Hydrogen sulfide inhibits epithelial-mesenchymal transition in peritoneal mesothelial cells

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Peritoneal fibrosis (PS) determines the long-term outcome of peritoneal dialysis (PD). We previously confirmed that hydrogen sulfide (H2S) inhibited PS, but its cellular mechanism was not fully elucidated. Epithelial-mesenchymal transition (EMT) of mesothelial cells (MCs) is an important cellular event of PS, we therefore investigated whether EMT can be affected by H2S in MCs. Rats were treated with 4.25% glucose PD fluids plus lipopolysaccharide for 28 days to produce PS, and NaHS (56 μg/kg.d) was given simultaneously. NaHS (56 μg/kg.d) reduced the deposition of collagen in the submesothelial zone compared with the PS group. In primarily cultured rat MCs, 4.25% -glucose PD fluid induced EMT in MCs featured as loss of ZO-1 and Cytokeratin, and increase of α-SMA, plasminogen activator inhibitor 1, fibronectin and TGF-β1 proteins. PD fluid also increased IL-6 and monocyte chemotactic protein-1 mRNA expressions as well as the phosphorylation of Smad2/3 and Smad3. NaHS (50–300 μmol/L) reversed the above alterations with the optimal dose at 100 μmol/L. Thus, exogenous H2S improves PS by inhibiting EMT in MCs. The anti-EMT effect of H2S is associated with the inhibition of inflammation and TGF-β1-Smad signal pathway.

Peritoneal fibrosis induced by the chronic stimulation of high glucose peritoneal dialysis fluid and frequent peritonitis is a major cause of ultrafiltration failure of peritoneal dialysis (PD)1. The pathological characteristic of peritoneal fibrosis consists of the loss of mesothelial cells (MCs), neovascularization, thickened submesothelial zone and the presence of myofibroblasts2. Interventions against these histological features are believed to ameliorate peritoneal fibrosis and improve the long term outcome of PD patients. However, effective treatments of peritoneal fibrosis are still limited.

Hydrogen sulfide (H2S) is the third endogenous gasotransmitter compared to carbon monoxide and nitric oxide3. The decreased plasma level of H2S in various fibrosis diseases provides the rationale of supplement of H2S in treating organ fibrosis4. Our previous work has confirmed that NaHS, a H2S donor inhibited the deposition of collagen fibers, inflammation and angiogenesis in the peritoneum of a chronic peritonitis rat model5, but the cellular mechanisms of H2S on peritoneal fibrosis has not been fully understood.

For the last twenty years, epithelial-mesenchymal transition (EMT) of peritoneal mesothelial cells has been used to explain the loss of MCs and the occurrence of myofibroblasts during peritoneal fibrosis6. Conventional PD fluids can stimulate the MCs to undergo EMT characterized by the disassembly of cellular tight junctions, increase of mesenchymal markers and the ability of invasion. As H2S is able to ameliorate peritoneal fibrosis, we hypothesize that H2S can inhibit EMT of MCs during peritoneal fibrosis. In this study, we examined the effect of H2S on EMT induced by 4.25% peritoneal dialysis fluid in primarily cultured rat MCs. The potential mechanisms of the anti-EMT effect of H2S were also explored.

Results

H2S reduced peritoneal fibrosis induced by chronic peritonitis in rats receiving PD. Masson-trichrome staining was used to assess the area of peritoneal fibrosis. Compared with the control group, injection with 4.25% peritoneal dialysate plus LPS considerably increased the amount of collagen (blue area) in the rats. Administration of NaHS (56 μg/kg.d) reduced the thickness of collagen fibers in the PD rats (Fig. 1).

Identification of primarily cultured rat peritoneal mesothelial cells. Primarily cultured cells of the third passage exhibited a polygonal cobblestone-like appearance. The cells expressed both epithelial marker
(cytokeratin) and mesenchymal marker (vimentin) (green color) indicating that these cells were peritoneal mesothelial cells (Fig. 2).

**H₂S alleviates the loss of epithelial markers induced by 4.25% peritoneal dialysate in peritoneal mesothelial cells.** Primarily cultured peritoneal mesothelial cells (RPMCs) were pretreated with various concentrations of NaHS (50–300 μmol/L) for 30 mins, followed by incubation with 4.25% glucose peritoneal dialysate solutions (PDFs) for 24 hours. Compared with the control group, 4.25% PDFs significantly decreased the expressions of ZO-1 and cytokeratin in RPMCs. Incubation with NaHS (50–300 μmol/L) reversed the downregulation of ZO-1 and cytokeratin induced by the 4.25% PDFs, with the optimal effect in the 100 μmol/L NaHS group (Fig. 3).

**H₂S decreases mesenchymal markers and profibrotic factors activated by 4.25% PDFs in RPMC.** After treatment with NaHS (50–300 μmol/L) for 30 mins, RPMCs were exposed to 4.25% glucose PDFs for 24 hours. Incubation with 4.25% PDFs increased the expressions of mesenchymal markers including α-SMA and profibrotic factors including PAI-1, fibronectin (FN) and TGF-β1, which were decreased by pretreatment with NaHS, especially in the 100 μmol/L NaHS group (Fig. 4).
**H₂S suppress the TGF-β₁ signal pathway activated by 4.25% PDFs in RPMCs.** RPMCs were treated with various concentrations of NaHS (50–300 μmol/L) for 30 mins, then exposed to the mixture of 4.25% glucose PDFs and culture medium by 1:1 for 1 h. 4.25% glucose PDFs stimulated the phosphorylation of Smad 3 and Smad 2/3, which were decreased by the addition of 50–300 μmol/L NaHS (Fig. 5).
H₂S reduces the inflammatory cytokines activated by 4.25% PDFs in RPMC. RPMCs were pretreated with or without NaHS (50, 100, 300 μmol/L) for 30 min. Cells were then treated with 4.25% glucose PDFs in culture medium (1:1) for 6 h. The cellular inflammatory cytokines including IL-6 and monocyte chemoattractant protein-1 (MCP-1) were determined by real-time PCR. IL-6 and MCP-1 were increased by the stimulation with 4.25% glucose PDFs, but were decreased after pre-treatment with NaHS. The optimal effect of NaHS on cytokines was observed at 100 μmol/L (Fig. 6).

Discussion
The present study demonstrated that high glucose peritoneal solution induced epithelial-mesenchymal transition (EMT) in primarily cultured peritoneal mesothelial cells. Administration with exogenous H₂S is able to reverse EMT in peritoneal mesothelial cells by inhibiting the TGF-β₁-smad3 signal pathway and the production of inflammatory cytokines including IL-6 and MCP-1. To the best of our knowledge, this is the first work to confirm the inhibitory effect of EMT of H₂S in peritoneal mesothelial cells.

Although a recent study using genetic labeling technique indicated that submesothelial fibroblasts were the major source of myofibroblast in peritoneal fibrosis, the potential role of EMT in peritoneal fibrosis could not be totally excluded. Animal and clinical studies have confirmed in situ evidence of EMT and interventions aiming at EMT also improved peritoneal fibrosis. Consistent with a previous study, our data showed that high glucose peritoneal dialysis fluid stimulated the mesothelial cells to undergo EMT characterized by the loss of epithelial tight junction molecules such as ZO-1 and cytokeratin as well as the increase of myofibroblast marker of α-SMA. Exogenous NaHS reversed such a phenotype shift in the mesothelial cells. NaHS also decreased the release of profibrotic factor including PAI-1 and TGF-β₁ and reduced the production of extracellular matrix protein including fibronectin. Although 300 μmol/L NaHS was not toxic to the mesothelial cells and effective in alleviating the EMT process in our study, the most effective dose of NaHS against the differentiation of mesothelial cells to myofibroblast was 100 μmol/L, which has been thought to produce a physiologically concentration of H₂S in many previous studies. Such a finding is also consistent with our previous work that relatively small dose of NaHS is preferable in the treatment of renal fibrosis in UUO animal model.

Peritoneal inflammation plays an important role in mesothelial cell EMT. It is believed that EMT is a pathological event responding to trauma and inflammatory insult. Numerous studies have confirmed that H₂S is able to inhibit inflammation in multiple organs. We previously found that lower dose of NaHS (5.6–56 μg/kg.d) was able to inhibit inflammation of UUO animal model by suppressing the MAPK signal pathway, while higher dose of NaHS (560 μg/kg.d) aggravated inflammation. The current study demonstrated that NaHS 100 μmol/L was effective to reduce cellular inflammatory cytokines of IL-6 and MCP-1 induced by 4.25% glucose PDFs, indicating that inhibitory effect of H₂S on EMT may contributable to the anti-inflammation property of H₂S.

TGF-β₁ was firstly proven to induce EMT in mammary epithelial cells. Even since then, a number of epithelial cell types such as renal tubular cells and alveolar epithelial cells have been shown to undergo EMT stimulated by TGF-β₁. TGF-β₁ can induce EMT through Smad dependent and independent signal pathway. In the Smad dependent way, TGF-β₁ induces the phosphorylation of Smad proteins including Smad 2 and Smad 3 that control the transcription of many fibrogenic genes of EMT including α-SMA, fibronectin, PAI-1 and MCP-1. Our results demonstrated that high glucose PDFs stimulated the production of TGF-β₁ as well as the phosphorylation of Smad 3 and Smad 2/3, while exogenous H₂S partially inhibited such an effect. These data support that the anti-EMT effect of H₂S in peritoneal mesothelial cells is associated with the inhibition of TGF-β₁-Smad signal pathway.
In conclusion, our data suggested that 4.25% glucose PDFs stimulated EMT of peritoneal mesothelial cells characterized by the loss of cell-cell adhesion protein including ZO-1 and the increase of parenchymal marker of α-SMA. Exogenous H$_2$S inhibited the EMT process induced by high glucose PDFs due to its anti-inflammation property as well as the inhibition of the TGF-β1-Smad signal pathway.

**Materials and Methods**

**Animal.** Eight-week-old male Sprague–Dawley (SD) rats weighing 200–240 g were purchased from Soochow University Laboratory Animal Center, and raised in an environment at 24 ± 2°C with a 12 hours light/dark cycle. All experimental protocols were approved by the Ethics Committee of the Second Affiliated Hospital of Soochow University. Animal experiment conforms to the international guidelines of use and care of laboratory animals.

**Dialysis animal model study.** To achieve peritoneal dialysis rats, 20 ml of 4.25% glucose PD fluid (Baxter International Inc., Chicago, Ill., USA) was administered to SD rats daily for 28 days, with 0.6 mg/kg of LPS (Sigma, St. Louis, Mo., USA) in the PD fluid on days 1, 3, 5, and 7. Rats were randomly divided into four groups: (1) control group (20 ml saline); (2) PD group; (3) PD + NaHS (Sigma, St. Louis, Mo., USA) group: (56 µg/kg/day NaHS daily in PD fluid with LPS); (4) NaHS group (56 µg/kg/day NaHS daily in 20 ml saline). All drugs were given intraperitoneally. On the 28th day, rats were sacrificed and the peritonea of the upper right abdominal wall were collected.

**Histological analysis.** Peritoneal tissue was fixed in 4% paraformaldehyde solution and embedded in paraffin. Sections were made at 4 µm thick, deparaffined and stained using Masson reagents according to the manufacturer’s manual (Solarbio, Perking, China). Sections were then observed under a light microscopy and the amount of collage (blue area) was quantified in five selected fields (200×).

**Cell culture study.** Rat peritoneal mesothelial cells (RPMCs) were isolated and cultured as previously described. Concisely, rat omentum were obtained from male SD rats weighing 130–140 g and digested with 0.125% trypsin- ethylenediamine tetracetic acid (EDTA) for 15 mins in incubator shakers (BiuBard,150 rpm, 7°C). RPMCs were then centrifuged at 1000 rpm for 5 mins and the cell pellets were suspended. The cells were then cultured in DMEM/F12 medium supplemented with 15% fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL Streptomycin (Invitrogen, Carlsbad, CA) and 0.5 ug/ml transferring (Sigma). Cells of the third passage were used for the experiments.

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**Figure 6.** Effect of H$_2$S on TGF-β1 signal pathway activated by 4.25% glucose PDFs in RPMCs. RPMCs were incubated with or without NaHS (50, 100, 300 µmol/L) for 30 min, followed by the stimulation of the mixture of 4.25% glucose PDFs and culture medium by 1:1 for 1 h. (a) Representative images of TGF-β1, phospho-Smad3, phospho-Smad2/3 are presented and (b) relative abundance of these proteins in each group are calculated. Data represent mean ± SD of three independent experiments. **P < 0.01 versus control group; *P < 0.05, **P < 0.01 versus 4.25% glucose PDFs group.
**Immunofluorescence Staining.** In order to identify RPMCs, monolayer cells were cultured on glass coverslips in complete culture medium to 70% confluence. Cells were fixed with 4% paraformaldehyde for 25 mins and permeabilized with 0.2% Triton X-100 for 5 mins. The coverslips were incubated with mouse anti-vimentin antibody or mouse anti-cytokeratin antibody (1:100 dilution; Santa Cruz Biotechnology, Inc., USA) overnight at 4°C. Coverslips were then incubated with second antibody (1:2,000 dilution; KPL, USA) for 1 h at room temperature and counter-stained with DAPI (1:500). Then coverslips were mounted in 80% glycerol in PBS, finally photographed with a Nikon fluorescence photomicroscope.

**Quantitative PCR.** The expression of RNA was evaluated by real-time RT-PCR in RPMCs. Total RNA was isolated from RPMC by the Trizol reagent (Life technologies, USA) according to the manufacturer’s instruction. After reverse transcription, RT-PCR amplification was performed using the SYBR Green Master Mix (Thermo Fisher Scientific). The RT-PCR amplification procedure was constitutive of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 1 min after a 3 min denaturation step at 94°C in a Applied Biosystem PCR System 9700 (Bio-RAD PTC-200, USA). The primer sequences of β-actin, MCP-1 and IL-6 were synthesized by (GenePharma, Shanghai, China). Primer sequences are: forward 5′-GTGCTATGTTGCTGACTTGCG-3′, reverse 5′-ATGCCAAGGATTTCCATACC-3′ (β-actin); forward 5′-CCCCACTGATACGGCTGAG-3′, reverse 5′-GGACTGATCCCATGATTCC-3′ (IL-6); forward 5′-GAGGTGTTCCCAAGAGAAG-3′, reverse 5′-TCAAGGTGCAGTTCC-3′ (MCP-1). Relative abundance of mRNA was normalized by β-actin RNA expression.

**Western Blotting.** A total of 20 μg protein was loaded and separated on 10% SDS-PAGE. After electrophoresis, proteins were transferred to a PVDF membrane and blocked with 5% milk/Tris-buffered saline and Tween-20 buffer. The membranes were incubated with Zo-1 (1:1000), cytokeratin (1:1000), plasmogen activator inhibitor I (PAI-1) (1:1000), fibroconnectin (1:1000), TGF-β1 (1:1000), phosphor-Smad3 (1:500) and phosphor-Smad2/3 (1:500) at 4°C overnight. Afterwards, the membranes were washed and incubated at room temperature for 1 h with the secondary antibody and developed with an ECL kit (Biological Industries, China). The image was captured with the GeneGenius imaging system (Syngene, Cambridge, UK). Band intensity was measured with the Image J software (Bethesda, MD). All primary and secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

**Statistical analysis.** Data are represented as mean ± S.D of three independent experiments. One-way ANOVA analysis was used to determine the variance among multiple groups. P < 0.05 was defined statistically significant.

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Author Contributions
Shengnan Cheng and Ying Lu conducted in vitro experiments. Yuanyuan Li and Luyan Gao finished the animal experiments. Huaying Shen analyzed the data and finished the figures. Kai Song was responsible for the design of the experiments and drafted the manuscript.

Additional Information
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