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

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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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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