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Hydrogen sulfide compensates nitric oxide deficiency in murine corpus cavernosum

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ABSTRACT

Erectile dysfunction (ED) is considered as a marker for cardiovascular diseases. Nitric oxide (NO) deficiency is the major cause of erectile dysfunction (ED). The role of hydrogen sulfide (H_2S) in erection has recently been recognized and is receiving attention as a pharmacological target. Several studies have focused on the effect of H_2S on NO-dependent relaxation, but the role of NO on H_2S in penile tissue has not been studied yet. Unlike NO, H_2S is mainly synthesized from smooth muscle cells rather than endothelial cells. We hypothesized that H_2S may compensate for the decreased NO bioavailability and may be beneficial in severe ED where endothelial dysfunction is present. Thus we studied the effect of NO deficiency on H_2S formation and vasorelaxation induced by L-cysteine, which is the substrate of the H_2S producing enzymes in mice corpus cavernosum (MCC).

NO deficiency induced by Nω-Nitro-L-arginine (L-NNA) was confirmed by the inhibition of acetylcholine-induced relaxation. L-cysteine, the substrate for the endogenous H_2S production, caused a concentration-dependent relaxation that was reduced by CBS/CSE inhibitor aminooxyacetic acid (AOAA) in MCC strips. L-NNA caused a significant increase in L-cysteine-induced relaxation, and this effect was reversed by AOAA. On the contrary, no change in relaxation to NaHS (exogenous H_2S donor) in MCC was observed. L-NNA increased H_2S formation stimulated by L-cysteine in wild type MCC but not in $CSE^{-/-}$ mice. In parallel, the expression of both cysthationine γ lyase (CSE) and 3-mercaptopyruvate sulphurtransferase (3-MST) was increased, whereas cysthationine-β synthase (CBS) was decreased in $eNOS^{-/-}$ MCC. We conclude that H_2S plays a compensatory role in the absence of NO by enhancing the relaxation induced by endogenous H_2S through CSE and 3-MPST in MCC, without altering downstream mechanisms. We suggest that H_2S-targeting drugs may provide the maintenance of compensatory treatment in ED patients. This may be more relevant in ED with severe endothelial dysfunction, as H_2S is mainly derived from smooth muscle.

1. Introduction

Erectile dysfunction (ED) not only limits the quality of life but also is a predictor of cardiovascular health [1]. Myocardial infarction occurs within 1–2 years after ED manifestation where the main problem is considered as disruption of vascular relaxation. Thus, it is important to define the mechanisms involved in the regulation of vascular tone in healthy and pathological conditions in penile tissue.

Decreased bioavailability of nitric oxide (NO) is the major cause of ED [2,3]. Recently, hydrogen sulfide (H_2S), a gasotransmitter generated by the enzymes cystathione-γlyase (CSE), cystathione-β synthase (CBS) and 3-mercaptopyruvate sulphurtransferase (3-MPST), has been recognized as a vasodilator in penile physiology [4–6]. There is accumulating data showing the crosstalk between H_2S and NO. Most of these studies focused on the effect of H_2S on NO-dependent relaxation; however, the role of NO on H_2S pathway is still unclear. There has been a study showing that exogenous H_2S-induced relaxation depends on NO bioavailability in rat aorta [7] but the effect of NO on endogenous H_2S pathway was not investigated

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in this study. In contrast, there is data which shows that NaHS-induced relaxation is augmented in brain microvessels [8] and not altered in penile tissue by L-NNA [9,10], suggesting a tissue and species specific crosstalk between H_2S and NO. Thus, we hypothesized that H_2S may compensate for the decreased NO bioavailability in penile tissue and may be beneficial in severe ED where endothelial dysfunction is present. Thus we aimed to investigate the role of H_2S in mice corpus cavernosum (MCC) under NO deficiency.

Although the discovery of phosphodiesterase (PDE) inhibitors acting on the NO/sGC pathway opened a new avenue in the treatment of ED, there are still non-responders, especially among diabetic ED patients where severe endothelial apoptosis, leading to NO deficiency, is present [11]. Therefore we used both genetic and pharmacological inhibition of eNOS to resemble ED with severe NO deficiency. We investigated the effects of NO deficiency on endogenous H_2S formation, molecular target for H_2S synthesis and endogenous H_2S-dependent vasorelaxation in MCC.

2. Material and methods

2.1. Animals and tissue preparation

The present study was approved by the Animal Experiment Local Ethical Committee of Ege University (2014-018) and the Animal Ethics Committee of the University of Naples "Federico II" (Italy) following the guidelines of Italian law (D.L. 26/2014) and both were in agreement with the European Union Community directive (UE Directive 63/2010) for experimental animal care and use. Animals were kept at a temperature of 23 ± 2 °C, a humidity range of 40–70% and 12 h light/dark cycles. Food and water were provided ad libitum. 25–28 g male CD1 mice (Charles River) were used for isolated organ bath experiments (n = 21) and H_2S assay (n = 8) with or without pharmacological inhibitor of eNOS, Nω-Nitro-L-arginine (L-NNA). $eNOS^{-/-}$ mice or their background C57Bl/6 mice were used for western blot experiments (n = 3) as well as H_2S assay (n = 3). CSE-ablated (KO) mice ($CSE^{-/-}$) were used for H_2S assay (n = 4). "n" is the number of tissues or homogenates used from separate animals. Two to three mice penile tissues were pooled for the western blot experiment or H_2S assay to get enough protein for each sample, respectively. Mice were anesthetized with isoflurane and euthanized. The penis was excised at its base with removal of the glans penis and connective and adventitial tissues along the shaft. The individual MCC strips were used for isolated organ bath experiments, western blot or H_2S assay.

2.2. Drugs and treatments

In some experiments isolated MCC strips or homogenates were incubated with NOS inhibitor, L-NNA (100 μM, 30 min) or CBS/CSE inhibitor aminooxyacetic acid (AOAA, 2 mM, 30 min). Unless otherwise stated, all the chemicals were obtained from Sigma.

2.3. Isolated organ bath experiments

MCC strips were mounted in a 5 ml bath of myograph for isometric force recording (Danish Myograph Technology, Aarhus, Denmark) coupled to a PowerLab 8/SP data acquisition system (Chart 5 Chart 5.0 software; ADInstruments, Colorado Springs, CO), and bathed in carboxygenated (95% O_2; 5% CO_2) modified Krebs–Ringer solution NaCl, 130 mM; $NaHCO_3$, 14.9 mM; dextrose, 5.5 mM; KCl, 4.7 mM; KH_2PO_4, 1.18 mM; $MgSO_4 7H_2O$, 1.17 mM and $CaCl_2 2H_2O$, 1.6 mM at 37 °C. Tissues were allowed to equilibrate for 90 min under a resting tension of 5 mN. Experiments were done in strips with endothelium as confirmed by relaxation of more than 40% to acetylcholine (ACh, 1 μM) after contraction with phenylephrine (Phe, 10 μM). In some experiments penile tissue was incubated with L-NNA (100 μM, 30 min) before pre-contraction. One concentration-response curve was obtained in each MCC. In the later series of experiments, relaxant responses to ACh (1 nM–100 μM), exogenous H_2S donor NaHS (1 μM–30 mM) or endogenous H_2S donor L-cysteine (10 μM –30 mM) were obtained in Phe-pre-contracted MCC. Data was calculated as% of relaxation toward a stable tone induced by Phe-induced precontraction.

2.4. Measurement of H_2S level by methylene blue assay

To evaluate the activity of CBS and CSE in MCC, H_2S formation was measured by methylene blue assay as previously described [12,13]. At least two pairs of MCC were pooled, homogenized with lysis buffer containing potassium phosphate buffer (100 mM, pH 7.4), sodium orthovanadate (10 mM) and protease inhibitors. Protein concentration was determined using the Bradford assay. Homogenate samples were added to a reaction mixture (total volume 500 μl) containing piridoxal- 5′-phosphate (2 mM, 20 μl) and saline (20 μl) or L-cysteine (10 mM, 20 μl) to measure basal and stimulated H_2S generation. In some experiments the tissues were incubated with L-NNA (100 μM) to evaluate the NO contribution in H_2S formation. The reaction was carried out at 37 °C for 40 min. Then, $ZnAc_2$ (1%, 250 μl) was added followed by trichloroacetic acid (10%, 250 μl) incubation to trap H_2S and to precipitate proteins. Subsequently, N-dimethyl-*p*-phenylendiamine-sulphate (DPD, 20 mM) in 7.2 M HCl and iron chloride ($FeCl_3$, 30 mM) in HCl (1.2 M) were added and the optical absorbance of the resulting solution was measured after 10 min at a wavelength of 650 nm. All samples were assayed in duplicate and H_2S concentration was calculated against a calibration curve of NaHS (3.9–250 μM). Data were calculated as nanomoles per milligram of protein per minute and expressed as fold increase over control of H_2S production.

2.5. Western blot analysis

Modified RIPA buffer (50 mMTris-HCl (pH 7.4),1% Triton X-100, 0.25% sodium deoxycholate, 150 mM NaCl,1 mM EDTA, 1 mmol/L PMSF, 10 μg/mL aprotinin, 20 μmol/L leupeptin, 50 mmol/L NaF) (Roche Applied Science, Italy) was used to homogenize MCC strips from wild type or $eNOS^{-/-}$ mice. After centrifugation of homogenates at 10,000 rpm for 10 min, protein concentration was determined by Bradford assay using bovine serum albumin (BSA) as standard. The Western blot was performed as previously described [14]; briefly 40 μg of the denatured proteins was separated on12%SDS/polyacrylamide gels and transferred to a PVDF membrane. The membranes were blocked by incubation in PBS containing 0.1% v/v Tween 20 and 5% non-fat dried milk for 1 h at room temperature and then incubated with mouse monoclonal antibody for CSE (1:1000; Abnova, Milan, Italy) or rabbit polyclonal antibody for CBS (1:1000; Santa Cruz Biotechnology, Inc.) or rabbit polyclonal antibody for MPST (Novus Biologicals) overnight at 4 °C. Membranes were extensively washed in PBS containing 0.1% v/v Tween-20 prior to incubation with horseradish peroxidse-conjugated secondary antibody for 2 h at room temperature. Following incubation, membranes were washed and developed using ImageQuant-400 (GE Healthcare, USA). The target protein band intensity was normalized over the intensity of the housekeeping protein GAPDH (1:5000, Sigma-Aldrich, Milan, Italy).

2.6. Statistics

Data was expressed as% relaxation of the Phe-induced tone. Concentration–response curves were fitted by sigmoid curves using the least squares method. All calculations were determined using a standard statistical software package (Prism5, Graphpad, San Diego, California, USA). Significance was accepted at $P < 0.05$.