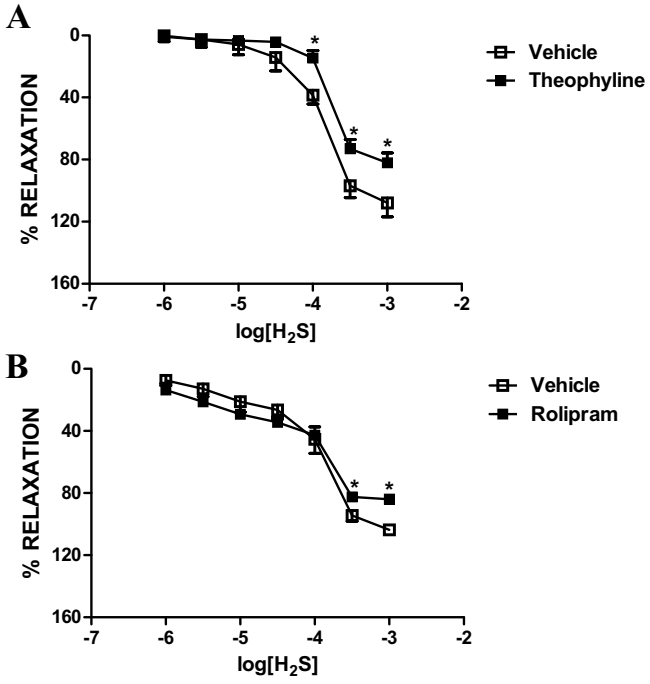

Fig. 2. The contribution of phosphodiesterase IV to exogenous H_2S-induced relaxations. The cumulative concentration-response curves showing the effects of theophylline (500 μM) **(A)**, and rolipram (1 μM) **(B)** on exogenous H_2S (1–1000 μM)-induced relaxations in mice cavernosal strips. All values are expressed as mean ± SEM ($n = 7$).*$p < 0.05$ significantly different from vehicle; one-way ANOVA and unpaired t-test followed by Bonferroni's comparison test.

there were no significant difference pD_2 values compared to control in the presence of theophylline and rolipram, respectively, 3.74 ± 0.14 to 3.58 ± 0.13 ($p > 0.05$, n = 7) and 3.77 ± 0.13 to 3.83 ± 0.13 ($p > 0.05$; n = 7).

The role of RhoA/Rho-kinase in H_2S-induced relaxation

To examine the whether relaxation to H_2S is mediated by RhoA/Rho-kinase signaling pathway, the influence of fasudil, a specific Rho-kinase inhibitor, on the relaxant response to H_2S was investigated. The maximal relaxant response to H_2S was markedly reduced in the presence of fasudil (3 μM) from $113.8 \pm 5.3\%$ to $83.9 \pm 6.3\%$ ($p < 0.05$; n = 7; Fig. 3). There was no significantly difference between pD2 values, 3.95 ± 0.08 and 3.98 ± 0.11 for control and fasudil, respectively ($p > 0.05$; n = 7).

Discussion

The present results suggest that PLA_2, COX, cytochrome P450, PDEIV and Rho-kinase pathway may be an important component of H_2S-induced relaxant responses in mouse corpus cavernosum tissues. Also, it seems that cytochrome P450 enzyme from these enzymes contributes most to H_2S-induced penile relaxation. Also, the inhibitors used in this study did not change pD_2 values while reduced maximum response, indicating there is no effect on sensitivity to H_2S-induced responses. On the other hand, it appears that LOX pathway is not involved in H_2S-induced relaxation.

Recently, several studies show that H_2S has a concentration-dependent dual effect on vascular smooth muscle [15,18–20]. At lower concentrations (10 μM), H_2S causes vasoconstriction, whereas at higher concentrations (100 μM), H_2S produces vasodilatation. Di Villa Bianca et al. has been shown that both effects are dependent on arachidonic acid generated by PLA_2 and cytochrome P450 but not by COX or LOX metabolites in mesenteric artery [15]. In the present study, in order to evaluate the involvement of arachidonic acid metabolites contributing to the formation of relaxation to H_2S in corpus cavernosum, we inhibited different steps in the arachidonic acid cascade by blocking PLA_2, COX, cytochrome P450 or LOX enzymes. We observed that pretreatment with OBAA, a PLA_2 inhibitor, indomethacin, a non-selective COX inhibitor and proadifen, a selective inhibitor of cytochrome P450 but not baicalein, a selective LOX inhibitor, significantly reduced relaxant responses to exogenous H_2S. Our results suggest that the H_2S-induced relaxant effect seems to involve the PLA_2, COX and cytochrome P450 enzymes in mice corpus cavernosum. Consistent with the present results, it has been recently shown that dexamethasone, a PLA_2 inhibitor, directly inhibits H_2S biosynthesis through the impairment of CBS and CSE expression in mesenteric and carotid artery of rats, suggesting the possible role of PLA_2 in H_2S-induced relaxation [21]. By contrast, the H_2S-induced relaxant response was not affected by LOX inhibitor, suggesting LOX enzyme is not responsible for relaxation to H_2S in mouse corpus cavernosum. Since, it has been demonstrated that impaired arachidonic acid metabolism with reduced release/effects of vasodilator prostaglandins from both COX-1 and COX-2 pathways, plays a key role in the pathogenesis of endothelial dysfunction of penile arteries [22]. Also, in human penile smooth muscle, the synthesis of relaxant prostanoids is promoted by arachidonic acid, since indomethacin prevented arachidonic acid-induced relaxations [23]. Consistent with this notion, recently COX-2 −10aa-PGIS, which converts arachidonic acid directly to prostacyclin, gene therapy improved erectile function after cavernous injury through antifibrotic and anti-apoptotic mechanism [24]. Furthermore, in the pig bladder neck smooth muscle, indomethacin and SC560, which are inhibitors of COX and COX-1, respectively, reduced the relaxation to GYY4137, H_2S donor, while NS398, a COX-2 selective inhibitor, did not affect the relaxation, suggesting COX-1 pathway derived prostanoids are involved [25]. We observed a decrease with proadifen, a selective inhibitor of cytochrome P450, on H_2S-induced relaxation. Since, it has been shown that epoxyeicosatrienoic acids are the essential metabolite of cytochrome P450-mediated metabolism of arachidonic acid, and that inhibition of epoxyeicosatrienoic acids synthesis decreases erectile function in rat corpus cavernosum, suggesting cytochrome P450 epoxygenase signaling pathway is another important vasorelaxation pathway required for normal erectile function in addition to NO signaling in penis [26]. Also, it has been suggested that reduction of epoxyeicosatrienoic acids inactivation may have therapeutic potential to prevent erectile dysfunction associated with diabetes and aging [27]. In contrast to the our results, Jubiter at al. has been reported that the inhibitors of cyclooxygenase and cytochrome P450 had no effect on increases intracavernosal pressure in response to Na_2S, suggesting arachidonic acid metabolites are not involved [28]. These differences may be explained by different route of administration, experimental model or exogenous source is used (such as NaHS or Na_2S). Di Villa Bianca et al. has been reported cytochrome P450 metabolites of arachidonic acid are involved in the vasodilator effect generated by higher concentrations of exogenous H_2S, suggesting that H_2S promotes the release of EDHF in the mesenteric circulation [15]. However, we recently show that exogenous H_2S-induced relaxations may be independent of the EDHF in mouse corpus cavernosum [11]. Further detailed additional experiment will be required to elucidate this.

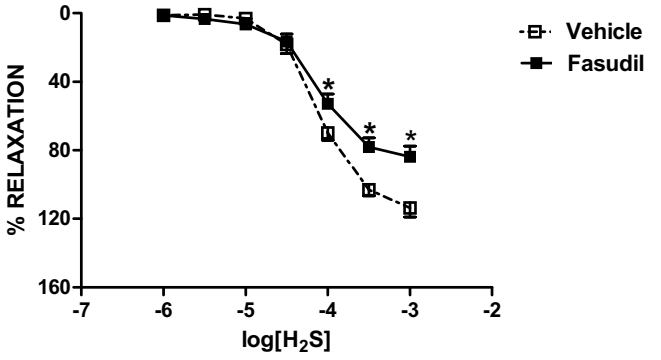

Fig. 3. The contribution of RhoA/Rho-kinase pathway to exogenous H_2S-induced relaxations. The cumulative concentration-response curve showing the effect of fasudil (3 μM) on exogenous H_2S (1–1000 μM)-induced relaxations in mice cavernosal strips. All values are expressed as mean ± SEM ($n = 7$). $^*p < 0.05$ significantly different from vehicle; one-way ANOVA and unpaired t test followed by Bonferroni's comparison test.

Cyclic nucleotides (cAMP and cGMP) are involved in the signal pathways most important for relaxation of corpus cavernosum. Adenylate cyclase and guanylate cyclase enzymes, responsible of the cyclic nucleotides synthesis, regulate the intracellular levels of cAMP and cGMP, and phoshodiesterases (PDEs) involves in their hydrolysis [29]. In human cavernosal tissue, at least 13 PDE isoenzymes have been identified, including PDEIII, PDEIV and PDEV [30,31]. PDEIV, cGMP-insensitive, hydrolyses selectively cAMP with high affinity and is specially inhibited by rolipram [32,33]. PDEV inactivates cGMP by hydrolyses cGMP to GMP [34]. Besides significance of the nitric oxide/cGMP-mediated mechanism, the cAMP signaling pathway is also involved in the regulation of tone of the erectile tissue. Previous studies have reported that adenylyl cyclase inhibitors reduced relaxant responses to exogenous H_2S-induced relaxations in isolated rat and rabbit corpus cavernosal tissues, and suggested that adenylyl cyclase/cAMP pathway play a role in these relaxations [35,36]. Supporting these data, we recently observed that the SQ22536 and

NEM, selective and non-selective adenylyl cyclase inhibitor, respectively, markedly reduced the exogenous H_2S-induced relaxations, suggesting that adenylyl cyclase plays a role in the relaxant effect of H_2S in mouse corpus cavernosum [11]. Therefore, in the present study we investigate the effect of PDEIV, selective cAMP hydrolyzer, on H_2S-induced relaxations by using rolipram and theophylline, selective and non-selective PDEIV inhibitors, respectively. The relaxant response to exogenous H_2S significantly reduced presence of theophylline and rolipram, suggesting H_2S-triggered relaxation is mediated, at least in part, by inhibition of PDEIV. The pre-incubation of cavernosal strips with theophylline reduced relaxations to H_2S with relatively greater than rolipram. Since, theophylline is also an adenosine receptor antagonist, blocked of adenosine activity may be proposed as a possible mechanism for theophylline's effects in H_2S-produced relaxation in mouse corpus cavernosum [37]. Further studies are needed to clarify this point.

Recently, it has been suggested that the relaxant effect of endogenously released and exogenously applied H_2S on rabbit gastric smooth cells involves inhibition of RhoA/Rho-kinase and PKC activities leading to stimulation of MLC_{20} phosphorylation, and inhibition of contraction [17]. The accumulated evidence in the literature suggests the importance of Rho-kinase activity in the maintenance of corporal vasoconstriction and penile *detumescense* [38–40]. Also, we have been recently reported that RhoA, ROCK1 and ROCK2 are expressed in the mouse corpus cavernosum smooth muscle and phenylephrine-induced contractions depends on RhoA/Rho-kinase-mediated Ca^{2+} sensitization [41]. To our knowledge, the role of RhoA/Rho-kinase pathway in relaxant responses produced by H_2S-induced has not been studied in cavernosal tissue. In this study, the contribution of RhoA/Rho-kinase pathway to H_2S-induced relaxation was investigated by using fasudil, a specific Rho-kinase inhibitor. We observed that fasudil significantly decreased H_2S-induced relaxations, suggesting the relaxant effect of H_2S may be due to, at least in part, the inhibition of Rho-kinase in corpus cavernosal tissue. Our observation with fasudil is important, as it is the first reported the contribution of Rho-kinase to H_2S-induced relaxations in corpus cavernosal tissue. In contrast to present results, Rho-kinase inhibitor Y27632 did not any effect on H_2S response in rat mesenteric small arteries [42]. Further additional experiments will be required to reveal the involvement of RhoA/Rho-kinase pathway to inhibitor effects of H_2S in corporal tissue.

In conclusion, these results suggest that PLA_2, COX, cytochrome P450, PDEIV and Rho-kinase pathway may be an important component of H_2S-induced relaxant responses in mouse corpus cavernosum tissues. However, it seems that LOX pathway is not involved in H_2S-induced relaxation. These observations may help to unravel the complex mechanism underlying the relaxant response of H_2S and may lead to the development of therapeutic approaches in the treatment of erectile dysfunction.

Conflict of interest

The authors declare that there are no conflict of interest.

Acknowledgment

The authors thank James Amoonarquah for English editing. This work was supported by Cukurova University Research Foundation (TSA-2016-6378).

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