Increased \( \text{H}_2\text{S} \) and its synthases in urothelial cell carcinoma of the bladder, and enhanced cisplatin-induced apoptosis following \( \text{H}_2\text{S} \) inhibition in EJ cells

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Abstract. \( \text{H}_2\text{S} \), synthesized by cystathionine \( \beta \)-synthase (CBS), cystathionine \( \gamma \)-lyase (CSE) and 3-mercaptoppyruvate sulfurtransferase (MPST), functions as a signalling molecule in mammalian cells. \( \text{H}_2\text{S} \) serves complex functions in physiological and pathological processes, including in bladder cancer. In the present study, \( \text{H}_2\text{S} \) production, the expression of the associated enzymes and the effect of \( \text{H}_2\text{S} \) on human urothelial cell carcinoma of the bladder (UCB) tissue and cell lines were evaluated, and whether decreasing \( \text{H}_2\text{S} \) levels influenced cell viability and tumour growth following treatment with cisplatin (CDDP) was assessed in UCB cells \textit{in vitro} and \textit{in vivo}. \( \text{H}_2\text{S} \) production and the expression of CBS, CSE and MPST in bladder tissue specimens and the UCB cell lines 5637, EJ and UM-UC-3 were analysed using a sulfur-sensitive electrode and western blotting. UCB cells were subjected to different treatments, and viability and protein expression were determined. \( \text{H}_2\text{S} \) production was inhibited to examine its influence on EJ cell tumour growth following CDDP treatment \textit{in vitro}. It was identified that CBS, CSE and MPST protein were up-regulated in UCB tissues and cells. The \( \text{H}_2\text{S} \) production and enzyme expression levels were the highest in UCB tissue and EJ cells.

The inhibition of endogenous \( \text{H}_2\text{S} \) biosynthesis decreased EJ cell viability and tumour growth in response to CDDP treatment. \( \text{H}_2\text{S} \) levels and the associated biosynthetic enzymes were increased in human UCB tissue and cells compared with adjacent tissue and normal cells, which may have increased the resistance to CDDP-induced apoptosis in UCB. Therefore, \( \text{H}_2\text{S} \) and its production may be an alternative therapeutic target for UCB.

Introduction

\( \text{H}_2\text{S} \), a signalling molecule in mammals and other taxa, is synthesized from L-cysteine by cystathionine \( \beta \)-synthase (CBS), cystathionine \( \gamma \)-lyase (CSE) and 3-mercaptoppyruvate sulfurtransferase (MPST) through one-carbon metabolism and the transsulfuration pathway (1,2). Endogenous \( \text{H}_2\text{S} \) and/or the associated enzymes have been observed to participate in a range of physiological and pathological processes, including vasodilation, smooth muscle relaxation, inflammation and tumorigenesis (3-7). The expression and activity levels of these enzymes in human urothelial cell carcinoma of the bladder (UCB) tissues and cell lines were determined in our previous study (8).

\( \text{H}_2\text{S} \) may exert various effects in disease through the activation or inhibition of ion channels, particularly through thiol groups, including metallothionein, thioredoxin, disulfide and, most importantly, glutathione (GSH) (9). GSH maintains a redox balance in cells by directly scavenging free radicals, including reactive oxygen species (ROS) and reactive nitrogen species (RNS), and by functioning as a cofactor for protective enzymes to decrease oxidative stress (10,11). Accordingly, there are a substantial number of studies regarding the association between UCB carcinogenesis and the aberrant activation of cellular signals or redox status, as reviewed by Wallerand \textit{et al} (12). Previous studies have revealed that \( \text{H}_2\text{S} \) is able to interact directly with free radicals and modulate oxidative stress to induce the activation of a range of tumorigenic pathways (13,14).

A number of studies have identified one-carbon metabolism and transsulfuration pathways, as well as variations of these pathways that may increase the risk of UCB (15,16).
These results have indicated H₂S as a target for the development of modulating agents for treatment, diagnosis and prognosis for urology oncologists (2). The aim of the present study was to assess CBS, CSE and MPST expression levels, and H₂S production, in human UCB tissue and cells, and to examine their functions in carcinogenesis. Owing to the lack of MPST-specific inhibitors, and as the homocysteine metabolism is affected by the CBS-specific inhibitors aminooxyacetic acid and hydroxylamine (1,2,15,16), a CSE-specific inhibitor was used to modulate H₂S biosynthesis in the present study.

Materials and methods

Tissue samples. Human UCB tumour specimens were obtained from 27 male patients that had received transurethral resection or radical cystectomy for UBC (mean age, 58.6 years; range, 51-70 years), and normal bladder tissue samples from 7 male patients that had received ureteral reimplantation or cystoscopic biopsy (mean age, 56.4 years; range, 47-68 years) at Chao Yang Hospital (Beijing, China), between August 2014 and March 2016. The present study was approved by Beijing Chao Yang Hospital's institutional research ethics board, including the use of human samples and animal experiments (approval no. AN-1405-002-100). Written informed consent was obtained from all patients enrolled in the present study.

All samples were confirmed and staged by two independent experienced pathologists according to the tumor-node-metastasis system (17). Table I lists the clinical and pathological characteristics of the enrolled patients with UCB. A total of 27 UCB tumour samples were used for western blot analysis, and 23 UCB tumor samples were used for determination of H₂S production; the normal bladder tissue samples were used as controls and examined for protein levels and H₂S production. Specimens analysed by western blotting were divided into three groups: ‘Norm’ group for the 7 normal bladder samples; non-muscle-invasive bladder cancer (NMIBC) group for 15 samples (stage Ta/T1); and muscle-invasive bladder cancer (MIBC) group for 12 samples (≥T2). Specimens analysed for the determination of H₂S production were also divided into the three groups as above, but with 11 samples in the NMIBC group.

Cell culture and reagents. The human high-grade UCB cell lines 5637, EJ and UM-UC-3 were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Beijing, China) and were maintained in a 37°C humidified incubator with 5% CO₂ and 95% O₂. The immortalized human normal bladder urothelium cell line SV-HUC-1 was cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (HyClone; GE Healthcare Life Sciences, Logan, UT, USA). The 5637, UM-UC-3 and EJ cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (both HyClone; GE Healthcare Life Sciences), 100 U penicillin G and 100 µg streptomycin (Lonza Group, Ltd., Basel, Switzerland).

Although the EJ cell line is reported to be contaminated, it is a derivative of T24 cells, which were also extracted from a bladder carcinoma. Therefore, this contamination issue was considered unlikely to affect the outcomes of the present study (18,19).

PBS, diaminobenzidine (DAB), EDTA, DAPI, L-cysteine, NaOH, cisplatin (DDDP), DL-propargylglycine (PAG) and NaHS were purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany).

Immunofluorescence. Immunohistochemical staining for CBS, CSE and MPST was performed with paraffin-embedded MIBC tissue sections, and SV-HUC-1 or EJ cell slides. PBS containing 0.2% Triton X-100 and 0.1% goat serum albumin (Santa Cruz Biotechnologies, Inc., Dallas, TX, USA) was used to preincubate 5-µm-thick sections for 30 min at room temperature, and the samples were boiled in 0.01% (w/v) EDTA (cat. no. sc-29092; Santa Cruz Biotechnology, Inc.) for 10 min in the microwave. Cells grown on coverslips were rinsed with PBS and fixed with 4% paraformaldehyde for 30 min, followed by a 30-min pre-incubation with 0.5% Triton X-100 (Nanjing Keygen Biotech Co., Ltd., Nanjing, China) in PBS buffer at 4°C. Primary antibodies against CBS (cat. no. H00000875-M02), CSE (cat. no. H0054414-M; diluted 1:200; Abnova, Taipei, Taiwan) and MPST (cat. no. sc-376168; dilution, 1:100; Santa Cruz Biotechnology, Inc.) were incubated with the tissue sections and cells on slides overnight at 4°C. The samples were rinsed twice with PBS containing 0.1% Triton X-100 (PBST; Nanjing Keygen Biotech Co., Ltd., Nanjing, China) followed by incubation for 1 h with a horseradish peroxidase (HRP)-conjugated goat IgG secondary antibody (cat. no. sc-2354; dilution, 1:100; Santa Cruz Biotechnology, Inc.) at room temperature. The cells on slides were washed twice with PBS, followed by incubation with a goat-anti-mouse secondary antibody conjugated to fluorescein isothiocyanate (cat. no. sc-2356; dilution, 1:50; Santa Cruz Biotechnology, Inc.) for 1 h at 37°C. Finally, the samples were stained with ≥98% (HPLC and TLC) DAPI (cat. no. D9542; Sigma-Aldrich; Merck KGaA) for 5 min at room temperature. Images were captured using a confocal microscope (Olympus Corporation, Tokyo, Japan; magnification x100).

Western blot assay. Human bladder tissue and cells were lysed in lysis buffer (Nanjing Keygen Biotech Co. Ltd.), and protein concentrations were determined with Bradford's method. A total of 50 µg protein from each sample was separated by 12% SDS-PAGE (Bio-Rad Laboratories, Inc. Hercules, CA, USA) and transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Inc.).

Fat-free milk powder (5%) was used to block the membranes in PBS for 60 min at room temperature, which were then incubated with the following antibodies: Monoclonal mouse anti-human CBS and CSE (both diluted 1:1,000; Abnova); polyclonal rabbit anti-human MPST (dilution, 1:500; Santa Cruz Biotechnology, Inc.), extracellular-signal-regulated kinase 1/2 (Erk1/2; cat. no. V114A), phosphorylated (p)-Erk1/2 (cat. no. 9101S), cleaved poly(ADP-ribose) polymerase (PARP) p85 (cat. no. G734A) (all diluted 1:1,000; Promega Corporation, Madison, WI, USA), B-cell lymphoma 2 (Bcl-2; cat. no. 3498S), Bcl-2-like 1 (Bcl-XL; cat. no. 2762S), Bcl-2-associated X (Bax; cat. no. 2774S), Bcl-2-associated agonist of cell death (Bad; cat. no. 9292S) and GAPDH (cat. no. 8884S) (all diluted 1:1,000; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. Following primary antibody incubation, the membranes were incubated with a horseradish peroxidase-conjugated goat IgG secondary antibody (cat. no. sc-2354; dilution, 1:2,000;
Table I. Clinical and pathological characteristics of the patients with urothelial cell carcinoma of the bladder.

| Characteristic     | Value          |
|-------------------|----------------|
| Sex, n            |                |
| Male              | 27             |
| Female            | 0              |
| Age, years (mean ± standard deviation) | 58.6±9.8 |
| Grade, n          |                |
| 1                 | 11             |
| 2                 | 9              |
| 3                 | 7              |
| Pathological stage, n |       |
| Ta                | 4              |
| T1                | 11             |
| T2                | 7              |
| T3                | 4              |
| T4                | 1              |

T, tumor.

Santa Cruz Biotechnology, Inc.). Enhanced chemiluminescence reagent (Pharmacia Biotech; GE Healthcare, Chicago, IL, USA) was used to detect signals, and a Kodak Image Station (Kodak, Rochester, NY, USA) was used for analysis and recording.

Determination of H$_2$S production. Determination of H$_2$S production was performed using a previously described method (20). Briefly, a 10-fold volume (w/v) of ice-cold potassium phosphate buffer (pH 6.8) was used to homogenize tissues and cells. Reactions were performed in custom-made glass chambers 1 cm in diameter and 2 cm in height in a Pyrex Erlenmeyer flask. Cryovial test tubes (size, 2 ml) containing 0.5 ml 1 M sodium hydroxide were inserted into the chambers. A mixture of 500 µl cell homogenate with 500 µl 50 mmol/l (pH 6.8) PBS and 1 ml reaction system solution [100 mmol/l (pH 7.4) PBS, 10 mmol/l L-cysteine and 2 mmol/l phosphate pyridoxine aldehyde] was prepared. The protein concentration of the sample was determined, and 2 ml mixed solution was transferred to the outer area of the flask. NaOH (1 mol/l) was added to the chamber of the flask, which was incubated for 90 min at 37˚C in a water bath. At the end of the reaction, 1 ml 50% trichloroacetic acid was added. The flask was incubated at 37˚C for 60 min, and the contents of the central chambers were transferred to a 12-well cell culture plate (Corning Incorporated, Corning, NY, USA) containing 1 ml antioxidant solution. A sulfide-sensitive electrode (PXS-270; Shanghai INESA Auto Electronic System Co., Ltd., Shanghai, China) was employed to determine the H$_2$S concentration of the solution using a standard curve. The H$_2$S activities are expressed in nmol/min x mg, adjusted to the protein concentration of the corresponding samples.

Cell viability test. Cells were seeded in 96-well plates (10,000 cells/well) and incubated at 37˚C for 24 h to reach 50-60% confluence. The cells were treated with CDDP (5 µg/l), CDDP + PAG (10 or 100 µM) or CDDP + NaHS (10 or 100 µM), incubated for 72 h, and then harvested for further analysis. Cell viability was assessed using the CellTiter 96 Aqueous Assay kit (Promega Corporation), according to the manufacturer's protocol, at different time intervals using a multiwell spectrophotometer (Bio-Rad Laboratories, Inc.).

Tumorigenesis assay. EJ cells (1x10$^7$ cells/mouse) were injected subcutaneously into the axillary area of 6-week-old male BALB/c-nu mice (10 mice per treatment), which were purchased from the Experimental Animal Center of Peking University Health Science (Haidian, China); the average weight of mice was 20.0±0.9 g. Mice had access to food and water ad libitum, and were kept at a temperature of 20-26˚C, a humidity of 30-70% and in a 12 h light/12 h dark cycle. CDDP (5 mg/kg), CDDP (5 mg/kg) + PAG (100 µM) or CDDP (5 mg/kg) + NaHS (100 µM) were injected into the abdominal cavity. Following sacrifice at intervals of 4 days, the tumour volumes were determined using the equation: Length x width$^2$ x 0.5. The study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Statistical analysis. A Student's t-test was performed to compare differences 2 groups. One-way analysis of variance followed by and Dunnnett's test was used to evaluate the statistical significance between ≥3 groups. Data are expressed as the mean ± standard deviation. Statistical analysis was performed using SPSS (version 17.0; SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to represent a statistically significant difference.

Results

Immunofluorescence and western blot analyses of CBS, CSE and MPST in tissue and cells. As determined by western blotting, the bladder tissue samples exhibited increasing CBS, CSE and MPST protein expression from normal tissue to NMIBC to MIBC (Fig. 1A-C). The protein levels for all three H$_2$S-associated enzymes in UCB tissue were increased compared with those in normal controls. However, between the NMIBC and the MIBC groups, the only significant difference was in the CSE level (P=0.023; Fig. 1B). The UCB sections (Fig. 1D) exhibited moderate to strong immunoreactivity for CBS, CSE and MPST. The highest expression among the cells was observed in EJ cells (Fig. 2). SV-HUC-1 normal bladder urothelium cells exhibited low to moderate immunoreactivity for CBS, CSE and MPST (Fig. 2A), whereas our previous study demonstrated moderate to strong immunoreactivity in EJ cells (Fig. 2A) (8).

H$_2$S productivity rate in bladder tissue and cells. The H$_2$S productivity rate was determined for bladder tissue samples and cells. UCB tissues exhibited increased H$_2$S productivity compared with in the normal control samples (normal samples vs. NMIBC, P=0.035; normal samples vs. MIBC, P=0.007); the different UCB groups also differed significantly (NMIBC vs. MIBC, P=0.021; Fig. 1E). In addition, decreased H$_2$S productivity was detected in the normal human urinary tract epithelial cell line SV-HUC-1 compared with the bladder
cancer cell lines derived from low-grade (5637) and invasive transitional (UM-UC-3 and EJ) cell carcinoma lines. To investigate the mechanism by which H$_2$S contributes to bladder cancer malignancy, EJ cells were employed as a model, as the rate of H$_2$S production was the highest in these cells (P=0.003; Fig. 2C). **P<0.01 vs. SV-HUC-1 cells. CBS, cystathionine β-synthase; CSE, cystathionine γ-lyase; MPST, 3-mercaptopyruvate sulfurtransferase; UCB, urothelial cell carcinoma of the bladder; NMIBC, non-muscle-invasive bladder cancer; MIBC, muscle-invasive bladder cancer; Norm, normal; T, tumor.

$H_2S$ levels affect CDDP cytotoxicity in EJ cells. The effects of altered H$_2$S levels on the cell viability following treatment with CDDP in EJ cells are presented in Fig. 3. Although an endogenous H$_2$S synthase CSE inhibitor (PAG) and exogenous H$_2$S donor (NaHS) alone did not induce any alteration in the viability of the EJ cells, treatment with PAG or NaHS affected the CDDP cytotoxicity (Fig. 3A and B). Increased H$_2$S levels using NaHS activated p-Erk1/2, Bcl-2 and Bcl-xL expression in CDDP-treated EJ cells, and down-regulated levels of Bax, Bad and cleaved PARP (Fig. 3C). By contrast, the endogenous H$_2$S synthase CSE inhibitor PAG up-regulated the expression...
of Bax, Bad and cleaved PARP, and decreased the levels of Bcl-2, Bcl-xL and p-Erk1/2 (Fig. 3C). The Erk1/2 expression level was unaffected by all treatments.

Combining CDDP with PAG inhibits UCB growth. CDDP + PAG treatment (tumor volume, 721.13±21.41 mm³; P=0.0019) and CDDP alone treatment (tumor volume, 768.64±29.06 mm³; P=0.0086) were associated with the significant suppression of EJ cell tumorigenesis compared with those derived from the control group (tumor volume, 1,112.02±52.13 mm³). CDDP + NaHS (tumor volume, 952.46±59.87 mm³; P=0.021) led to a moderate suppression of EJ cell tumorigenesis (Fig. 4A and B).

Discussion

Our previous study identified that endogenous H₂S, and the associated enzymes CBS, CSE and MPST, are highly expressed in human UCB tissues and cell lines (8), which is consistent with a number of other studies (1,2,7,9,21). To
the best of our knowledge, the present study is the first to demonstrate that increased levels of H$_2$S exert cytoprotective effects on UCB cells treated with CDDP. Furthermore, these results demonstrate that the H$_2$S production and CSE expression levels were significantly different between the NMIBC and MIBC groups. This result suggests that H$_2$S production and CSE expression may serve as biomarkers for urologists to determine a prognosis. Although addition of the H$_2$S donor NaHS or the H$_2$S synthase inhibitor PAG did not result in changes to EJ cell viability, altered H$_2$S levels affected the viability of CDDP-treated EJ cells. The cytotoxicity of CDDP in EJ cells was mitigated by high levels of H$_2$S or its biosynthetic enzymes, which appeared to involve the activation of the Erk1/2 signalling pathway and interruption of the intrinsic mitochondrial apoptosis pathway. We hypothesise that H$_2$S participates in additional cellular events in the carcinogenesis of the urothelium, which warrants further studies.

H$_2$S counteracted the cytotoxicity of CDDP in UCB cells, which is of considerable interest as altering the H$_2$S level alone did not elicit any effect on cell viability in the present study. GSH, the most important scavenger of ROS and RNS in the human body, and a widely studied molecule, is a product of the H$_2$S biosynthesis pathway (1,10,11,22). The catenation and interchalcon bond formation between H$_2$S and thiol-containing substances may facilitate the metabolism and recycling of thiol compounds, including those containing disulfide bridges in cellular redox signalling, and GSH (9,22); GSH is up-regulated to serve complex, although controversial, functions in UCB (23,24). Previous studies have identified that H$_2$S is able to stimulate GSH synthesis (9) and that the anomalous expression of GSH-associated enzymes, including glutathione synthetases and $\gamma$-glutamylcysteine synthetase, is involved in tumorigenesis and chemoresistance in UCB (10,11,23-26). Therefore, up-regulated H$_2$S biosynthesis in UCB may serve similar functions to those of GSH in UCB. Another previous study indicated that PAG enhanced the effect of CDDP on bladder tumours in a murine model (27).

The increased expression of CBS, CSE and MPST in human UCB raises questions regarding the function of the level of H$_2$S in urothelial carcinogenesis. H$_2$S interacts with nitric oxide (NO) and carbon monoxide (CO), which together create a complex network that contributes to inflammation and carcinogenesis (2,14). NO and CO have been demonstrated to participate in oxidation-reduction processes, and to promote angiogenesis via cGMP-mediated or non-cGMP-mediated pathways, similar to vascular endothelial growth factor (VEGF) and hypoxia-inducible factor-1$\alpha$ (HIF-1$\alpha$), in UCB (28,29). These results suggest a potential function for H$_2$S in angiogenesis through cross-talk between these gaseous molecules in UCB. Accumulating evidence indicates that H$_2$S acts on ion channels, including ATP-sensitive potassium channels (9,14). Additionally, H$_2$S functions in signal transduction, including in the mitogen-activated protein kinase, VEGF, insulin-like growth factor, phosphoinositide 3-kinase/protein kinase B, nuclear factor-xB, signal transducer and activator of transcription 3, nuclear factor erythroid-derived 2-related factor 2 and HIF-1$\alpha$ signalling pathways (2,29-31). Given that disrupted cell signalling contributes to the initiation of UCB (12,32), the possible interaction between an increase in H$_2$S and the activation of aberrant cellular signals in UCB is plausible and warrants further research.

Genetic analysis of the function of one-carbon metabolism and transsulfuration pathways in bladder cancer has produced notable results. For example, several CSE single-nucleotide polymorphisms (SNPs) may be associated with an increased risk of bladder cancer (15,16). SNPs may lead to abnormal transcription and translation, affecting the expression or function of the encoded protein (33). Although SNPs in the CSE gene in patients with bladder cancer have been identified, analysis of the CSE mRNA and protein expression levels and catalytic activity has not been reported. Therefore, it is not possible to compare between these previous studies and the present study. Nevertheless, the present study may provide a clue regarding how alterations in H$_2$S-associated enzymes may contribute to UCB tumorigenesis.

The endogenous signalling molecule H$_2$S and its associated biosynthetic enzymes CBS, CSE and MPST are expressed at increased levels in human UCB, including in bladder tumour tissue and cell lines. However, clinical trials of the approach reported in the present study are not possible at present, as the drugs that inhibit H$_2$S production are not suitable for use in the clinic. Regardless, the inhibition of H$_2$S levels enhanced CDDP-induced apoptosis in UCB cells in vitro, and this may represent a new therapeutic target or diagnostic marker for UCB.

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Availability of data and materials

The analysed datasets generated during the study are available from the corresponding author, on reasonable request.

Authors' contributions

NX had full access to all the data in the study and is responsible for the integrity of the data and the accuracy of the data analysis. WW and JG were major contributors in writing the methods and statistical analysis. JG and FY performed data acquisition. MW and FY analysed and interpreted data. All authors have read and approved the manuscript.

Ethics approval and consent to participate

The present study was approved by the Beijing Chao Yang Hospital's institutional research ethics board, including the use of human samples and animal experiments (protocol no.
AN-1405-002-100). Informed consent was obtained from all patients enrolled in the study.

Consent for publication

The study was performed with the patients' informed consent for publication.

Competing interests

The authors declare that they have no competing interests.

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