Original Paper

Exogenous H₂S Inhibits Autophagy in Unilateral Ureteral Obstruction Mouse Renal Tubule Cells by Regulating the ROS-AMPK Signaling Pathway

Qinghai Chen^a Shiliang Yu^a Kuo Zhang^a Zuobiao Zhang^a Chao Li^a
Bingpeng Gao^a Weihua Zhang^b Yan Wang^a

^aDepartment of Urology, The First Clinical Medical School of Harbin Medical University, Harbin,

^bDepartment of Pathophysiology, Harbin Medical University, Harbin, China

Key Words

Hydrogen sulfide • Obstructive nephropathy • Renal epithelial cells • Autophagy • Oxidative stress

Abstract

Background/Aims: The induction of excessive autophagy by increased levels of oxidative stress is one of the main mechanisms underlying unilateral ureteral obstruction (UUO)-induced vascular endothelial cell dysfunction. Hydrogen sulfide (H₂S) has been shown to have an anti-oxidative effect, but its mode of action on excessive autophagy in vascular endothelial cells is unclear. **Methods:** Surgery was used to induce UUO in male C57BL/6 mice as an *in vivo* model. Human renal epithelial cells (HK-2) were treated with H₂O₂ as an *in vitro* model. NaHS was used as an exogenous H₂S donor. Transmission electron microscopy was applied to observe the structure of renal autophagosomes. The expression of proteins related to autophagy and apoptosis was detected by western blot analysis *in vivo* and *in vitro*. Flow cytometry (DCFH-DA) was used to examine the levels of intracellular reactive oxygen species (ROS). The terminal deoxynucleotidyl transferase dUTP nick end labeling assay was used to detect cell apoptosis. Compound C was used to analyze the association of AMPK with autophagy. **Results:** Compared with the sham group, in which the ureter was exposed but not ligated, the cell apoptosis index, number of autophagosomes, protein expression of microtubule-associated protein 1 light-chain 3 (LC3)-II/I, beclin-1, and p-AMPK/AMPK were significantly increased in the UUO group. On the other hand, p62, cystathionine β-synthase, and cystathionine γ-lyase protein expression levels and H₂S concentration were significantly decreased ($p < 0.05$). These alterations were ameliorated by the addition of NaHS ($p < 0.05$). Similar results were observed *in vitro*. By using the AMPK inhibitor compound C, it was indicated that AMPK was involved in ROS-induced autophagy. In addition, using tissue from patients with obstructive nephropathy,

Q. Chen and S. Yu contributed equally to this work.

Yan Wang
and Weihua Zhang

Dept. of Urology, The First Clinical Medical School of Harbin Medical University
Harbin, Heilongjiang 150001 (China)
E-Mail yymnwangyan@126.com; zhangwh116@126.com

excessive autophagy was observed by an increased LC3-II/LC3-I ratio. **Conclusion:** NaHS-treatment may exert a protective effect on mouse kidney against UUO by suppressing the ROS-AMPK pathway. ROS-AMPK-mediated autophagy may represent a promising therapeutic target for obstructive nephropathy.

© 2018 The Author(s)
Published by S. Karger AG, Basel

Introduction

Obstructive nephropathy is characterized by renal dysfunction and an increase of interstitial fibrosis and tubular apoptosis [1]. Histological studies suggest that tubular dilation precedes the inflammatory response and interstitial fibrosis. Obstructive nephropathy is typically associated with an increase of free radicals and/or impaired antioxidant defense, resulting in the overproduction of reactive oxygen species (ROS), which contribute to the onset, progression, and pathological consequences of obstructive nephropathy [2].

Oxidative stress leading to mitochondrial damage and the induction of autophagy-dependent cell death and apoptosis is an important mechanism for tubular decomposition in obstructive nephropathy [3]. Autophagy is a self-degrading process that can be activated under certain circumstances, such as starvation, hypoxia, ischemia/reperfusion, stress, and infection. Under a low level of injury, autophagy precedes apoptosis and plays a role in cell survival by degrading long-lived proteins and subcellular organelles and re-establishing homeostasis [4]. Autophagy plays an important role in regulating cell death. When excessive levels of autophagy are activated due to severe injury, it may be detrimental and responsible for non-apoptotic cell death [5]. Autophagy induced by unilateral ureteral obstruction (UUO) has a renoprotective role in the obstructed kidney at the early stage [6]. Under the presence of persistent UUO, oxidative stress may induce proximal tubule epithelial cell death by the induction of excessive autophagy and apoptosis [7]. Autophagy induced by oxidative stress in proximal tubules does not play a protective mechanism, but contributes to cell death under the presence of persistent UUO.

Hydrogen sulfide (H₂S) represents the third gasotransmitter along with nitric oxide and carbon monoxide. H₂S is able to diffuse freely across cell membranes in a receptor-independent manner and activates various cellular targets. Several studies found that H₂S may possess antioxidant effects [8]. Endogenous H₂S plays various physiological and pathological roles in the kidney. Recently, H₂S was shown to exert antifibrotic effects in obstructive nephropathy and inhibit the proliferation and differentiation of renal fibroblasts *in vitro* and *in vivo*. The antifibrotic effects of H₂S occur via anti-inflammatory and anti-oxidative mechanisms [9].

As mentioned above, ROS-induced cell damage plays a pivotal role in the pathogenesis of UUO injury. However, to date, no studies have examined the relationship between ROS and autophagy in a UUO model. It has been reported that autophagy is mediated by ROS in many cell types [10]. ROS-induced autophagy can be inhibited by antioxidant drugs, such as N-acetyl cysteine (NAC), catalase (CAT), glutathione, melatonin, and vitamin E [11]. In the present study, we explored whether H₂S could ameliorate renal injury induced by UUO through the regulation of autophagy, and what the underlying mechanisms were. Our findings uncovered a previously unknown role for oxidative stress in the pathogenesis of UUO injury that promoted autophagic cell death and delineated a crucial protective function for H₂S in UUO.

Materials and Methods

Animal model

Male C57BL/6 (18–20 g, 4–6 weeks old) mice were supplied by the Animal Research Center at the Second Clinical Medical School of Harbin Medical University (Harbin, China). All surgical procedures and care administered to the animals were approved by the institutional ethics committee. This study complied with the criteria in the Guide for the Care and Use of Laboratory Animals by the US National Institutes of Health (NIH).

A UUO model was induced in male C57BL/6 mice by ligation of the left ureter, as described previously [3]. NaHS (5.6 µg/kg/day) was employed as a donor of H₂S [12]. The mice were divided randomly into four groups: (1) sham surgery group (only free ureteral ligation, intraperitoneal injection of normal saline);

(2) sham surgery with NaHS group; (3) UUO group (UUO and intraperitoneal injection of saline); and (4) UUO with NaHS group. NaHS (which was prepared immediately before use) and normal saline were given intraperitoneally once per day starting at 3 days before surgery and continuing from 1 day to 2 weeks after surgery. Tissues were taken every day after UUO from the UUO-induced kidney for a period of 7 days. The above treatment did not induce any significant side effects such as decreased food intake or abnormal behavior. Each experimental group included a minimum of six mice. For biochemical and histological study, the kidneys were frozen in liquid nitrogen immediately after extraction and perfusion-fixed.

Histology and immunohistochemistry

Changes in renal morphology were examined in formalin-fixed, 4-μm paraffin sections by hematoxylin and eosin staining. Histopathological changes were studied under a light microscope. The degree of tubulointerstitial injury was defined as tubular dilatation and/or atrophy, cell infiltration, or cellular edema.

For transmission electron microscope examination, kidney tissue was cut into small pieces and then fixed in 2.5% glutaraldehyde, post-fixed in 1% osmium tetroxide, dehydrated in an ascending alcohol series, and embedded in epoxy resin. Ultrathin sections were cut and stained with uranyl acetate and lead citrate. The samples were observed under a transmission electron microscope (JEM 1210; JEOL Ltd., Tokyo, Japan) at 80 or 60 kV onto electron microscope film (ESTAR Thick Base; Eastman Kodak Co., Rochester, NY) and printed onto photographic paper. We randomly selected 20–30 fields under low magnification (×1000) from each kidney, and digital images with scale bars were taken. Using AxioVision 4.0 software, the number of autophagic vacuoles per unit cytoplasmic area of 100 μm was evaluated, sectioned, and photographed under a transmission electron microscope according to routine procedures (JEOL Ltd., Tokyo, Japan). The histopathological scoring analysis was performed blindly as described previously [13].

The evaluation of renal morphology also included Doppler ultrasonography with a measurement of the size of the kidney, the thickness of the renal cortex, and the degree of separation of the renal pelvis. All Doppler examinations were performed by one examiner.

Terminal deoxynucleotidyl transferase dUTP nick end labeling assay

DNA fragmentation indicative of apoptosis was examined using the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. All renal cortices were fixed by immersion for at least 24 h in 10% buffered formaldehyde phosphate, and subsequently dehydrated and embedded in paraffin wax. The TUNEL assay was performed using deparaffinized sections (5 μm thick) with an *in situ* cell apoptosis detection kit (MK1020; Boster, Wuhan, China) according to the manufacturer's instructions.

H₂S concentration in mouse renal tissue and blood serum

The concentration of H₂S in renal tissue was measured by spectrophotometry. After mixing with 1 mL phosphate-buffered saline (PBS), 10 mg mouse renal tissue or blood serum was placed into a 2-mL Eppendorf tube, followed by the addition of a 1% zinc acetate solution (0.125 mL), 0.15 mL double distilled water, 0.1 mL tissue specimen and standards, 20 mM phenylenediamine phthalate solution (0.067 mL), and 30 mM ferric chloride solution (0.067 mL). The tissue was immersed in a water bath at 25°C for 20 min and centrifuged at 2374 × *g* for 5 min. Absorbance was measured at a wavelength of 670 nm using a microplate reader. Protein concentration was determined using a bicinchoninic acid kit. The concentration of H₂S in renal tissue (μmol/g protein) is the ratio of the concentration of H₂S in all specimens and total protein concentration.

Cell culture and treatment

Human proximal tubule HK-2 cells were purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Science. HK-2 cells were cultured in 1640 medium (HyClone, Beijing, China) supplemented with 10% fetal bovine serum (FBS; Gibco, Invitrogen, Carlsbad, CA) and 1% penicillin and amoxicillin in a humidified atmosphere of 5% CO₂ in air at 37°C. The cells were seeded on 6-well culture plates and grown to 60–80% confluence in complete medium containing 10% FBS for 20 h. The cells were then cultured in serum-free medium after being washed twice with serum-free medium.

The cultured HK-2 cells were divided randomly into four groups: control group; H₂O₂ (200 μM) group, H₂O₂ (200 μM) + NaHS (40 μM) group; and NaHS (40 μM) group. Compound C (20 μM, an inhibitor of adenosine monophosphate-activated protein kinase [AMPK]) was also used to investigate the role of AMPK. In addition, 3-MA (0–200 μmol/L, an inhibitor of autophagy) dissolved in DMSO was added at 8 h before NaHS stimulation. In the control group, the cells were treated with 1640 medium only. In the proliferation studies, 10% FBS served as a stimulator and the cells were incubated in serum-free medium for 24 h before the experiments.

Western blot analysis of total protein extracted from UUO mouse kidney and HK-2 cells

Protein samples (20–80 mg) from kidney tissue and primary HK-2 cells were separated by 8–12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred onto a polyvinylidene fluoride membrane (Millipore, Bedford, MA). After blocking in 5% milk/Tris-buffered saline and Tween-20 buffer, the membrane was incubated individually with antibodies against the following proteins of interest: AMPK/p-AMPK (1:500), cystathionine γ -lyase (CSE; 1:500), cystathionine β -synthase (CBS; 1:500), p62 (1:1000), beclin-1 (1:1000), microtubule-associated protein 1 light-chain 3 (LC3; 1:1000), CAT (1:1000), superoxide dismutase (SOD; 1:1000), Bax (1:1000), Bcl-2 (1:1000), cytochrome c (1:1000), caspase-3 (1:1000), and caspase-9 (1:1000), overnight at 4°C (all antibodies were purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The membrane was then washed and incubated with an appropriate horseradish peroxidase-conjugated secondary antibody for 1 h. Visualization was carried out using an Advanced Chemiluminescence Kit (GE Healthcare, Buckinghamshire, UK). Band density was quantified by ImageJ software (NIH, Bethesda, MD). β -Actin was used as an internal control.

Measurement of cytosolic and mitochondrial ROS

Cytosol-specific staining with DCFH was applied to measure cytosolic ROS production in HK-2 cells. HK-2 cells were divided into the four groups mentioned above and incubated in 6-well plates for 12 h followed by pre-treatment with different reagents for various periods of time. Then, the cells were washed with PBS and incubated with pre-warmed PBS containing DCFH dye (at a final working concentration of 10 μ M) for cytosolic detection at 37°C for 30 min. The same process was used to detect cellular ROS. DCFH fluorescence was measured using excitation and emission wavelengths of 480 and 535 nm, respectively.

Statistical analysis

Data are expressed as the mean $\pm$ standard error. Statistical analysis was performed by one-way analysis of variance. Differences between individual groups were analyzed using Student's *t* test. *p* < 0.05 was considered statistically significant and *p* < 0.01 was considered very significant.

Results

Effects of UUO on histological alterations in mice

Hematoxylin and eosin staining (Fig. 1A) indicated the presence of obvious edema, hypertrophy, and cell swelling in the UUO group compared with the sham group. Doppler ultrasound analysis demonstrated that the size of the kidney and the degree of separation of the renal pelvis were increased in the UUO group, while the thickness of the renal cortex was decreased (Fig. 1B). These results indicate the successful induction of UUO in our model. Meanwhile, these histopathological and ultrasonography alterations were markedly attenuated by NaHS treatment.

NaHS ameliorates the decrease of H₂S concentration and CBS and CSE expression in the obstructed kidney

To investigate whether UUO affects the level of endogenous H₂S, we examined the concentration of H₂S in plasma and renal tissue and the expression of the H₂S-producing enzymes CBS and CSE. Compared with the concentration of H₂S in renal tissue and blood among the sham groups, H₂S concentration was significantly decreased in the UUO group in a time-dependent manner (*p* < 0.05). The H₂S concentration in the UUO+NaHS group was significantly elevated compared with the corresponding UUO group (*p* < 0.05) (Fig. 2A, B).

In vivo, we observed that, compared with the contralateral and sham-operated kidneys, UUO injury reduced CBS and CSE expression in obstructed kidneys in a time-dependent manner (Fig. 2C). The same phenomenon was observed in tissue from patients with obstructive nephropathy (Fig. 2D). To assess the effect of exogenous H₂S supplementation on renal fibrosis, we treated UUO mice with NaHS (5.6 μ g/kg/day). Compared with the UUO mice, NaHS increased the expression of CBS and CSE. We also found that CBS and CSE expression was significantly decreased in H₂O₂-treated HK-2 cells *in vitro*, which was also attenuated by NaHS (Fig. 2E). These results demonstrate that UUO reduces endogenous H₂S levels, which could be increased by NaHS administration.

Fig. 1. NaHS treatment reverse the UUO renal damage both in morphology and ultrasonography. Comparison of rat renal tissues from different groups. A) Light microscope images (HE, $\times 400$) shows the pathological changes of the renal tissue ($n=7$). Obvious edema, hypertrophy, and cell swelling were found in UUO model compared with sham group. The histopathological alterations were markedly alleviated by NaHS treatment; B)

Ultrasonography examination demonstrated that the degree of separation of the renal pelvis was increased, while the thickness of the renal cortex was decreased in UUO model, however these alterations were markedly attenuated by NaHS treatment. * $P \leq 0.05$ compared with the sham group; ** $P \leq 0.01$ compared with the sham group; # $P \leq 0.05$ compared with the UUO group; ## $P \leq 0.01$ compared with the UUO group.

H_2S inhibits apoptosis and oxidative stress induced by UUO in vivo and in vitro

TUNEL staining showed that the number of apoptotic renal tubule epithelial cells was significantly higher in the UUO group than in the control group. These harmful effects were essentially abolished by the addition of NaHS (Fig. 3A). Western blot analysis also demonstrated that the expression of Bax, cleaved caspase-3, cleaved caspase-9, and cytochrome c protein was significantly upregulated in UUO mice at day 7 ($p < 0.05$). Bcl-2 is known to play an anti-apoptotic role in renal tubule damage [14]. In this UUO model, Bcl-2 protein expression was markedly decreased. However, the expression of Bax, cleaved caspase-3, cleaved caspase-9, and cytochrome c was significantly lower in UUO+NaHS mice than in UUO mice ($p < 0.05$), whereas Bcl-2 expression was upregulated significantly in UUO+NaHS mice compared with UUO mice ($p < 0.05$; Fig. 3B–E). Similar results were observed in H_2O_2 -treated cells, which imitate obstructed renal cells, *in vitro* (Fig. 4).

It has been reported that H_2S is capable of suppressing ROS production; thus, we hypothesized that exogenous H_2S may protect cells by inhibiting ROS production. On the basis of this hypothesis, we applied DCFH to examine total ROS production *in vitro*. Total ROS production was significantly increased in the H_2O_2 -treated group, whereas such an increase was suppressed by NaHS treatment (Fig. 5A). The effect of H_2S on the redox status of obstructed kidney was also determined using the UUO model after 7 days. It is known that SOD and CAT protect against superoxide-mediated cytotoxicity by catalyzing O_2^- to form H_2O_2 , which is one of the most important cellular protective mechanisms. Thus, we detected the expression of SOD and CAT in the obstructed kidney by western blot analysis. The levels of CAT and SOD were decreased in the UUO model; however, treatment with NaHS abolished the decrease of SOD and CAT induced by UUO injury (Fig. 5B).

Increased autophagy activity induced by UUO is related to ROS in vivo and in vitro

By using transmission electron microscopy, large numbers of double-membraned autophagosomes were observed near cell nuclei in the UUO group, whereas a small number of autophagosomes was discovered in the UUO+NaHS group. In contrast, no autophagosomes were detected in the sham groups (Fig. 6A). To confirm further that autophagy follows UUO, we examined the expression of LC3-II. The conversion of LC3-I to LC3-II is considered a standard marker of autophagy [15]. The ratio of LC3-II/LC3-I and p62 protein levels have been used to evaluate the level of autophagy in many studies [16]. Western blot analysis

Fig. 2. Concentration of H₂S, and CBS, CSE expression are decreased in the obstructed kidney, which could be ameliorated by NaHS. A) H₂S concentration in rat blood in different groups (n = 10). *P<0.05 compared with the sham group; ** P<0.01 compared with the sham group; #P<0.05 compared with the UUO group. B) H₂S concentration in rat renal tissues in different groups (n = 10) ,*P<0.05 compared with the sham group. C) Protein expressions of CBS and CSE in renal tissue. Data are expressed as mean±SD of three repeat experiments. NaHS treatment up-regulates CBS and CSE expression; *P<0.05 compared with the sham group; ***P<0.001 compared with the sham group; ##P<0.01 compared with the UUO group; ###P<0.001 compared with the UUO group. D) Protein level of CSE and CBS in the tissue of obstruction kidney patients. *P<0.05 compared with the control group. E) Similar results were obtained during in vitro experiment. *P<0.05 compared with the control group; #P<0.05 compared with the H₂O₂ group; ###P<0.01 compared with the H₂O₂ group.

showed that the LC3-II/LC3-I ratio was significantly increased at day 7 in the UUO model, whereas p62 expression was decreased ($p < 0.05$) (Fig. 6B, C) and this phenomenon could be reversed by NaHS. Beclin-1 is involved in the initiation of autophagosome formation by forming a multiprotein complex [17]. We also confirmed that beclin-1 protein expression and LC3-II/LC3-I ratio were significantly higher in tissue from patients with obstructive nephropathy than in the normal tissue group (Fig. 6E).

To confirm further whether UUO has an effect on autophagy *in vitro* similar to that observed *in vivo*, we evaluated autophagy in HK-2 cells following treatment with H₂O₂. H₂O₂ treatment significantly induced autophagy in HK-2 cells, as indicated by the diminished levels of p62 protein as well as the increased expression of LC3-II and beclin-1 (Fig. 7A). The ROS scavenger NAC was further applied to explore the role of oxidative stress in autophagy. As expected, NAC treatment effectively reversed the H₂O₂-induced decrease of p62 protein levels (Fig. 7B). These results indicate that increased levels of ROS are highly correlated with the induction of autophagy in obstructive nephropathy.

Fig. 3. UUO induced apoptosis in vivo can be inhibited by H₂S. A) Detection of apoptosis levels in rats from different groups using TUNEL stain ($\times 400$). NaHS treatment has anti-apoptosis effect on UUO renal tissue. * $P \leq 0.05$ compared with the sham group; *** $P \leq 0.001$ compared with the sham group; B-E): Western blot results. Protein expressions of BAX(B), Bcl-2(C), Cytochrome C(D), cleaved caspase-3 and cleaved caspase-9(E) in the renal tissue. Data are expressed as mean $\pm$ SD with three repeat experiments. * $P \leq 0.05$ compared with the sham group; ** $P \leq 0.01$ compared with the sham group; *** $P \leq 0.001$ compared with the sham group; ## $P \leq 0.01$ compared with the UUO group; ### $P \leq 0.001$ compared with the UUO group.

Fig. 4. UUO-induced apoptosis in vitro can be inhibited by H₂S. Western blot: The expression levels of protein Bax(A), caspase-9 (B), caspase-3(C) and Cytochrome C(D) in the HK-2 cells. Data are expressed as mean $\pm$ SD of three repeat experiments. NaHS treatment has anti-apoptosis effect on H₂O₂ treated-HK-2 cells. ** $P \leq 0.01$ compared with the sham group; *** $P \leq 0.001$ compared with the sham group; # $P \leq 0.05$ compared with the H₂O₂ group; ## $P \leq 0.01$ compared with the H₂O₂ group; ### $P \leq 0.001$ compared with the H₂O₂ group.

Fig. 5. UUO-induced oxidative stress can be inhibited by H₂S. A) The level of oxidative stress in different intervention groups of HK-2 cells was measured by flow cytometry. ROS production significantly increased in the H₂O₂-treated group whereas these changes were suppressed by NaHS treatment. ** P≤0.01 compared with the sham group, #P≤0.05 compared with the H₂O₂ group; B) CAT and SOD expression level in rats from different groups. ***P≤0.001 compared with the sham group; ##P≤0.01 compared with the UUO group; ###P≤0.001 compared with the UUO group.

Effects of H₂S on UUO-induced autophagy in HK-2 cells

To explore the effects of H₂S on autophagy, we examined the expression of LC3-II, beclin-1, and p62, specific markers of autophagic vacuoles, in the UUO model by western blot assay. In the UUO model kidney cortex, LC3-II and beclin-1 expression was significantly up-regulated in the model group, whereas H₂S suppressed this elevation of LC3-II and beclin-1. p62 expression was significantly down-regulated in the model group and was reversed by H₂S treatment (Fig. 6B, C, D).

To investigate further the effects of H₂S *in vitro*, protein was extracted from treated HK-2 cells. The expression of LC3-II, p62, and beclin-1 was analyzed by western blotting. Compared with the control, the protein levels of LC3-II and beclin-1 were significantly increased in H₂O₂-treated cells, which was accompanied with decreased p62 expression. However, when the cells were incubated with NaHS following H₂O₂ treatment, these phenomena were reversed (Fig. 7). These data demonstrate that the renoprotective effect of H₂S is closely related to its inhibition of autophagy in the UUO kidney.

AMPK-dependent signaling pathways mediate ROS-induced autophagic activity

AMPK plays an important role in energy metabolism, which can also be triggered by oxidative stress [18]. AMPK activation is a well-known down-regulator of mammalian target of rapamycin (mTOR) activation, which is a key negative regulator for the suppression of autophagy. To further determine whether the AMPK pathway is involved in UUO-induced

Fig. 6. UUO led to increased autophagy activity in vivo. A) The structure of autophagosomes in the renal tissues of rats from different groups. The white arrows represent the autophagosomes with bilayer membrane structures; B) The LC3 II/I levels in rats from different groups (Western blot result); C) P62 protein levels in rats from different groups (Western blot result); D) Beclin1 protein levels in rats from different groups (Western blot result); E) The LC3, II/I and Beclin1 protein levels in the human tissue of obstruction kidney patients and normal patients. * $P \leq 0.05$ compared with the sham group; ** $P \leq 0.01$ compared with the sham group; *** $P \leq 0.001$ compared with the sham group; ## $P \leq 0.01$ compared with the UUO group; ### $P \leq 0.001$ compared with the UUO group.

autophagy, we administered the AMPK inhibitor compound C (50 μ M) to HK-2 cells exposed to H₂O₂. Western blot analysis demonstrated that AMPK phosphorylation was increased in UUO mice and H₂O₂-treated cells. In addition, both were reversed by compound C (Fig. 8). Additionally, the formation of autophagosomes marked by beclin-1 using western blot analysis in HK-2 cells also demonstrated that compound C could decrease the H₂O₂-induced increase in beclin-1 expression (Fig. 8). Meanwhile, the H₂O₂-induced down-regulation of p62 expression was significantly increased in the presence of compound C (Fig. 8). The above results indicate that the AMPK signaling pathway is involved in ROS-induced autophagy in HK-2 cells.

H₂S inhibits autophagy by regulating the AMPK signaling pathway

Autophagy can be enhanced by activation of the AMPK signaling pathway [19]. The ratio of p-AMPK to AMPK was detected to confirm the mechanism of the activation of autophagy, and showed that UUO or H₂O₂ treatment increased this ratio, whereas NaHS treatment attenuated this increase (Fig. 8). To further investigate whether the AMPK signaling pathway played a role in UUO-induced autophagy, compound C was also applied. Our results showed that compound C had the same effect as NaHS, and regulated the changes of p62 and beclin-1 expression (Fig. 8). In summary, our data suggest that H₂S inhibits obstructive nephropathy-induced autophagy through regulation of the AMPK signaling pathway.

Fig. 7. UUO led to increased autophagy activity invitro, which is related to ROS. A) LC3 II/I, Beclin1 and P62 protein levels in HK-2 cells in different groups (Western blot). H₂O₂ treatment significantly induced autophagy activity, as indicated by diminished levels of p62 protein as well as increased levels of LC3-II and Beclin 1. B) NAC treatment could effectively reverse the H₂O₂-induced decrease of p62 protein levels. *P≤0.05 compared with the sham group; **P≤0.01 compared with the sham group; ***P≤0.001 compared with the sham group; ##P≤0.01 compared with the UUO group; ###P≤0.001 compared with the UUO group.

Discussion

In this study, HK-2 cells were exposed to H₂O₂ to examine the mechanism by which UUO induces autophagy in renal tubule epithelial cells, and to investigate the association between UUO-induced autophagy and ROS generation, as well as the effects of H₂S on this process. Our results indicate that NaHS, a donor of H₂S, alleviated the excessive activation of autophagy by attenuating the activation of the ROS-AMPK pathway. We provide the first evidence that the renoprotection effect of H₂S in obstructive nephropathy is associated with autophagy.

It has been demonstrated that UUO promotes ROS production via mineralocorticoid receptor-dependent mechanisms, and the inhibition of ROS synthesis can prevent the progression of proteinuria and ameliorate renal injury. This suggests the importance of oxidative stress in UUO-induced renal injury [20]. Our study shows that the expression of CSE, CBS, SOD, and CAT and the levels of endogenous H₂S were decreased in UUO mice, while the expression of pro-apoptotic proteins was elevated and the number of TUNEL-positive cells was increased. This indicates the modulatory effect of H₂S on the cellular antioxidant system of the kidney. H₂S, a gasotransmitter, has attracted wide attention in recent years because of its protective effect in the liver, kidney, heart, lung, and other organs [21, 22]. Many studies have also demonstrated the protective effect of H₂S against kidney injury [23, 24]. H₂S has been shown to exert a wide range of physiological and cytoprotective functions *in vivo*. Among these functions, its role in oxidative stress has been one of the main foci of research over the

Fig. 8. UUO-induced autophagic activity is mediated by AMPK dependent signaling pathways. A and B): AMPK phosphorylation was effectively overexpressed in UUO rats and H₂O₂-treated cells, which could be reversed by Compound C; C and D) Compound C attenuated H₂O₂-induced alteration of Beclin 1 and p62 protein level. *P≤0.05 compared with the sham group; **P≤0.01 compared with the sham group; ***P≤0.001 compared with the sham group; ##P≤0.01 compared with the H₂O₂ group; ###P≤0.001 compared with the H₂O₂ group.

years. Many studies have revealed the underlying mechanisms for the antioxidant effect of H₂S. Under an environment of 37°C and pH 7.4, over 80% of H₂S molecules can dissolve in water and dissociate into H⁺, HS⁻, and S²⁻ ions. HS⁻ is powerful one-electron chemical reductant and has a remarkable capacity to scavenge ROS. It has also been reported that H₂S can stimulate cellular enzymatic or non-enzymatic antioxidants to scavenge free radicals. H₂S donors include sulfide salts such as sodium sulfide, NaHS, garlic-derived compounds, and 1, 2-dithiole-3-thiones. Among these donors, NaHS has been used widely. The concentration of NaHS in our study was determined based on our previous experiments. In the present study, we also found that NaHS supplementation provides anti-oxidative and anti-apoptosis effects *in vivo* and *in vitro*. This finding is consistent with a previous study showing that H₂S can serve as a ROS scavenger [25]. These results indicate that exogenous H₂S is capable of protecting the function of renal epithelial cells in an obstructive nephropathy environment.

Autophagy has been demonstrated to be involved in many physiological and pathological processes. When autophagy destroys the cytosol and organelles beyond a certain threshold, autophagic cell death will occur. Accumulating evidence has suggested that intracellular ROS play important roles in the regulation of autophagy [26, 27]. The autophagy process is regulated by autophagy-related genes, among which beclin-1 is needed for the autophagy vesicle nucleation step. The conversion of the soluble form of LC3 (LC3-I) to the autophagic vesicle-associated form (LC3-II) is used as a marker of autophagy. Thus, the expression of beclin-1 and the ratio of LC3-II/LC3-I are used to evaluate the level of autophagy in most studies. In our study, an elevated LC3-II/LC3-I ratio was observed in tissue from patients

with obstructive nephropathy, indicating that autophagy participates in its pathogenesis. However, it has not been reported whether H₂S could attenuate autophagy in a UUO model. Thus, we examined the LC3-II/LC3-I ratio and beclin-1 and p62 expression, three specific markers of autophagic vacuoles in UUO mice and H₂O₂-treated cells. The results showed that H₂S reversed the elevation of LC3-II and beclin-1, as well as the reduction of p62 induced in the UUO model *in vivo* and *in vitro*. Our results are the first to demonstrate that H₂S plays a protective role in the kidney by inhibiting autophagy in a UUO model. We found that NAC treatment had a similar effect on autophagy to NaHS, which suggested that ROS play a crucial role in the activation of excessive autophagy. Thus, we proved that UUO-induced autophagy in renal epithelial cells is associated with ROS [3], even though the underlying mechanism still remains unknown.

There are many signaling cascades involved in autophagy regulation in response to different stimuli in intracellular and extracellular environments [28]. Among them, the master regulator serine/threonine kinase mTOR is known as the classical pathway [29]. As a key mediator of mTOR, AMPK has been demonstrated to be a positive regulator of autophagy by negatively regulating mTOR activity [30, 31]. AMPK is a major regulator of energy homeostasis. Adenosine triphosphate depletion can activate AMPK to decompose fatty acids and induce autophagy [32]. However, the continuous activation of AMPK results in excessive autophagy, which plays an important role in apoptosis [33]. In addition, AMPK is usually activated in response to a variety of extracellular and intracellular stresses, such as oxidative stress [31, 34]. ROS can also stimulate the activity of AMPK [35].

To elucidate the mechanism by which ROS enhanced autophagy, the levels of AMPK and p-AMPK were examined in the present study. We found that p-AMPK was elevated in the UUO model and in H₂O₂-treated HK-2 cells, indicating that ROS are located upstream of AMPK. We further investigated the association between ROS and AMPK by using compound C, a specific AMPK inhibitor. Our western blot data revealed that the changes in the expression of p62 and beclin-1 induced by H₂O₂ could be reversed in the presence of compound C. These results triggered us to hypothesize that the autophagy observed in the UUO model is linked to the accumulation of intracellular ROS, which leads to the activation of AMPK and in turn the activation of autophagy. These results provide additional evidence to studies showing that the ROS-AMPK pathway is an important mediator of autophagy [36, 37].

In conclusion, the results of the present study reveal a key role for H₂S in the regulation of oxidative stress and autophagy in obstructive kidney disease. Endogenous H₂S may participate in the reduction of excessive autophagy by inhibiting the ROS-AMPK pathway. NaHS could increase the levels of endogenous H₂S and could therefore be considered a therapeutic target in obstructive nephropathy. These findings provide a novel insight and may assist in the development of novel treatments for kidney diseases.

Acknowledgements

This study was supported by the Nature Science Youth Foundation of Heilongjiang Province (no. QC2010010), Education Foundation of Heilongjiang Province (no. 12541545), and Young Talent of Harbin Science Bureau (no. 2016RAQYJ169).

Disclosure Statement

No conflict of interests exists.

References

- 1 Klahr S, Morrissey J: Obstructive nephropathy and renal fibrosis. *Am J Physiol Renal Physiol* 2002;283:861-875.
- 2 Qin J, Xie YY, Huang L, Yuan QJ, Mei WJ, Yuan XN, Hu GY, Cheng GJ, Tao LJ, Peng ZZ: Fluorofenidone inhibits nicotinamide adeninedinucleotide phosphate oxidase via PI3K/Akt pathway in the pathogenesis of renal interstitial fibrosis. *Nephrology (Carlton)* 2013;18:690-699.

- 3 Xu Y, Ruan S, Wu X, Chen H, Zheng K, Fu B: Autophagy and apoptosis in tubular cells following unilateral ureteral obstruction are associated with mitochondrial oxidative stress. *Int J Mol Med* 2013;31:628-636.
- 4 Nishida K, Yamaguchi O, Otsu K: Crosstalk between autophagy and apoptosis in heart disease. *Circ Res* 2008;103:343-351.
- 5 Luo S, Rubinsztein DC: Apoptosis blocks Beclin 1-dependent autophagosome synthesis: an effect rescued by Bcl-xL. *Cell Death Differ* 2010;17:268-277.
- 6 Kim WY, Nam SA, Song HC, Ko JS, Park SH, Kim HL, Choi EJ, Kim YS, Kim J, Kim YK: The role of autophagy in unilateral ureteral obstruction rat model. *Nephrology (Carlton)* 2012;17:148-159.
- 7 Li L, Zepeda-Orozco D, Black R, Lin F: Autophagy is a component of epithelial cell fate in obstructive uropathy. *Am J Pathol* 2010;176:1767-1778.
- 8 Yang G, An SS, Ji Y, Zhang W, Pei Y: Hydrogen sulfide signaling in oxidative stress and aging development. *Oxid Med Cell Longev* 2015; DOI:10.1155/2015/357824.
- 9 Song K, Wang F, Li Q, Shi YB, Zheng HF, Peng H, Shen HY, Liu CF, Hu LF: Hydrogen sulfide inhibits the renal fibrosis of obstructive nephropathy. *Kidney Int* 2014;85:1318-1329.
- 10 Medvedev R, Ploen D, Spengler C, Elgner F, Ren H, Bunten S, Hildt E: HCV-induced oxidative stress by inhibition of Nrf2 triggers autophagy and favors release of viral particles. *Free Radic Biol Med* 2017;110:300-315.
- 11 Ravikumar B, Sarkar S, Davies JE, Futter M, Garcia-Arencibia M, Green-Thompson ZW, Jimenez-Sanchez M, Korolchuk VI, Lichtenberg M, Luo S, Massey DC, Menzies FM, Moreau K, Narayanan U, Renna M, Siddiqi FH, Underwood BR, Winslow AR, Rubinsztein DC: Regulation of mammalian autophagy in physiology and pathophysiology. *Physiol Rev* 2010;90:1383-1435.
- 12 Liu J, Wu J, Sun A, Sun Y, Yu X, Liu N, Dong S, Yang F, Zhang L, Zhong X, Xu C, Lu F, Zhang W: Hydrogen sulfide decreases high glucose/palmitate-induced autophagy in endothelial cells by the Nrf2-ROS-AMPK signaling pathway. *Cell Biosci* 2016; DOI:10.1186/s13578-016-0099-1.
- 13 Becknell B, Carpenter AR, Allen JL, Wilhide ME, Ingraham SE, Hains DS, Mchugh KM: Molecular Basis of Renal Adaptation in a Murine Model of Congenital Obstructive Nephropathy. *PLoS One* 2013;8:e72762.
- 14 Miyajima A, Chen J, Lawrence C, Ledbetter S, Soslow RA, Stern J, Jha S, Pigato J, Lemer ML, Poppas DP, Vaughan ED, Felsen D: Antibody to transforming growth factor-beta ameliorates tubular apoptosis in unilateral ureteral obstruction. *Kidney Int* 2000;58:2301-2313.
- 15 Zhang H, Kong X, Kang J, Su J, Li Y, Zhong J, Sun L: Oxidative stress induces parallel autophagy and mitochondria dysfunction in human glioma U251 cells. *Toxicol Sci* 2009;110:376-388.
- 16 Jiao H, Zhang Z, Ma Q, Fu W, Liu Z: Mechanism underlying the inhibitory effect of apelin-13 on glucose deprivation-induced autophagy in rat cardiomyocytes. *Exp Ther Med* 2013;5:797-802.
- 17 Nishida K, Yamaguchi O, Otsu K: Crosstalk between autophagy and apoptosis in heart disease. *Circ Res* 2008;103:343-351.
- 18 Kemp BE, Stapleton D, Campbell DJ, Chen ZP, Murthy S, Walter M, Gupta A, Adams JJ, Katsis F, van Denderen B, Jennings IG, Iseli T, Mitchell BJ, Witters LA: AMP-activated protein kinase, super metabolic regulator. *Biochem Soc Trans* 2003;31:162-168.
- 19 Alers S, Löffler AS, Wesselborg S, Stork B: Role of AMPK-mTOR-Ulk1/2 in the regulation of autophagy: cross talk, shortcuts, and feedbacks. *Mol Cell Biol* 2012;32:2-11.
- 20 Qin J, Mei WJ, Xie YY, Huang L, Yuan QJ, Hu GY, Tao LJ, Peng ZZ: Fluorofenidone Attenuates Oxidative Stress and Renal Fibrosis in Obstructive Nephropathy via Blocking NOX2 (gp91phox) Expression and Inhibiting ERK/MAPK Signaling Pathway. *Kidney Blood Press Res* 2015;40:89-99.
- 21 Yang CT, Zhao Y, Xian M, Li JH, Dong Q, Bai HB, Xu JD, Zhang MF: A novel controllable hydrogen sulfide-releasing molecule protects human skin keratinocytes against methylglyoxal-induced injury and dysfunction. *Cell Physiol Biochem* 2014;34:1304-1317.
- 22 Avanzato D, Merlino A, Porrera S, Wang R, Munaron L, Mancardi D: Role of calcium channels in the protective effect of hydrogen sulfide in rat cardiomyoblasts. *Cell Physiol Biochem* 2014;33:1205-1214.
- 23 Ling Q, Yu X, Wang T, Wang SG, Ye ZQ, Liu JH: Roles of the Exogenous H₂S-Mediated SR-A Signaling Pathway in Renal Ischemia/ Reperfusion Injury in Regulating Endoplasmic Reticulum Stress-Induced Autophagy in a Rat Model. *Cell Physiol Biochem* 2017;41:2461-2474.
- 24 Li L, Xiao T, Li F, Li Y, Zeng O, Liu M, Liang B, Li Z, Chu C, Yang J: Hydrogen sulfide reduced renal tissue fibrosis by regulating autophagy in diabetic rats. *Mol Med Rep* 2017;16:1715-1722.
- 25 Kasinath BS: Hydrogen sulfide to the rescue in obstructive kidney injury. *Kidney Int* 2014;85:1255-1258.
- 26 Navarro-Yepes J, Burns M, Anandhan A, Khalimonchuk O, del Razo LM, Quintanilla-Vega B, Pappa A, Panayiotidis MI, Franco R: Oxidative stress, redox signaling, and autophagy: cell death versus survival. *Antioxid Redox Signal* 2014;21:66-85.
- 27 Pant K, Saraya A, Venugopal SK: Oxidative stress plays a key role in butyrate-mediated autophagy via Akt/mTOR pathway in hepatoma cells. *Chem Biol Interact* 2017;273:99-106.

- 28 He C, Klionsky DJ: Regulation mechanisms and signaling pathways of autophagy. *Annu Rev Genet* 2009;43:67-93.
- 29 Jung CH, Ro SH, Cao J, Otto NM, Kim DH: mTOR regulation of autophagy. *FEBS Lett* 2010;584:1287-1295.
- 30 Shang L, Wang X: AMPK and mTOR coordinate the regulation of Ulk1 and mammalian autophagy initiation. *Autophagy* 2011;7:924-926.
- 31 Kim J, Kundu M, Viollet B, Guan KL: AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. *Nat Cell Biol* 2011;13:132-141.
- 32 Hardie DG: AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy. *Nat Rev Mol Cell Biol* 2007;8:774-785.
- 33 Shi X, Zhu H, Zhang Y, Zhou M, Tang D, Zhang H: XuefuZhuyu decoction protected cardiomyocytes against hypoxia/reoxygenation injury by inhibiting autophagy. *BMC Complement Altern Med* 2017;17:325.
- 34 Kim DE, Kim Y, Cho DH, Jeong SY, Kim SB, Suh N, Lee JS, Choi EK, Koh JY, Hwang JJ, Kim CS: Raloxifene induces autophagy-dependent cell death in breast cancer cells via the activation of AMP-activated protein kinase. *Mol Cells* 2015;38:138-144.
- 35 Jung SN, Yang WK, Kim J, Kim HS, Kim EJ, Yun H, Park H, Kim SS, Choe W, Kang I, Ha J: Reactive oxygen species stabilize hypoxia-inducible factor-1 α protein and stimulate transcriptional activity via AMP-activated protein kinase in DU145 human prostate cancer cells. *Carcinogenesis* 2008;29:713-721.
- 36 Xu Z, Huang CM, Shao Z, Zhao XP, Wang M, Yan TL, Zhou XC, Jiang EH, Liu K, Shang ZJ: Autophagy Induced by Areca Nut Extract Contributes to Decreasing Cisplatin Toxicity in Oral Squamous Cell Carcinoma Cells: Roles of Reactive Oxygen Species/AMPK Signaling. *Int J Mol Sci* 2017;18 pii:E524.
- 37 Pu T, Liao XH, Sun H, Guo H, Jiang X, Peng JB, Zhang L, Liu Q: Augmenter of liver regeneration regulates autophagy in renal ischemia-reperfusion injury via the AMPK/mTOR pathway. *Apoptosis* 2017;22:955-969.