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Role of endogenous hydrogen sulfide in neurogenic relaxation of rat corpus cavernosum☆

Mehdi Ghasemi [a,b], Ahmad R. Dehpour [c], Kevin P. Moore [a], Ali R. Mani [a,d,*]

[a] Department of Medicine, UCL Medical School, University College London (UCL), London, UK
[b] Department of Neurology, Johns Hopkins School of Medicine, Baltimore, USA
[c] Department of Pharmacology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran
[d] Department of Physiology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran

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ABSTRACT

Relaxation of corpus cavernosum during penile erection is mediated by a non-adrenergic non-cholinergic (NANC) neurotransmission and by the endothelium via the release of nitric oxide. Hydrogen sulfide (H_2S) is an endogenous gaseous mediator which is a potent vasodilator and a neurotransmitter. This study was initiated to characterize the role of H_2S in NANC neurogenic transmission in rat corpus cavernosum. The expression of H_2S producing enzymes was assessed using RT-PCR as well as Western blotting and showed the expression of cystathionine γ-lyase (CSE) in rat corporal tissue. Homogenates from rat corpus cavernosum convert L-cysteine to H_2S and this was partially inhibited by a CSE inhibitor, propargylglycine. Electrical stimulation of corporal tissue strips caused NANC relaxation. This neurogenic relaxation was significantly enhanced by inhibition of CSE by propargylglycine indicating that endogenously produced H_2S may have a negative regulatory role in neurogenic relaxation of rat corpus cavernosum. To investigate this further we used physiologically relevant concentrations of exogenous NaHS, and showed that nanomolar concentrations could inhibit corporal relaxation induced by a nitroxyl (HNO) donor (Angeli's salt) but not with nitrosonium (NO^+) or NO donors. This suggests that an interaction between endogenously produced H_2S and nitroxyl (HNO) might be involved in erectile function.

1. Introduction

Endogenous gaseous mediators such as nitric oxide (NO) and carbon monoxide play important roles both in health and disease. In recent years, interest has been directed towards hydrogen sulfide (H_2S) as the third gaseous mediator, which is an endogenous vasodilator [1,2] and a neurotransmitter within the nervous system [3]. Aside from that present in the circulation, a significant amount of H_2S is produced in various tissues [4]. Two pyridoxal-5′-phosphate-dependent enzymes, cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE), are responsible for the endogenous production of H_2S in mammalian tissues [5–7]. Although expression of CBS is more abundant in liver and neuronal system, CSE is the dominant H_2S-generating enzyme in the cardiovascular system [8–11]. The main substrate for the generation of endogenous H_2S is L-cysteine [9,12,13]. Activation of either CSE or CBS employs an α,β-elimination reaction to convert L-cysteine to H_2S, pyruvate and ammonium [4,9,14].

$$\text{L} - \text{Cysteine} \rightarrow H_2S + \text{pyruvate} + NH_4^+$$

Penile erection is regulated by a complex neurological control of the corpus cavernosum. Locally, the relaxation of corpus cavernosum (i.e. penile erection) is mediated by a non-adrenergic non-cholinergic (NANC) neurogenic mechanism and by the endothelium via the release of an endothelium-derived relaxing factor such as NO [15]. In vitro, electrical stimulation of isolated corpus cavernosum strips elicits a neurogenic, frequency-dependent relaxation that is resistant to adrenergic and cholinergic blockers. Thus, it is characterized as a NANC-mediated response. Nitric oxide or its related species seems to be the main NANC transmitter which mediates the relaxation of corpus cavernosum [15,16].

In recent studies, the involvement of H_2S in facilitating erectile function has been reported in rabbits and primates [17,18]. Srilatha et al. showed that intra-cavernous injection of sodium hydrogen sulfide (NaHS, an H_2S donor) resulted in significant increases in cavernous pressure in primates [17]. Another study by the same group of investigators also showed that NaHS is able to relax the

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* Corresponding author at: Department of Medicine, UCL Medical School, University College London (UCL), London, UK. Fax: +44 0207 433 2871.
E-mail address: rmhaalm@ucl.ac.uk (A.R. Mani).

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rabbit corpus cavernosum in vitro [18]. Moreover, both CBS and CSE inhibitors markedly increased the noradrenergic contractile neurotransmission of the corporal strips to field stimulation indicating the possible inherent inhibition of the relaxant H_2S formation [18]. In a recent study a functionally active L-cysteine/H_2S pathway has also been described in human corpus cavernosum [19]. Although recent studies have suggested that H_2S is produced within the corporal tissue and is able to relax the corpus cavernosum smooth muscle, it is not known whether or not H_2S is involved in the neurogenic relaxation of corpus cavernosum as a NANC mediator.

Nitrogen oxide research in the biological system has primarily focused on the effects of NO and higher oxidation products such as nitrosonium ion (NO^+). However, nitroxyl (HNO), the one-electron-reduction product of NO, has recently been shown to have unique and potentially physiological and pharmacological properties [20–22]. It is speculated that endogenous HNO is produced by a NO synthase (NOS)-dependent mechanism [23]. Nitroxyl is very reactive towards nucleophiles such as thiols [20–22]. Thus, HNO is able to target signaling pathways distinct from $NO^•$ with an ability to react with thiols and possibly H_2S. Recent reports are in agreement with interaction between HNO and H_2S in cardiomyocytes [24]. However the interaction between HNO and endogenous H_2S remains to be studied in other biological systems such corpus cavernosum.

The aim of the present study was to examine the possible role of hydrogen sulfide in NANC relaxation of rat corporal tissue in vitro. Since the available data on the expression of CBS and CSE as well as the endogenous production of H_2S in the corpus cavernosum is limited to rabbits and primates, we initially tried to confirm the expression and function of H_2S-producing enzymes in rat corpus cavernosum and then we investigated the possible role of H_2S in the NANC neurogenic relaxation of rat corporal tissue. We also studied the interaction between an HNO donor (Angeli's salt) and physiologically relevant concentrations of hydrogen sulfide in isolated rat corporal tissue.

2. Materials and methods

2.1. Drugs

Phenylephrine hydrochloride, guanethidine sulfate, atropine sulfate, N^G-L-nitro-arginine methyl ester (L-NAME), DL-propargylglycine (PAG), oxamic acid, L-cysteine, pyridoxal-5′-phosphate, [1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one] (ODQ), ammonium chloride, sodium pyruvate, sodium hydrogen sulfide (NaHS), sodium nitroprusside (SNP), S-nitrosoglutathione (GSNO), glibenclamide, MDL12,330A hydrochloride were purchased from Sigma Chemical Company (Bristol, UK). The nitroxyl (HNO) donor, Angeli's salt was purchased from Cayman Chemical (Ann Arbor, USA). All drugs were freshly dissolved in distilled water except ODQ which was dissolved in 15% dimethyl sulfoxide (DMSO).

2.2. Animals

Male Sprague-Dawley rats (body weight 200–250 g) were obtained from the Comparative Biology Unit at the UCL Medical School (Royal Free Campus, London, UK). The animals were housed in a light-controlled room with a 12 h day/night cycle and were given free access to food and water. All animal procedures were in accordance with Home Office (UK) recommendations. Each separate experimental group consisted of six animals.

2.3. mRNA extraction and reverse transcription polymerase chain reaction (RT-PCR) procedure

Corpus cavernosum, liver and brain were obtained from anesthetized rats and immediately immersed in liquid nitrogen. Total RNA was extracted from tissue homogenate using RNeasy Fibrous Tissue mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. Further elimination of genomic DNA was performed by DNase (Promega, Madison, WI, USA) after RNA extraction. First strand cDNA was then generated using Moloney murine leukaemia virus reverse transcriptase. Oligonucleotide primers used for PCR amplification of rat CBS, CSE and β-actin were as follows:

a. CSE (NM017074), PCR product: 579 bp, Forward: 5′-CGCACAAATTGTCCACAAAC, Reverse: 5′-GCTCTGTCCTTCTCAGGCAC
b. CBS (NM012522), PCR product: 559 bp, Forward: 5′-ATGCTGCAGAAAGGCTTCAT, Reverse: 5′-GTGGAAACCAGTCGGTGTCT
c. β-Actin (NM031144), PCR product: 453 bp, Forward: 5′-AGAGGGAAATCGTGCGTGACA, Reverse: 5′-ACATCTGCTGGAAGGTGGACA

PCR reactions comprised of 1 μl of cDNA template, 10 pmol each of forward and reverse primers and optimized TaqPCR mastermix (Promega, Madison, WI, USA) in a total reaction volume of 25 μl. After initial 5 min incubation at 94 °C, PCRs were performed using a 1 min annealing step, followed by a 1 min elongation step at 72 °C and a 45 s denaturation step at 94 °C. Forty PCR cycles were performed for amplification of CSE and CBS cDNAs, 25 cycles for β-actin cDNA, followed by a final elongation for 10 min at 72.0 °C. PCR products were separated by electrophoresis through a 1% agarose gel and detected by ethidium bromide staining.

2.4. Western blotting

Corpus cavernosum and liver (positive control) were obtained from rat and were immediately frozen in liquid nitrogen. Snap frozen tissues were homogenized in ice-cold RIPA buffer containing protease inhibitors (protease inhibitor mixture from Roche, Mannheim, Germany), 50 mM Tris (pH 8), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS. Homogenates were then centrifugation at 10,000 × *g* for 10 min. After determining the protein concentrations of the supernatants (Bradford assay with BSA as standard), 10 μg protein of each sample was fractionated by SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane. After blocking with Tris buffered saline (10 mM Tris, 100 mM NaCl) containing 0.1% Tween-20 for 1 h, the membranes were incubated overnight with rabbit anti-CSE antibody (1:200 rabbit polyclonal antibody from Santa Cruz Biotechnology, Santa Cruz, CA, USA). After 4 times washing, the membranes were incubated with anti-rabbit IgG alkaline phosphatase-linked antibody (1:5000 dilution, Perbio Science, Cramlington, UK). Alkaline phosphatase was detected using a BCIP/NBT developing kit (Promega, Madison, USA).

2.5. Assay of cavernous tissue H_2S synthesis

Cavernous tissue H_2S synthesizing activity was determined essentially as described elsewhere [17] with some modifications. 50 mg of each frozen tissue preparation was homogenized in 0.2 ml ice-cold potassium phosphate buffer (100 mM, pH 7.4). Homogenates were added in a reaction mixture (total volume 500 μl) containing 20 μl of 2 mM pyridoxal 5′-phosphate, 20 μl of 10 mM L-cysteine, and 30 μl of saline. The reaction was performed in parafilmed tubes and initiated by transferring tubes from ice to a water bath at 37 °C. After incubation for 75 min, 250 μl of 1% zinc acetate was added followed by 250 μl of 10% trichloroacetic acid. Subsequently, 133 μl of 20 mM N,N-dimethyl-p-phenylendiamine-sulfate (DPD) in 7.2 M HCl and 133 μl of 30 mM $FeCl_3$ in

1.2 M HCl were added, and the absorbance of the solution was measured after 10 min at a wavelength of 670 nm. The H_2S synthesis inhibitors PAG (20 mM), oxamic acid (20 mM), or a combination of both were added 5 min before addition of L-cysteine (10 mM) and pyridoxal 5′-phosphate (2 mM). All samples were assayed in duplicate, and H_2S concentrations were calculated against a calibration curve of NaHS (3.12–500 μM). Results were expressed as nmoles per milligram of protein per min and calculated as mean ± SEM from 4 or 5 specimens.

2.6. Organ bath studies

2.6.1. The effect of exogenous H_2S on relaxation of corpus cavernosum

Corpora cavernosa strips were excised from rats as previously described [25–27]. They were mounted separately in 25-ml organ chambers with one end tied to a tissue holder and the other to a wire connected to a force transducer (ADInstrument, Australia). The chambers contained Krebs-bicarbonate solution (containing in mM: NaCl, 118.1; KCl, 4.7; KH_2PO_4, 1.0; $MgSO_4$, 1.0; $NaHCO_3$, 25.0; $CaCl_2$, 2.5; glucose, 11.1, pH 7.4) at 37 °C equilibrated with 95% oxygen and 5% carbon dioxide. The corporal strips were allowed to equilibrate under 0.5 g resting tension and after equilibration for 60 min, a concentration–response curve to 1–1000 μM NaHS was obtained by using cavernosal strips precontracted with phenylephrine (7.5 μM). To assess the involvement of NO/cGMP pathway, we incubated the strips for 20 min with 100 μM L-NAME (a non-selective NOS inhibitor) or 10 μM ODQ (a specific guanylyl cyclase inhibitor) before NaHS challenge. K_{ATP} channel involvement in NaHS-induced relaxation was assessed by incubating cavernosal strips with 10 and 100 μM glibenclamide for 20 min. Additionally, the involvement of adenylyl cyclase pathway was evaluated by incubating the strips for 20 min with 10 μM MDL12,330A (an adenylyl cyclase inhibitor).

In order to study the interaction between hydrogen sulfide and nitric oxide species (NO, NO^+ or HNO) on relaxation of rat corpus cavernousm, the effect of incubation with low concentrations of NaHS (30 nM, 300 nM and 30 μM) were examined on concentration-dependent relaxation induced by either NO, NO^+ or HNO donors (GSNO, SNP and Angeli's salt respectively; 0.1–1000 μM).

2.6.2. NANC neurogenic relaxation

In experiments that neurogenic relaxation of the tissue strips was evaluated, electrical field stimulation (EFS) was applied at 10 Hz frequency via two parallel platinum electrodes on either side of the corpus strips using a constant voltage stimulator (Digitimer, UK). In these experiments, atropine (1 μM, to produce cholinergic blockade) and guanethidine (5 μM, to inhibit norepinephrine release) were always present in the bathing medium to obtain NANC conditions. Effects of L-NAME (100 μM), ODQ (10 μM), PAG (2 mM) or oxamic acid (2 mM) on EFS-induced relaxations were examined by adding these agents 20 min before the relaxant responses to EFS.

2.7. Statistical analysis

The data are expressed as mean ± standard error of the mean (SEM). Statistical analysis of the data was performed by analysis of variance (ANOVA) followed by Newman–Keuls post hoc test. Statistical significance was considered when $P < 0.05$.

3. Results

3.1. CSE and CBS expression in the corporal tissue

To examine the expression of CSE and CBS in corporal tissue, RT-PCR was performed on rat corpus cavernosum and both brain and liver tissues were served as positive controls. β-Actin was used as house keeping gene. As shown in Fig. 1A, CSE mRNA was detectable in corporal tissue as well as the brain and liver samples. This was further confirmed by western blot analysis which showed the expression of a 45 kDa protein in corporal homogenate that reacted with anti-CSE antibody (Fig. 1B). However, we were unable to show the expression of CBS mRNA in the corporal tissue using RT-PCR.

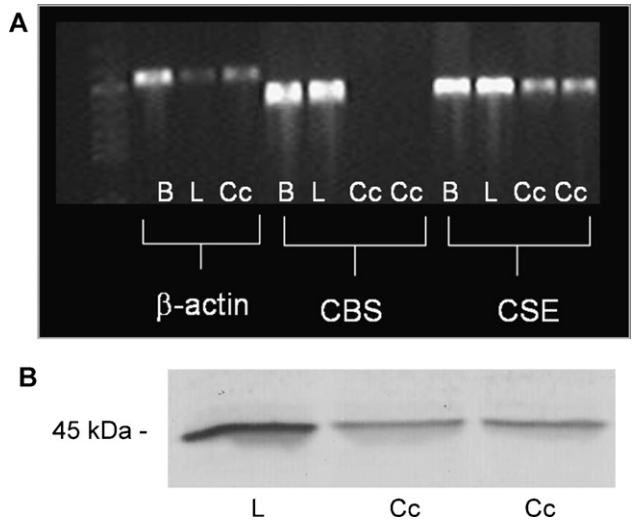

Fig. 1. (A) Expression of cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE) and β-actin in rat corpus cavernosum (Cc) and in rat brain (B) and liver (L; as positive controls). RNA was isolated from rat corpus cavernosum and analyzed by RT-PCR for β-actin, CBS and CSE. The DNA bands were fractionated on agarose gels and subjected to ethidium bromide staining. (B) Western blot analysis of cystathionine γ-lyase protein in two rat corporal tissue strips (Cc). Protein extracted from rat liver (L) was used as the positive control for cystathionine γ-lyase immune-reactivity assay.

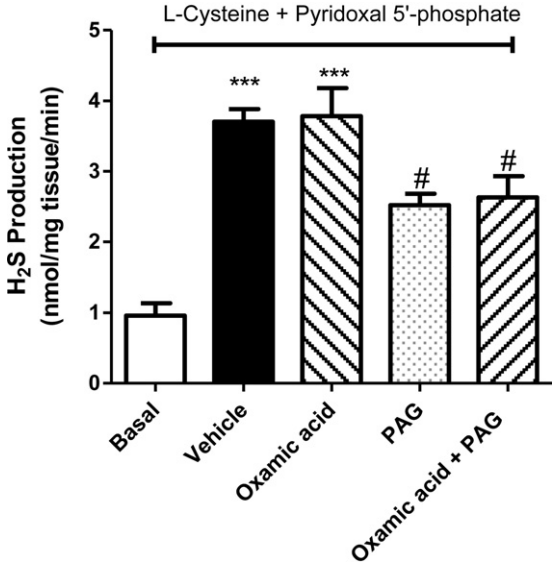

Fig. 2. Rat corpus cavernosum homogenate produced H_2S under basal conditions (black bar). Incubation of rat corpus cavernosum homogenate with L-cysteine (10 mM) and pyridoxal 5′-phosphate (2 mM) caused a significant ~3.5-fold increase in the H_2S production compared with basal values ($^{***}P < 0.001$). PAG (20 mM) alone or plus oxamic acid (20 mM) significantly ($^{\#}P < 0.05$ compared with vehicle group) inhibited the increase in H_2S production stimulated with L-cysteine (10 mM) and pyridoxal 5′-phosphate (2 mM). However, oxamic acid (20 mM) alone did not alter the H_2S production in the tissue homogenates. Data represent the mean ± SEM from 4 or 5 different rat specimens.

3.2. Cavernosal H_2S synthesis

Rat corpus cavernosum generated detectable amounts of H_2S (Fig. 2). The formation of H_2S was increased by approximately 3.5-fold over basal values after incubation of tissue homogenates with L-cysteine (the CBS/CSE substrate) and pyridoxal 5′-phosphate (co-factor for the CBS/CSE). The CSE inhibitor PAG significantly inhibited the increase in H_2S production stimulated with L-cysteine and pyridoxal 5′-phosphate. However, the CBS inhibitor oxamic acid alone did not alter the H_2S production in the tissue homogenates. Thus, rat corporal tissue is capable of synthesizing H_2S in the presence of L-cysteine and pyridoxal 5′-phosphate.

3.3. Organ bath studies

In the presence of stable tone elicited by phenylephrine, the H_2S donor NaHS (1–1000 μM), caused a concentration-dependent relaxation in the isolated corpus cavernosum in vitro (Fig. 3A), but only at high non-physiological concentrations. To assess further the potential involvement of NO/cGMP pathway in the NaHS effect, the strips were incubated with the NOS inhibitor L-NAME or the guanylate synthase inhibitor ODQ before adding NaHS. Neither L-NAME nor ODQ had a significant effect on the NaHS-induced relaxation of isolated strips (Fig. 3B). However, the adenylyl cyclase inhibitor MDL12,330A significantly reduced the relaxant responses to NaHS, but at only at high non-physiological concentrations (Fig. 3C). There was a significant difference ($P < 0.05$) between the maximal relaxant responses to NaHS in the presence or absence of MDL12,330A (78 ± 6.15 and 99.98 ± 3.02%, respectively). As shown in Fig. 3D, glibenclamide at 100 μM but not 10 μM significantly decreased the NaHS-induced relaxation of isolated corporal tissue strips ($P < 0.05$). There was a significant difference between the maximal relaxant responses to NaHS in the presence or absence of 100 μM glibenclamide (74.00 ± 9.58 and 99.98 ± 3.02%, respectively). Thus, although H_2S could lead to relaxation of the corpus cavernosum, this was only at high non-physiological concentrations. The next question was whether more physiological levels of hydrogen sulfide could affect relaxation or corpus cavernosum and thus erectile function.

Corporal strips, pre-contracted with phenylephrine in the presence of guanethidine and atropine, were relaxed by electrical field stimulation at frequency of 10 Hz (Fig. 4A). The neurogenic relaxations were significantly ($P < 0.01$) increased 20 min after incubation with PAG (which inhibits CSE), but not by oxamic acid (which inhibits CBS) alone (Fig. 4A and B). At this frequency, the NANC relaxation was significantly ($P < 0.001$) inhibited by pre-incubation by both the NOS inhibitor L-NAME and the guanylyl cyclase inhibitor ODQ in the absence or presence of PAG, suggesting that electrically NANC relaxation is a mediated by a NOS dependent mechanism (Fig. 4B).

As shown in Fig. 5A, in phenylephrine-precontracted corporal strips, SNP, GSNO or Angeli's salt caused a concentration-dependent relaxation in isolated corporal strips. ODQ significantly ($P < 0.001$) inhibited the relaxant responses to SNP, GSNO and Angeli's salt; consistent with involvement of cGMP (Fig. 5). Addition of hydrogen sulfide as NaHS (30 nM, 300 nM and 30 μM) did not significantly alter the relaxant responses to either SNP or GSNO (Fig. 6A and B). However, and in contrast, NaHS (30 nM, 300 nM and 30 μM) significantly inhibited the relaxation induced by the HNO donor (Angeli's salt) as shown in Fig. 6C. This might be taken to suggest that HNO is predominantly involved in erectile function rather than NO per se.

4. Discussion

Nitric oxide from cavernosal nerves/vascular endothelium plays a crucial role in initiating and maintaining intracavernous pressure increase, and penile vasodilation (erection). There are

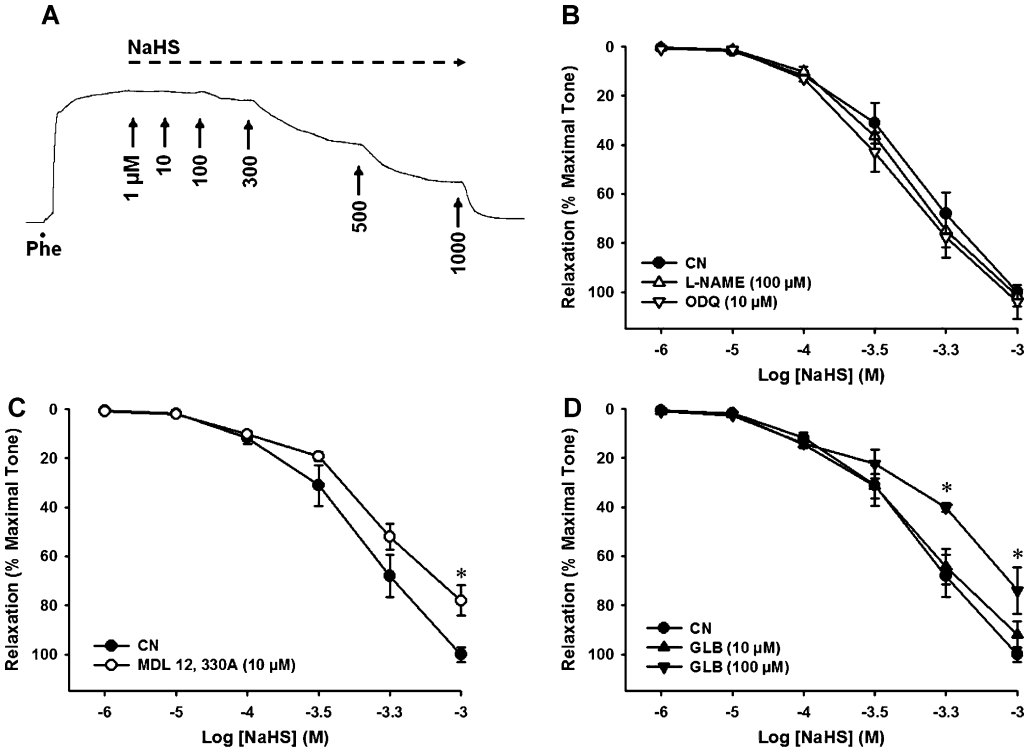

Fig. 3. (A) The H_2S donor NaHS (1–1000 μM)-induced relaxation in rat cavernosal tissue pre-contracted with 7.5 μM phenylephrine (Phe). (B) Effects of L-NAME (100 μM) and ODQ (10 μM) on NaHS (1–1000 μM)-induced relaxation of cavernosal tissue pre-contracted with phenylephrine (7.5 μM). (C) Effects of MDL12,330A (10 μM) on NaHS (1–1000 μM) induced relaxation of cavernosal tissue pre-contracted with phenylephrine (7.5 μM). (D) Effects of glibenclamide (GLB, 10 and 100 μM) on NaHS (1–1000 μM) induced relaxation of cavernosal tissue pre-contracted with phenylephrine (7.5 μM). Data represent the mean ± SEM. *$P < 0.05$ compared with control (CN) group.

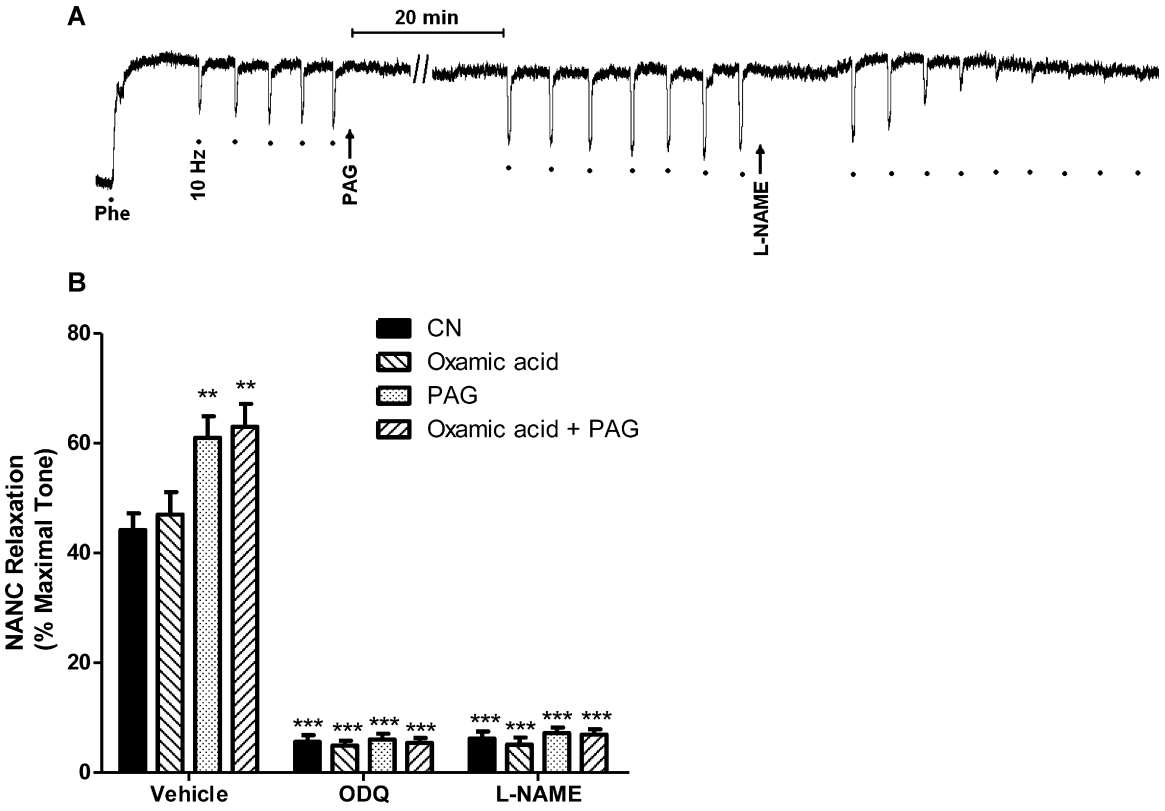

Fig. 4. (A) Effect of PAG (2 mM) on the NANC relaxation of isolated corpus cavernosum in response to electrical field stimulation (EFS, 10 Hz) in cavernosal tissue pre-contracted with 7.5 μM phenylephrine (Phe). (B) Effect of PAG (2 mM), oxamic acid (2 mM), or their combination on the NANC relaxation of isolated corpus cavernosum in response to electrical field stimulation (EFS, 10 Hz) in cavernosal tissue pre-contracted with 7.5 μM phenylephrine in the presence or absence of either L-NAME (100 μM) or ODQ (10 μM). $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with control (CN) group.

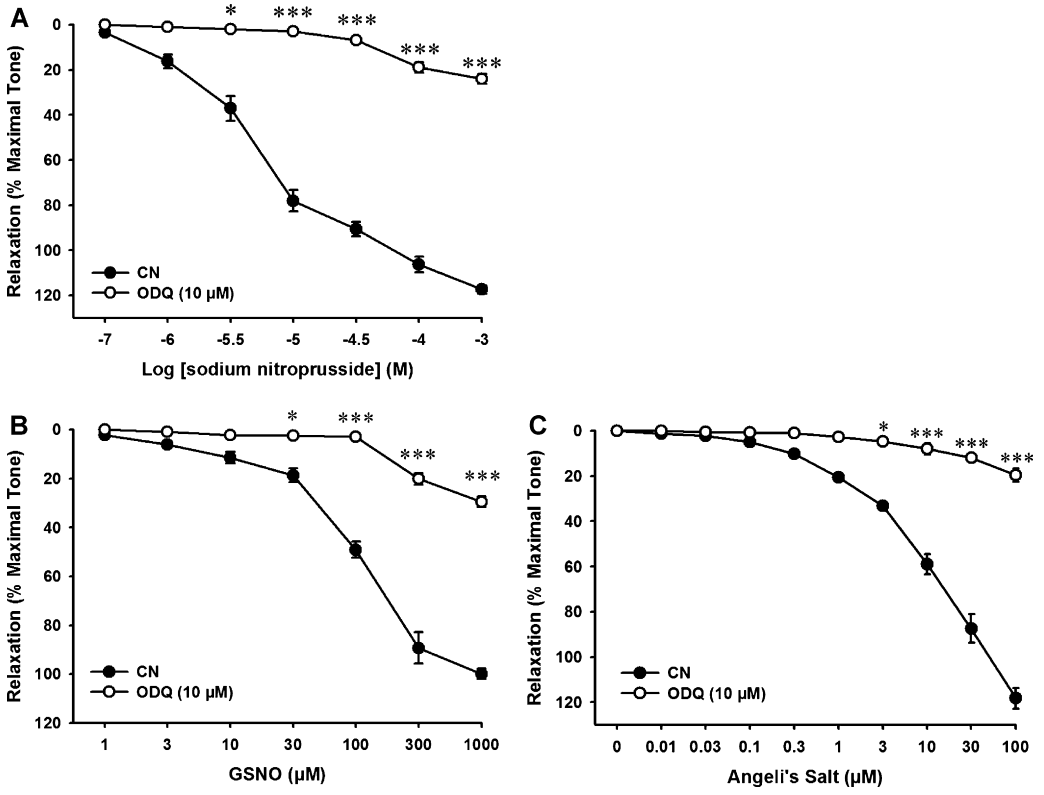

Fig. 5. Relaxant effects of the NO^+ donor sodium nitroprusside (0.1–1000 μM; A), NO donor GSNO (0.1–1000 μM; B) and HNO donor Angeli's salt (0.01–100 μM; C) on the cavernosal tissue strips pre-contracted with phenylephrine (7.5 μM) in the presence or absence of the guanylyl cyclase inhibitor ODQ (10 μM). Data represent the mean ± SEM. $^{*}P < 0.05$ and $^{***}P < 0.001$ compared with control (CN) group.

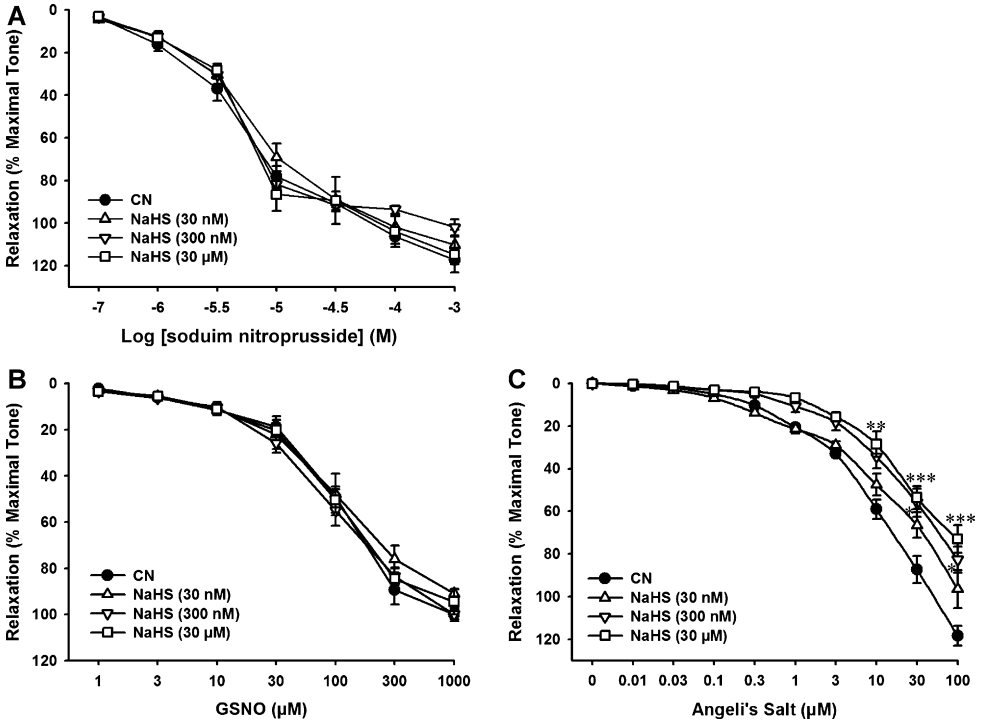

Fig. 6. Effects of NaHS (30 nM, 300 nM and 30 μM) on the sodium nitroprusside (0.1–1000 μM; A), GSNO (0.1–1000 μM; B) and Angeli's salt (0.01–100 μM; C)-induced relaxation of cavernosal tissue strips pre-contracted with phenylephrine (7.5 μM). Data represent the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with control (CN) group.

many data that show these are dependent on cGMP formed by soluble guanylyl cyclase by NO in smooth muscle cells [28–31]. Experimental evidence points to possible crosstalk between the NO and H_2S, in various physiological systems including NO dependent pathways [11,32,33]. H_2S is endogenously produced by two enzymes, CBS and CSE, of which CBS is predominantly expressed in liver, kidney and the central nervous system, but CSE is the main H_2S-synthesizing enzyme in the vasculature [14]. In the present study we demonstrated the expression of CSE but not CBS in rat corpus cavernosum. These data together with the recent studies [17–19] suggest a physiologic role for H_2S in the rat corpus cavernosum functioning. Thus, in the next step, we demonstrated that rat corpus cavernosum tissue homogenates generated detectable amounts of H_2S, which was increased by approximately 3.5-fold over basal values after incubation of tissue homogenates with L-cysteine (the CBS/CSE substrate). This result is similar to recent studies which have shown that H_2S could be generated in both rabbit and human corpus cavernosum [17–19]. However, it is noteworthy that previous study by d'Emmanuele di Villa Bianca et al., demonstrated the expression of both CBS and CSE in the human corpus cavernosum and the inhibitors of either CSE or CBS significantly decreased the generation of H_2S in the human cavernosal tissues [19] whilst in our study only the CSE inhibitor PAG but not the CBS inhibitor oxamic acid was able to significantly decrease the H_2S generation in the rat cavernosal homogenates tissues. These differences in results suggest that although the CSE enzyme is expressed in both rat and human corpus cavernosum, the presence of CBS may have a physiologic role in human cavernosal tissues but not rat tissue.

In the present study, we evaluated the effects of a CSE inhibitor on NANC relaxations induced by EFS in isolated rat cavernosal tissue strips. We found that the CSE inhibitor PAG caused a significant increase in the NANC relaxation induced by EFS at frequency of 10 Hz (Fig. 4A and B). At this frequency, the NANC relaxation is mainly related to NO release and was virtually blocked by both the NOS inhibitor L-NAME and the guanylyl cyclase inhibitor ODQ (Fig. 4B), suggesting that the effect of PAG in the corpus cavernosum is mediated by NO/cGMP pathway. To our knowledge, there is scant data regarding the effects of either the CSE or CBS inhibitors on NANC relaxation. The present result shows that inhibition of endogenous H_2S formation leads to enhanced neurogenic relaxation of the corpus cavernosum. Since H_2S does not have a direct constrictor effect in corporal tissue, this observed effect might be due to either inhibition of NO synthesis by endogenous H_2S or a direct chemical reaction between H_2S and a NOS product. There is evidence that NaHS inhibits three isoforms of NOS activity and decreased the conversion of [^{3}H]-arginine into [^{3}H]-citrulline by recombinant NOS [32,34]. According to these results, we suggest that the potentiating effect of PAG on NO-mediated NANC relaxation in rat cavernosal strips might be due to the possible inhibitory effect of H_2S on NOS activity or a direct chemical reaction between H_2S and an endogenous nitrogen oxide species such as HNO.

It is generally assumed that the primary product of NOS isoforms is the nitric oxide radical ($NO^{\bullet}$). However, growing evidence suggests that the one-electron reduced form of nitric oxide, nitroxyl anion (NO^- or HNO after protonation at physiologic pH), may be a natural co-product [23,35]. It appears that endogenously formed HNO exert widespread signaling functions along with $NO^{\bullet}$ in the cardiovascular and autonomic nervous systems [35,36]. Like $NO^{\bullet}$, HNO induces vasodilatation, inhibits platelet aggregation, and limits vascular smooth muscle cell proliferation [36–38]. However, the role of HNO in NANC relaxation of corpus cavernosum in not understood. Our results showed that exogenous HNO (released from Angeli's salt) is able to relax pre-contracted rat corpus cavernosum in a concentration dependent manner. This relaxation was completely blocked with ODQ showing that cGMP is involved in HNO-induced relaxation. Nitroxyl is very reactive towards nucleophiles (such as thiols) and quickly dimerizes to non-biologically active intermediates [22]. Thus, HNO is able to target signaling pathways distinct from $NO^{\bullet}$ with an ability to react with thiols and possibly H_2S. To investigate

this further we used physiologically relevant concentrations of exogenous NaHS, and showed that nanomolar concentrations could inhibit corporal relaxation induced by a nitroxyl donor (Angeli's salt) but not with nitrosonium (NO^+) or $NO^\bullet$ donors (Fig. 6). This suggests that an interaction between H_2S and nitroxyl (HNO) might be involved in neurogenic relaxation of corporal tissue. This observation may also explain how CSE inhibition caused a significant increase in neurogenic relaxation of rat corpus cavernosum. We were unable to measure the concentration of HNO anion following EFS in our experimental setting. Thus, most of our discussion on interaction between endogenously formed HNO and H_2S is based on indirect experiments on NANC relaxation of cavernosal tissue during electrical stimulation. Although the recent reports are in agreement with interaction between HNO and H_2S in cardiovascular system [24], further studies are required to confirm a role for endogenous HNO in physiology of penile erection. In present study we studied the relationship between H_2S and nitric oxide in healthy rats and only at the level of interaction with HNO. Interactions between hydrogen sulfide and nitric oxide are thought to exist at several other levels [39]. For instance Filipovic et al., have recently shown that H_2S interacts with peroxynitrite to form sulfinyl nitrite which is a novel nitric oxide donor [40]. These types of interactions can be investigated in future in pathologic conditions associated with formation of peroxynitrite (e.g. diabetes, cirrhosis).

H_2S is the first identified gaseous opener of K_{ATP} channels in vascular smooth muscle [1]. In isolated vascular smooth muscle cells, it directly increases K_{ATP} channels currents and hyperpolarizes membrane [6]. Considering this fact, we examined the involvement of K_{ATP} channel in the NaHS-induced relaxation in the rat isolated corpus cavernosum. Our data showed that the K_{ATP} channel blocker glibenclamide at 100 μM attenuated the relaxant responses to NaHS, indicating a role for K_{ATP} channels in the relaxant effects of NaHS in the rat corpus cavernosum. Recent study consistently demonstrated that glibenclamide (150 μM) significantly decreased the relaxation caused by NaHS in human corpus cavernosum [19]. It is noteworthy that the H_2S-induced relaxation is only partially blocked by glibenclamide in the present study and previous study in human corporal tissue which is in line with other experiments on different tissues such as rat aorta [6,32] and resistance mesenteric arteries [41], implying involvement of additional mechanisms independent of K_{ATP} channels. On the other hand, some studies have indicated that these channels do not seem to contribute to H_2S relaxation of either gastrointestinal (e.g. guinea-pig ileum) or reproductive smooth muscle [42], nor do they mediate responses in either the trout urinary bladder [43] or mouse aorta [32] because glibenclamide was ineffective in both tissues. This discrepancy in results might be explained by the fact that previous studies mostly used the lower concentrations of glibenclamide (e.g. 10 μM) in their experiments. Similarly, 10 μM glibenclamide failed to attenuate the NaHS-induced relaxation in rat cavernosal strips in our study. Additionally, although the exact reasons for species differences in the inhibitory effects of glibenclamide are still open to question, it is noteworthy that the use of glibenclamide at doses higher that 10 μM should be avoided in consideration of its non-selective actions, including inhibition of Na^+–K^+ pumps, L-type Ca^{2+} channels and certain Cl^- channels [44,45]. Therefore, considering our data together with previous reports, more detailed studies are clearly needed to verify whether K_{ATP} channels are involved in H_2S-induced relaxation in the corpus cavernosum. Besides, we and others observed that only pharmacological concentrations of H_2S are able to induce relaxation of pre-contracted corpus cavernosum. The physiological relevance of this relaxation is questionable since a number of studies have now shown that H_2S is produced at nanomolar concentrations, and not the micromolar concentrations originally reported using the methylene blue assay [46]. Therefore, physiological concentration of H_2S is less likely to induce relaxation of corpus cavernosum. In contrast, endogenous H_2S may have an inhibitory role in NOS-dependent neurogenic NANC relaxation of rat corporal tissue.

In summary, in the present study we found that: (i) the CSE but not the CBS enzyme is expressed in the rat corpus cavernosum, (ii) supra-physiological concentrations of an H_2S donor, NaHS, caused a concentration-dependent relaxation in isolated rat corpus cavernosum strips through a cAMP-dependent pathway, (iii) nanomolar concentrations of NaHS could inhibit corporal relaxation induced by a nitroxyl (HNO) donor, and (iv) inhibition of endogenous H_2S production was associated with enhanced neurogenic NANC relaxation in rat cavernosal tissues. These observations suggest that endogenous H_2S may have a negative regulatory role in neurogenic relaxation of rat corpus cavernosum. Our finding may help to unravel the complex mechanisms underlying the pathophysiology of penile erection and may provide a new insight into the treatment of erectile dysfunction.

Conflict of interest

The authors state no conflict of interest.

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