Hydrogen Sulfide-Induced Hypometabolism Prevents Renal Ischemia/Reperfusion Injury

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ABSTRACT

Hydrogen sulfide (H2S) can induce a hypometabolic, hibernation-like state in mammals when given in subtoxic concentrations. Pharmacologically reducing the demand for oxygen is a promising strategy to minimize unavoidable hypoxia-induced injury such as ischemia/reperfusion injury during renal transplantation. Here we show that H2S reduces metabolism in vivo, ex vivo, and in vitro. Furthermore, we demonstrate the beneficial effects of H2S-induced hypometabolism in a model of bilateral renal ischemia/reperfusion injury using three different treatment strategies. The results demonstrate striking protective effects on survival, renal function, apoptosis, and inflammation. A hypometabolic state induced by H2S might have therapeutic potential to protect kidneys that suffer from hypoxia.


The toxicity of hydrogen sulfide (H2S) has long been studied because of its involvement in deadly industrial and agricultural accidents.1 Recently, an unknown property of the gas was revealed, namely the ability to induce hypometabolism in naturally nonhibernating mammals.2,3 Mice exposed to subtoxic concentrations of gaseous H2S rapidly and reversibly enter a hibernation-like state. During H2S treatment, metabolic parameters rapidly decrease: an approximately 60% reduction in carbon dioxide (CO2) production and oxygen (O2)-consumption within minutes of exposure (Figure 1A), which can decline even further to more than 90%.2,4 In addition, the core body temperature decreases to near-ambient temperature and heart rate and breathing frequency are significantly lower.3 The demand for O2 is reduced to such an extent that H2S-treated mice can survive in 5% O2 for over 6 h, whereas untreated controls die within 15 min.4 In vitro, H2S can reversibly reduce mitochondrial O2 consumption5,6 and mitochondrial membrane potential (Figure 1B). Ex vivo, H2S can reduce O2 consumption and total ATP content of the isolated perfused kidney (Figure 1, C and D). H2S also has antioxidant capacity, either by direct scavenging of reactive O2 or nitrogen species or indirectly by increasing cellular glutathione levels.3 We hypothesized that a state of extremely low metabolism induced by exposure to gaseous H2S would provide protection during periods of ischemia and reperfusion by reducing the demand for O2.

To investigate the protective potential of H2S-induced hypometabolism, we used a model of bilateral renal ischemia/reperfusion in the mouse. We evaluated four different treatment regimens (Figure 1E), comparing pretreatment, post-treatment, and pre- and post-treatment with 100 ppm H2S (n = 6 to 7 per group). In both pretreated groups, C57BL/6 mice were first treated with H2S for 30 min to induce hypometabolism. Our initial experiments showed that the induction of hypometabolism typically takes place within the first 10 min of exposure (Figure 1A). After the pretreatment period, renal blood flow was interrupted for 30 min by placing nontraumatic vascular clamps over both renal pedicles. To study the effects of H2S on reperfusion damage alone, post-treatment with H2S started 5 min before removal of the clamps and lasted for 35 min. The pre- and post-treatment group received H2S starting 30 min before ischemia until 30 min after reperfusion. To separate the effects of H2S from the already well known protective effects of hypothermia, core body temperature of all animals was maintained at 37°C during and after the procedure. Induction of hypometabolism in H2S-treated animals was confirmed by lowered breathing frequency and CO2 production, measured using closed-system respirometry (Figure 1, F and G).

Bilateral ischemia caused excessive re-
nal damage in the control group, leading to an impaired 3-d survival caused by renal failure (Figure 2A). Both groups in which mice were pretreated with H2S had 100% survival after 3 d (**P < 0.001), whereas mice that only received H2S during reperfusion showed similar survival to the control group (P = NS). Serum creatinine and urea measurements were performed to quantify the renal function loss associated with bilateral renal ischemia/reperfusion. Control and post-treatment animals showed highly elevated levels of creatinine and urea (Figure 2B, Supplementary Figure 1), whereas animals pretreated with H2S had only slightly higher levels than sham-operated animals (P = NS). These measurements indicate massive renal failure in the control and post-treatment groups, which is the most likely cause of the diminished survival in these groups.

We assessed structural renal damage in periodic acid–Schiff-stained sections and found a similar pattern to the renal function measurements, as expected. Massive acute tubular necrosis was detected in control animals at day 1, whereas mice in both pretreated groups had no or minimal renal damage (Figure 2, D, J through N, Supplementary Figure 3). Post-treatment with H2S showed a significant reduction in tubular damage compared with controls, although it was not as extensive as in pretreated animals. After 3 d, a similar pattern was seen (Supplementary Figure 2). Post-treatment did not have significant protective effects at this time point, although these results are confounded to some extent, because animals with large amounts of renal damage had already deceased at this point.

Active Caspase3 staining using immunohistochemistry indicated that ischemia/reperfusion injury (IRI)-induced apoptosis is also prevented by H2S pretreatment. (Figure 2C, Supplementary Figure 4). A less pronounced but statistically significant effect was seen in the post-treatment group. Real-time PCR measurements showed that mRNA expression of proapoptotic Bax was 2.5 times higher in control kidneys compared with sham-operated animals (Supplementary Figure 5A). Expression was not significantly increased in animals pretreated with H2S. The expression of anti-apoptotic BCL-2 did not differ between groups (Supplementary Figure 5B), indicating that the anti-apoptotic effects of H2S are not mediated through induction of BCL-2 mRNA expression. Whether H2S directly or indirectly inhibits increased expression of Bax is not clear. Transmission electron microscopy...
of a few samples implies that H2S treatment protected against loss of mitochondrial integrity and mitochondrial swelling (Supplementary Figure 6). In literature, proapoptotic as well as anti-apoptotic effects of H2S are described,5,7–9 and it is not known whether H2S can directly modulate apoptotic pathways, or that increased mitochondrial integrity and reduced mitochondrial stress caused by reduced mitochondrial activity caused the reduction in Caspase 3 activity in the post-treatment group.

We studied the inflammatory component of IRI by immunohistochemical staining for Mac-1 (CD11b, which is present on macrophages, monocytes, granulocytes, and natural killer cells10) and Ly-6G (which is expressed on mature granulocytes). (Figure 2, E through I, Supplementary Figure 7). The influx of Mac-1 and Ly-6G-positive cells was greatly reduced by H2S pretreatment (P < 0.05) but was not significantly affected by post-treatment.

These results indicate that the reduction in metabolism before ischemia is highly protective in reducing ischemia-induced injury with predictable onset, such as during transplantation or surgical intervention. The mechanism of H2S-induced hypometabolism is unknown as of yet but is most likely mediated through reversible inhibition of complex IV (cytochrome oxidase),11 the terminal enzyme of the mitochondrial electron transport chain. Inhibition of this complex might be the mechanism of the reduction in mitochondrial membrane potential caused by H2S treatment. It seems unlikely that H2S directly and effectively

**Figure 2.** H2S-induced hypometabolism prevents mortality and renal damage after renal ischemia. (A) Three-day survival of animals after reperfusion. (B) Renal function as measured by serum creatinine after 1 d of reperfusion. *P < 0.05 versus control, †P < 0.001 versus sham. (C) Apoptosis after 1 d of reperfusion was scored in sections stained for active Caspase 3 using immunohistochemistry. Apoptotic tubular cells were counted at 400× magnification in ten nonoverlapping fields (***P < 0.001). (D) Structural damage as assessed in periodic acid–Schiff-stained sections after 1 d of reperfusion. *P < 0.05 versus control, †P < 0.001 versus control, ‡P < 0.001 versus sham. (E) Influx of leukocytes and granulocytes into the renal interstitium was scored in sections stained for Mac-1 (solid bars) or Ly-6G (dashed bars) using immunohistochemistry. *P < 0.05 versus control. (F through I) Representative photomicrographs of Ly-6G stained sections. (J through N) Representative periodic acid–Schiff-stained renal sections with necrotic area artificially colored red, indicating the extent of necrotic damage found in each group. [For B through E: sham (n = 5), control (n = 7), and H2S-treated groups (n = 9).]
inhibits necrotic, apoptotic, and inflammatory pathways after an ischemic insult. The observation that protection is greatest when H$_2$S is given before and during, but much less when given directly after the hypoxic period, supports the notion that the reduction in O$_2$ demand during hypoxia prevents the activation of these detrimental pathways. The moderate effects of H$_2$S in the post-treatment group could be caused by the inhibition of reactive O$_2$ species production by decreasing mitochondrial activity. Protection could also be mediated through direct antioxidative action, or increased glutathione levels caused by H$_2$S.

Recent literature shows beneficial effects of gaseous H$_2$S on survival in models of hypoxia and hemorrhagic shock. Other groups have studied the protective effects of soluble forms of H$_2$S (such as sodium hydrosulfide or sodium sulfide) in models of ischemia. These studies show beneficial effects of H$_2$S on renal, cardiac, hepatic, and pulmonary ischemia. One paper suggests an association between H$_2$S treatment and reduced activation of multiple signal transduction molecules, such as p38, ERK, and JNK; however, a direct relationship between H$_2$S and kinase activation was not proven. We found that phosphorylation of ERK1/2 was stimulated by ischemia in our model, but no modulation was seen in H$_2$S-treated animals (Supplementary Figure 8). Our study shows a novel relation between H$_2$S treatment and hypometabolism, which has not been previously investigated. The protective effects of H$_2$S treatment posthypoxia are less pronounced in our experiments. However, a recent paper indicated that injection of sodium sulfide just before reperfusion in a model of myocardial infarction caused a great reduction in infarct size and protected mitochondrial integrity and function. This indicates that post-treatment with H$_2$S might still be a promising intervention in cutting back on the detrimental effects of hypoxia after the event. We conclude that hypometabolism induced by gaseous H$_2$S is a novel treatment regimen with high therapeutic potential in reducing renal damage associated with ischemic insults.

**CONCISE METHODS**

**Animals**

Male, 6- to 8-wk-old C57BL/6 mice and 250- to 300 g Fischer F344 rats (Harlan, The Netherlands) were housed under standard conditions. Experimental procedures were in agreement with institutional and legislator regulations and approved by the local committee for animal experiments.

**H$_2$S Treatment and Respirometry**

H$_2$S treatment and measurement of animal O$_2$ production was performed using an advanced, modular respirometry system (TR-3 system, Sable Systems, Las Vegas, NV). Compressed air and 500 ppm H$_2$S/nitrogen (Air Products, Amsterdam, The Netherlands) were mixed in a 4:1 ratio, producing a 100 ppm H$_2$S/17% O$_2$ mixture.

**IRI Protocol**

Renal ischemia/reperfusion in mice was performed as described previously. In short, both renal pedicles were clamped for 30 min using nontraumatic vascular clamps through the midline abdominal incision under general anesthesia [0.07 ml/10 g mouse of 1.25 mg/ml midazolam (Roche Diagnostics Corp), 0.08 mg/ml fentanyl citrate, and 2.5 mg/ml fluanison (Janssen Pharmaceutica)]. Core body temperature was maintained at 37°C in all groups using heat pads and lamps. Mice were terminated after 1 and 3 h of reperfusion, and samples were collected.

**Isolated Perfused Kidney**

The isolated perfused kidney setup for rat kidneys was described previously. Renal O$_2$ consumption was measured in pre- and post-treatment samples taken from an injection port 2 cm before the kidney and from the renal vein, respectively. In these samples, pO$_2$ was measured using an ABL700 blood gas analyzer (Radiometer Medical, Denmark).

**In Vitro Studies**

The NRK-52E (ATCC, Manassas, VA) proximal tubular cell line was used for in vitro experiments. Effects of H$_2$S on mitochondrial activity were measured using the mitochondrial membrane potential sensitive fluorescence probe JC-1 (Invitrogen, The Netherlands). Cells were loaded with 15 μg/ml of JC-1 and after basal fluorescence measurement on a spectrophotometer (Victor 3, PerkinElmer, The Netherlands), sodium hydrosulfide (a donor of H$_2$S in solution) was added to the medium. After 20 min of stimulation, the plate was remeasured and the relative change in the red/green fluorescence ratio was calculated.

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**DISCLOSURES**

None.

**REFERENCES**


Supplemental information for this article is available online at http://www.jasn.org/.
Supplementary Figure 1 - Renal function as measured by serum urea after 1 day of reperfusion. *p<0.05 vs. Control, †p<0.01 vs. Sham
Supplementary Figure 2 - (A) Renal function as measured by serum creatinine and (B) serum urea after 3 days of reperfusion. *p<0.05 vs. Control. (C) Structural damage as assessed in PAS-stained sections after 3 days of reperfusion. **p<0.001 vs. Control.
Supplementary Figure 3 - Representative photomicrographs of PAS stained renal tissue from all groups. Sham kidneys show normal morphology, while control kidneys show necrosis (arrows) and cast deposition (arrowheads). Normal morphology is seen in kidneys from the pre- and posttreatment group. Kidneys from the pretreatment group show only minimal necrosis, while posttreatment kidneys show intermediate damage.
Supplementary Figure 4 - Representative photomicrographs of active Caspase 3 stained renal tissue, showing greatly reduced numbers of active Caspase 3 positive tubular cells in all treated groups. Arowheads indicate active Caspase 3 positive cells.
Supplementary Figure 5 - (A) BAX mRNA expression and (B) BCL-2 mRNA expression after 1 day of reperfusion as assessed by quantitative Real Time PCR. (C) The ratio of the pro-apoptotic BAX vs. anti-apoptotic BCL-2 expression. *p<0.05 vs. Control
Supplementary Figure 6 - Transmission electron microscopy images of a tubule in a control and a pre- and posttreated kidney. The controls showed large, swollen mitochondria, loss of mitochondrial integrity and loss of mitochondrial crypts (arrowheads), while pre- and posttreatment kidneys showed normal mitochondrial morphology with normal crypts.
Supplementary Figure 7 - (A) Interstitial macrophages and (B) interstitial granulocytes after three days of reperfusion in kidney sections stained using immunohistochemistry. *p<0.05 vs. Control.
Supplementary Figure 8 - Representative photomicrographs of renal sections from all treated groups, stained for phosphorylated ERK1/2 using immunohistochemistry. Increased activation compared to sham levels is seen in all groups exposed to IRI, however, no modulation by H2S treatment could be detected.