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ORIGINAL ARTICLE

Further evidence of endogenous hydrogen sulphide as a mediator of relaxation in human and rat bladder

Jun-Wei Gai[1,2,*], Wasilijiang Wahafu[3,*], Hui Guo[4], Miao Liu[5], Xu-Chang Wang[1,2], Yun-Xiang Xiao[1,2], Liang Zhang[6], Zhong-Cheng Xin[2,7] and Jie Jin[1,2]

We investigated the expression of hydrogen sulphide (H_2S) in human and rat lower urinary tract (including bladder, prostate and urethra) tissues, and we sought to determine whether H_2S induces relaxation of human and Sprague–Dawley (SD) rat bladder strips. Human normal lower urinary tract tissue was obtained for the evaluation of endogenous H_2S productivity using a sulphide-sensitive electrode and for the analysis of the expression levels of all three synthases of endogenous H_2S, cystathionine β-synthase (CBS), cystathionine γ lyase (CSE) and 3-mercaptopyruvate sulphur transferase (MPST, as known as 3-MST) by Western blot assay. CBS, CSE and MPST were located in human sample slides by immunohistochemistry. Human and male adult SD rat bladder strips were tested for H_2S function with a transducer and recorded. All experiments were repeated six times. The endogenous H_2S productivity and the H_2S synthases had various distributions in the human and rat lower urinary tract tissues and were located in both epithelial and stromal sections. *L*-cysteine (*L*-Cys, a substrate of CBS, CSE and MPST) elicited relaxation in a dose-dependent manner on human bladder strips pre-contracted by acetylcholine chloride. This effect could be diminished by the ATP-sensitive potassium ion (K_{ATP}) channel blocker glibenclamide (GLB), the CSE inhibitor DL-propargylglycine (PPG) and the CBS inhibitor hydroxylamine (HA). H_2S and its three synthases were present in the human and rat lower urinary tract tissues and relaxed human and rat bladder strips, which implied that endogenous H_2S might play a role in physiological function and pathological disorders of the lower urinary tract symptoms (LUTS) or overactive bladder (OAB).

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INTRODUCTION

Hydrogen sulphide (H_2S), which is endogenously catalysed by cystathionine β-synthase (CBS), cystathionine γ lyase (CSE) derived from *L*-cysteine (*L*-Cys) and 3-mercaptopyruvate sulphur transferase (MPST, as known as 3-MST) through one-carbon metabolism and transsulphuration pathway, have been identified as novel non-adrenergic and non-cholinergic signal transmitters.[1–3] Various mammalian tissues have been proven to produce H_2S. Endogenous H_2S can relax mammalian cardiovascular, corpus cavernosum, vas deferens smooth muscle and human bladder.[4–7] Bucci *et al.*[8] even noted that H_2S was involved in testosterone vascular effects, which made H_2S more attractive for urological researchers. Our previous study had also revealed the expression and characterisation of H_2S in human prostatic tissue and cell lines.[9] Although Fusco *et al.*[10] demonstrated the generation and function of endogenous H_2S and its synthases in human bladder tissue, they did not analyse the expression of MPST in human bladder tissue and the function of H_2S on rat bladder tissue, whereas the latter remains controversial.

Dysfunctional lower urinary smooth muscle, such as bladder detrusor contraction and relaxation, is of great importance in lower urinary tract symptoms (LUTS), often resulting from damaged bladder detrusor function in aged men.[11] Alleviating detrusor function remains difficult for urologists.[12,13] Usually patients with LUTS are treated with α-adrenergic blockers or anti-cholinergic drugs to control undesired urinary smooth muscle contraction,[14,15] whereas newly emerging mechanisms or hypotheses provide urological researchers with alternative therapeutic options.[16]

In this present study, we analysed the endogenous H_2S productivity and expression levels of CBS, CSE and MPST in human and rat bladder, prostate and urethra tissue, respectively, and explored the effects of H_2S on the tone of bladder strips from humans and Sprague–Dawley (SD) rats.

MATERIALS AND METHODS

Tissue samples and animals

Informed consent and samples were obtained from 56 patients with bladder cancer (mean age: 64.3 years, age range: 54–76 years) who had

[1]Department of Urology, Peking University First Hospital, Beijing 100034, China; [2]The Institute of Urology, Peking University, Beijing 100034, China; [3]Department of Urology, PLA General Hospital, Beijing 100853, China; [4]Department of Urology, Tsinghua University First Hospital, Beijing 100016, China; [5]Hedong Center for Disease Control and Prevention, Tianjin 300151, China; [6]Department of Orthopaedic Surgery, Beijing Jishuitan Hospital, Fourth Clinical College of Peking University, Beijing 100035, China and [7]Andrology Center, Peking University First Hospital, Beijing 100034, China
* These authors contributed equally to this work.
Correspondence: Professor ZC Xin (xinzc@bjmu.edu.cn) and Professor J Jin (Jinjie@vip.163.com)
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undergone radical cystoprostatectomy in Peking University First Hospital from October 2009 to December 2011. Bladder tissue samples were removed and confirmed to be cancer-free by experienced urological pathologists. Adult male SD rats (8 months old) weighing 350–450 g were purchased from the Experimental Animal Center of Peking University Health Science. This study was approved by the Institutional Research Ethical Board. Freshly removed human and rat tissues were both fixed in paraformaldehyde solution for 24 h and then paraffin-embedded for immunohistochemistry or frozen immediately and stored in liquid nitrogen. Human bladder strips were prepared according to a published article.[10]

Immunohistochemistry

The expression and location of CBS, CSE and MPST in the human bladder, prostate and urethra tissues were examined by immunohistochemistry. Tissue samples were formalin-fixed for 24 h and then paraffin-embedded. Slides of 5 μm thickness were prepared and dewaxed. After the antigens were retrieved, the slides were treated with 3% hydrogen peroxide for 15 min and incubated with normal goat serum for 30 min to block nonspecific-binding sites. The slides were incubated with primary antibody against CBS (Abnova, Taiwan, China), CSE (Abnova) and MPST (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1 : 100 overnight at 4 °C. The slides were then washed with phosphate-buffered saline containing 0.1% v/v Tween-20 and incubated with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (Santa Cruz Biotechnology) for 20 min at room temperature. Finally, the slides were treated with peroxidase-conjugated streptavidin and stained with DAB. The results were photographed by optical microscope (Olympus, Tokyo, Japan).

Western blot semiquantity assay

Human and rat tissue samples were prepared in RIPA buffer with protease and phosphatase inhibitors (BD Pharmingen, San Diego, CA, USA). Protein samples (50 μg) were separated in 10% sodium dodecyl sulphate–polyacrylamide gels (Sigma-Aldrich, St Louis, MO, USA) and transferred to a nitrocellulose membrane (BD Pharmingen). The membranes were blocked with non-fat milk in Tris-Buffered Saline with Tween (BD Pharmingen) and probed with primary antibodies against CBS or CSE at 1 : 1000 and against MPST at 1 : 500, followed by secondary goat anti-mouse IgG antibodies (Santa Cruz Biotechnology). The signals were developed by ECL reagent (GE Healthcare Bioscience, Piscataway, NJ, USA) and then recorded. The results were semi-quantifiably assayed by a KODAK Image Station (Kodak, Rochester, NY, USA).

Endogenous H_2S productivity measurements

The measurements of the H_2S contents were performed as previously mentioned.[17] The tissue samples were homogenised in a 10-fold volume (w/v) of an ice-cold potassium phosphate buffer (pH=6.8). The reaction was performed in a 25-ml Erlenmeyer Pyrex flask, and within the flask, there was a specially made glass chamber (diameter 1 cm and height 2 cm). Cryovial test tubes (2 ml) were used as the centre wells, each containing 0.5 ml of 1 mol l^{-1} NaOH. The sulphur-sensitive electrode (PXS-270, Shanghai, China) was used to evaluate the endogenous H_2S productivity. A preparation of 500 μl of tissue homogenate mixed with 500 μl of pre-cooled 50 mmol l^{-1} pH 6.8 phosphate-buffered saline and 1 ml of reacting system solution (100 mmol l^{-1} pH 7.4 phosphate-buffered saline, 10 mmol l^{-1} *L*-Cys (Sigma-Aldrich), 2 mmol l^{-1} phosphate pyridoxine aldehyde) was made. Then, 2 ml of the mixed solution was moved to the flask outer room after the sample protein concentration was determined, and 1 ml of 1 mol l^{-1} NaOH was added to the central room of the bottle. The sealed flask was incubated at 37°C water bath for 90 min, and then 1 ml of 50% trichloroacetic acid was added to end the reaction. The incubation of the whole flask was continued at 37°C for 60 min. The contents were transferred from the central wells to the corresponding wells of a 12-well cell culture cluster (Corning, Corning, NY, USA), each containing 1 ml of the antioxidant solution. Subsequently, the solution was measured with the sulphide-sensitive electrode to calculate the H_2S contents against a standard curve. After adjusting with the protein concentration in the corresponding samples, the endogenous H_2S productivity was expressed in nmol min^{-1} mg^{-1} and calculated as the mean±s.d.

Organ bath experiments

Human bladder samples from donor patients or SD rat bladder strips were immediately placed in oxygenated 4 °C Krebs solution. Each human or rat bladder prepared tissue was a longitudinal strip of approximately 2 mm×10 mm and randomly divided into different treated groups.

The strips were transferred to a 25 ml organ bath container, filled with oxygenated Krebs solution at 37.0 °C, and connected to force transducers. After 40 min of equilibration, the initial tension of strips was adjusted to precisely 1 g. The strips were then pre-contracted with 20 mmol l^{-1} acetylcholine chloride (Sigma-Aldrich) in Krebs solution. When the contraction reached its peak and was stabilised, the experiments were performed. The relaxation degrees were expressed as the percentage of the initial tension.

Statistical analysis

The values are represented as the means±s.d. and were analysed using LSD *post hoc* test by SPSS 17.0 software. $P<0.05$ was considered statistically significant.

RESULTS

CBS, CSE and MPST expression in human bladder, prostate and urethra tissues

Human sections of smooth muscle layer presented higher expression levels of CSE, compared with those of CBS and MPST (**Figure 1**), which were confirmed by Western blot (**Figure 2a**). The SD rat tissue presented different expression patterns of CBS, CSE and MPST (**Figure 2b**) by Western blot assay.

H_2S productivity in human and rat bladder, prostate and urethra tissues

The H_2S productivities of human bladder, prostate, and urethra were 42.5±4.7, 27±2.8 and 14.4±4.9 nmol min^{-1} mg^{-1}, respectively (**Figure 2c**). The H_2S productivities of SD rat bladder, prostate, and urethra were 143.3±12.2, 50±4.7 and 117.1±15.7 nmol min^{-1} mg^{-1}, respectively (**Figure 2d**).

H_2S-induced relaxation on human and SD rat bladder strips was blocked by glibenclamide (GLB), DL-propargylglycine (PPG) and hydroxylamine (HA)

NaHS (the exogenous H_2S donor; Sigma-Aldrich) and *L*-Cys (the endogenous substrate of the H_2S synthases; Sigma-Aldrich) plus 10^{-6} mol l^{-1} 5′-pyridoxal phosphate as the coenzyme all relaxed the bladder strips in a dose-dependent manner. This effect could be diminished by GLB (a KATP channel inhibitor; Sigma-Aldrich), PPG (a specific CSE inhibitor; Sigma-Aldrich) and HA (a specific

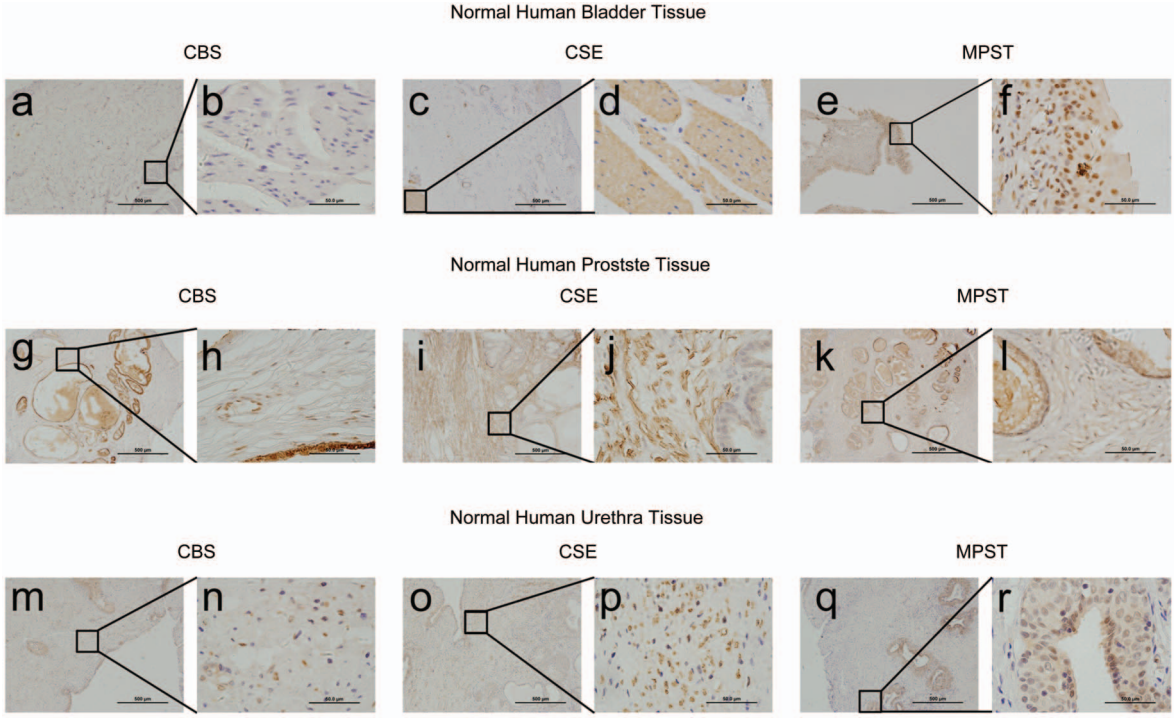

Figure 1 Immunohistochemical staining, protein levels and H_2S productivities of CBS, CSE and MPST in cancer-free human bladder, prostate and urethra tissues. The urothelial cell layers showed little or no staining of CBS, CSE, and MPST, whereas the bladder muscular layer showed high signals of CSE and MPST. CBS, cystathionine β-synthase; CSE, cystathionine γ lyas; MPST, 3-mercaptopyruvate sulphur transferase. For a, c, e, g, i, k, m, o and q, scale bar=500 μm. For b, d, f, h, j, l, n, p and r, scale bar=50 μm.

CBS inhibitor; Sigma-Aldrich) (**Figure 3**). The strips were pre-contracted with 20 mmol l^{-1} Ach in Krebs solution and then incubated with different doses of GLB (25–75 μmol l^{-1}) before the NaHS-induced relaxation. In addition, the strips were incubated with different doses of PPG (25–75 μmol l^{-1}) or different doses of HA (25–75 μmol l^{-1}) before the *L*-Cys-induced relaxation. GLB, 50 μmol l^{-1} and 75 μmol l^{-1}, attenuated the NaHS (10^{-3} mol l^{-1}) relaxation effects on the human and SD rat bladder strips significantly (P=0.0165, P=0.0061, P=0.0089 and P=0.0066, respectively) (**Figure 3a and 3d**). PPG at 75 μmol l^{-1} attenuated the *L*-Cys (10^{-3} mol l^{-1}) relaxation on the human and SD rat bladder strips significantly (P=0.0315 and P=0.0403, respectively) (**Figure 3b and 3e**), but HA decreased the relaxation effect of *L*-Cys on the strips in both species (**Figure 3c and 3f**).

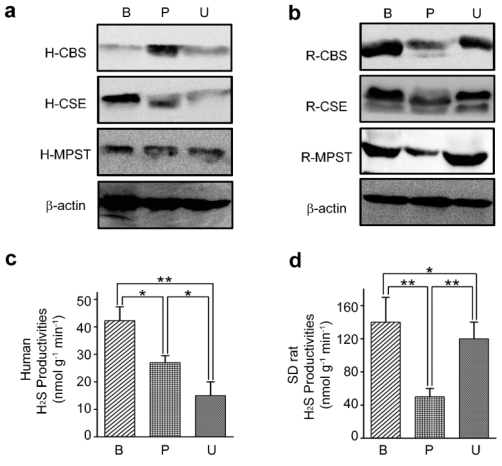

Figure 2 Protein expression levels of CBS, CSE and MPST varied in normal human and SD rat bladder, prostate and urethra tissues (**a** and **b**), which was confirmed by the H_2S productivity analysis (**c** and **d**). The relative protein expression levels were calculated by normalisation to the GAPDH expression level. The H_2S productivity results are expressed as the means±s.d. (*P<0.05 and **P<0.01). CBS, cystathionine β-synthase; CSE, cystathionine γ lyas; MPST, 3-mercaptopyruvate sulphur transferase.

DISCUSSION

Recent studies showed that H_2S and its synthases CBS and CSE existed in human bladder tissue and contributed to the relaxing effect of sildenafil on the human dome of the detrusor, which was independent of the traditional adrenergic and cholinergic receptor to some extent.[10,16,18,19] Given that dysfunction of the bladder detrusor partially contributes to LUTS, confirming and further exploring the expression and function of this newly gaseous signal in urination might be worthwhile. Our study demonstrated that not only CBS and CSE but also MPST were present in human and rat bladder tissue. Moreover, there were different expression patterns of H_2S and its synthases in the bladder, prostate, and urethra tissues between human and SD rat. In the present study, we observed that exogenous and endogenous H_2S could relax acetylcholine chloride-induced bladder strips.

Previous studies showed that exogenous H_2S could elicit various responses in rat and trout bladder with different pre-treatments.[20–22] We observed in the midst of H_2S, the relaxation of smooth muscle pre-contracted with acetylcholine chloride. Streng *et al.*[20] investigated the transient receptor potential vanilloid receptor 1 and showed that it might be involved in the contraction induced by NaHS when rat bladder strips were electrically stimulated. Patacchini *et al.*[21] reported

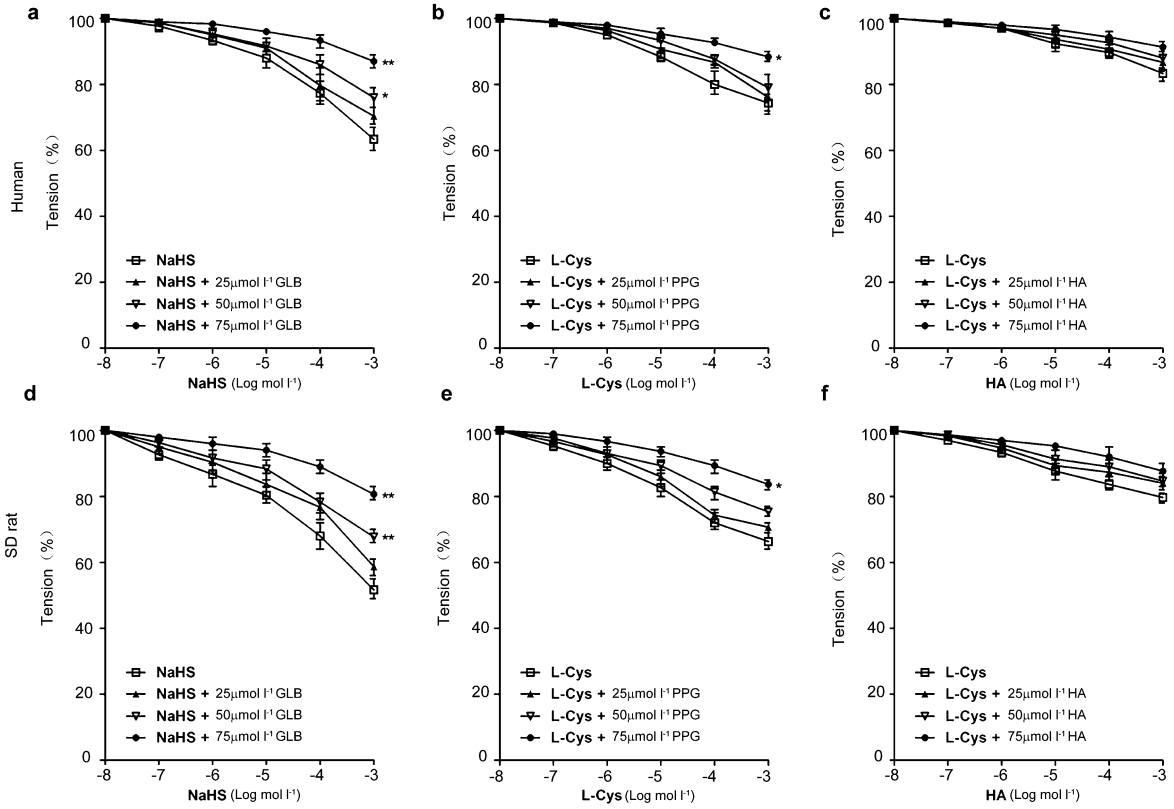

Figure 3 H_2S-induced relaxation on human and SD rat bladder strips was blocked by GLB, PPG and HA, as shown in NaHS-relaxed human (**a**) and rat (**d**) bladder strips, in a dose-dependent manner. PPG attenuated the *L*-Cys-induced relaxation of strips of human (**b**) and rat (**e**) bladder in a dose-dependent manner. HA diminished *L*-Cys-induced relaxation of strips of human (**c**) and rat bladder in a dose-dependent manner (**f**). All data are expressed as the means±s.d. (**P*<0.05 and ***P*<0.01). GLB, glibenclamide; HA, hydroxylamine; *L*-Cys, *L*-cysteine; PPG, DL-propargylglycine; SD, Sprague–Dawley.

that intravesical NaHS instillation increased protamine sulphate pre-treated rat bladder pressure. Dombkowski *et al.*[22] explored the H_2S effects on trout bladder smooth muscle and suggested that H_2S could produce a dose-dependent relaxation in unstimulated and carbachol pre-contracted bladders and inhibit spontaneous contractions. KCl, adrenergic and cholinergic agonists would usually be chosen to pre-contract smooth muscle in not only LUT but also cardiovascular vessels, gastrointestinal tract, and corpus cavernosum tissues.[2,5,6,7,23,24] Taken together, we proposed that the effect of H_2S on human and rat bladder smooth muscle tone might play various roles, depending on different pretreatments.[20,21] In our study, exogenous and endogenous H_2S relaxed smooth muscle that was pre-contracted with acetylcholine chloride. This effect was diminished by GLB, PPG and HA. HA is a specific CBS inhibitor that blocks the biosynthesis of H_2S *de novo* prior to CSE and MPST,[25] which might explain why HA produces a strong effect on restraining the endogenous biosynthesis of H_2S.

H_2S has been reported to be an endogenous activator targeting K_{ATP} channels and relaxing vascular smooth muscle mainly *via* opening the K_{ATP} channels on the membrane of muscular cell, whereas this effect could be attenuated by the K_{ATP} channel blocker GLB.[19] Although endogenous H_2S and its synthases have only been studied in human bladder tissue, K_{ATP} channels have been postulated to affect detrusor function since the 1980s.[10,26] Numerous studies showed that there are different K_{ATP} channels present in the human bladder muscular layers.[27] Our study further proved the expression and function of H_2S and its synthases CBS, CSE and MPST in human and rat bladder, prostate and urethra, which reinforced and complemented the study of Fusco *et al.*[10] Although the expression and function of H_2S in human LUT tissue have been studied widely, the development of new drugs based on H_2S or its synthases for the treatment of dysfunctional detrusor remains a challenge.

In summary, we demonstrated that endogenous H_2S and its synthases CBS, CSE and MPST are present in human and rat LUT tissue and that it modulated the tone of human and rat bladder strips. Our results indicated that H_2S and its synthases may be a newer therapeutic target to treat LUTS induced by undesired bladder contractions, such as OAB.

AUTHOR CONTRIBUTIONS

JWG participated in the design of the study, carried out the studies and drafted the manuscript. WW, HG, ML, XCW and LZ carried out the studies. YXX, ZCX and JJ participated in the design of the study. All authors read and approved the final manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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