Hydrogen sulfide attenuates hepatic ischemia-reperfusion injury: role of antioxidant and antiapoptotic signaling

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Experimental evidence suggests that liver I/R injury is biphasic: early I/R injury occurs with the initiation of an inflammatory cascade involving numerous reactive oxygen species, reactive nitrogen species (14, 38), chemokines, and cytokines (10, 28, 38), followed by neutrophil-mediated hepatic injury occurring at 6–24 h of reperfusion (18, 20). Additionally, a consequence of I/R injury is the disruption of intracellular energy metabolism and enzyme function, resulting in a depletion of ATP and an accumulation of intracellular sodium and edema (11), suggesting that the mitochondria play a role in the pathology of I/R injury (31). The role of necrosis was considered vital for I/R injury in the past, but it is now known that apoptosis also plays a significant role in cellular damage after I/R injury (12, 20).

Hydrogen sulfide (H2S) has long been considered as a toxic environmental pollutant emerging from sewers, marshes, and volcanic eruptions. Recently, H2S has been recognized alongside nitric oxide and carbon monoxide as an endogenously produced gaseous signaling molecule (29, 36, 39). In mammalian cells, H2S is produced by two heme containing enzymes, cystathionine β-synthase and cystathionine γ-lyase, the activity of which depends on pyridoxal 5′-phosphate (36, 39). Cystathionine β-synthase is primarily responsible for production of H2S in the central nervous system (8), while cystathionine γ-lyase is primarily expressed in peripheral tissues, including vascular and nonvascular smooth muscle (30, 36, 37, 48).

Since the recent discovery that H2S is a powerful physiological signaling molecule, experimental studies (30) have begun to characterize its biological profile. H2S promotes vascular smooth muscle relaxation and induces vasodilation of isolated blood vessels (3, 27, 48). H2S has also been shown to inhibit leukocyte-endothelial cell interactions in vivo (45) indicating an anti-inflammatory action. It has also become evident that H2S is a potent antioxidant (24, 40, 44) and under more chronic conditions upregulates antioxidant defenses (23, 24). It has also been demonstrated that H2S effectively inhibits apoptosis of a number of cell types (6, 32, 35), and this effect has been shown to promote cytoprotection.

Previous studies (1, 6, 35, 47) have provided insights into the protective actions of H2S in the setting of hypoxia-reoxygenation and I/R injury. However, to date the potential cytoprotective effects of H2S have not been evaluated in hepatic I/R injury. The aim of present study was to investigate the effects of an exogenous administration of H2S on the severity of I/R injury in an in vivo murine model of hepatic I/R injury.
**MATERIALS AND METHODS**

**H₂S donor.** Sodium sulfide (Na₂S, IK1001) was produced by Ikaria (Seattle, WA) by using H₂S gas (Matheson, Newark, CA) as a starting material. Na₂S was formulated to pH neutrality and iso-osmolality. Na₂S (stock solution at 0.55 mg/ml and 7.1 mM) was diluted in normal saline (100 μl) or H₂S donor (0.3, 1, and 2 mg/kg) in a final volume of 100 μl was injected intravenously into the inferior vena cava using a 32-gauge needle at 5 min before reperfusion.

**Animals.** Mice (C57BL6/J) were used for the study. All mice were male, 8–10 wk of age, and were purchased from Jackson Laboratories. All experimental procedures were approved by the Institute for Animal Care and Use Committee at Albert Einstein College of Medicine and complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**Hepatic I/R protocol.** The hepatic I/R protocol has been described previously (5). In the present study, mice were subjected to 60 min of hepatic ischemia and either 1, 5, or 24 h of reperfusion. Mice were anesthetized with xylazine (8 mg/kg) and ketamine (100 mg/kg), constituted in normal saline and administered intravenously. Core body temperature of the mice was monitored continuously during the entire period of surgery using a rectal probe, and a heat lamp was used to maintain the core body temperature at 37 ± 0.4°C. A midline laparotomy incision was performed to expose the liver. A microaneurysm clamp was applied to the hepatic artery and portal vein resulting in ischemia of the left lateral and median lobes of the liver. The procedure leads to segmental (70%) hepatic ischemia so as to prevent mesenteric venous congestion by allowing portal decompression through the caudate and right lobe of the liver. The liver was then repositioned back to its original position and kept moist throughout the period of ischemia (60 min) using gauze soaked with normal saline. Mice were injected with heparin (100 U/Kg) to prevent clotting during the period of ischemia. Mice were then centrifuged to remove platelets and blood clots. Postoperatively, mice were anesthetized again as described in the study. Intravenous administration of H₂S donor at doses of 0.3 and 1.0 mg/kg before reperfusion limited serum elevations of the liver transaminases AST and ALT in a dose-dependent manner after 60 min of ischemia and 5 h of reperfusion (Fig. 1, A and B). Specifically, the 0.3 mg/kg dose reduced serum AST by 50.2% (P < 0.05) and ALT by 41.1% (P < 0.05). The 1.0 mg/kg dose significantly reduced serum AST by 70.8% (P < 0.01) and serum ALT by 68.6% (P < 0.001). In sharp contrast to lower doses, 2.0 mg/kg of the H₂S donor increased serum AST by 151% and ALT by 124%, compared with the vehicle. Additionally, doses higher than 2 mg/kg were associated with significantly higher mortality (data not shown).

**Lipid hydroperoxide assay.** Quantification of lipid peroxidation was done to assess the extent of hepatic tissue oxidative injury. Lipid peroxidation results in the formation of highly unstable and reactive hydroperoxides of both saturated and unsaturated lipids. Hepatic tissue was collected for glutathione analysis at 1, 5, or 24 h of reperfusion. Tissue levels of reduced glutathione (GSH) and oxidized glutathione (GSSG) were measured using a commercially available kit (Cayman Chemicals). The GSH-to-GSSG ratio was calculated for each sample according to manufacturer’s recommendations.

**Western blot analysis.** Western blot analysis was performed to evaluate the protein expression of cleaved caspase-3, Bcl-2, thioredoxin-1 (Trx-1), and 90-kDa heat shock protein (HSP-90) as described previously (16). Briefly, livers (ischemic lobes only) of sham, vehicle plus I/R and H₂S plus I/R from nondiabetic mice were excised after 5 and 24 h after hepatic I/R. Samples were homogenized in 1 ml of ice-cold RIPA lysis buffer. Homogenates were then centrifuged at 1,300 g to remove any cellular debris. The pellet was discarded, and the supernatant was again centrifuged at 16,000 g for 30 min at 4°C. The resulting supernatant (cytosolic fraction) was collected. The resulting pellet was resuspended in 300 μl of RIPA lysis buffer. Protein assay was done with the use of Bio-RAD DC protein assay as per recommendations from manufacturer. An equal amount of protein (25 μg) was loaded into each well and separated on 7–12% polyacrylamide gel (7% for HSP-90, 12% for cleaved caspase 3, Bcl-2, and Trx-1). Protein was transferred to immunoblot polyvinylidene difluoride membrane and then blocked in 5% milk in Tris-buffered saline Tween-20 at room temperature. Membranes were then incubated with primary antibodies (mouse anti-Trx-1, 1:2,000; mouse anti-HSP-90, 1:5,000; mouse anti-cleaved caspase 3, 1:500, and mouse anti-Bcl-2, 1:1,500) overnight at 4°C. Membranes were then washed and incubated with horseradish peroxidase-linked anti-rabbit IgG (1:10,000) at room temperature. Membranes were then washed, followed by incubation with ECL reagents (Amersham), and then exposed to film. Membranes were then stripped and incubated with either mouse anti-α-tubulin (1:10,000) or anti-cytochrome c oxidase (COX-IV; 1:20,000) overnight at 4°C. Membranes were then washed and incubated with horseradish peroxidase-linked anti-rabbit or anti-goat secondary (1:10,000), followed by incubation with ECL (Amersham) reagents, and then exposed to film. Anti-α-tubulin antibodies were purchased from Santa Cruz Biotechnology, and all other antibodies were purchased from Cell Signaling Technology. Densitometric analysis was performed using Image J software from the National Institutes of Health.

**Statistical analysis.** Data were analyzed by one-way ANOVA with post-Turkey multiple comparison test and two-way ANOVA with post hoc Bonferroni analysis wherever appropriate using Prism software (San Diego, CA). Data are reported as means ± SE. P values <0.05 were considered to be statistically significant.

**RESULTS**

**H₂S attenuated hepatic I/R injury.** Intravenous administration of H₂S donor at doses of 0.3 and 1.0 mg/kg before reperfusion limited serum elevations of the liver transaminases AST and ALT in a dose-dependent manner after 60 min of ischemia and 5 h of reperfusion (Fig. 1, A and B). Specifically, the 0.3 mg/kg dose reduced serum AST by 50.2% (P < 0.05) and serum ALT by 41.1% (P < 0.05). The 1.0 mg/kg dose significantly reduced serum AST by 70.8% (P < 0.01) and serum ALT by 68.6% (P < 0.001). In sharp contrast to lower doses, 2.0 mg/kg of the H₂S donor increased serum AST by 151% and ALT by 124%, compared with the vehicle. Additionally, doses higher than 2 mg/kg were associated with significantly higher mortality (data not shown). Thus 1 mg/kg was considered the most effective dose for hepatocellular protection and was investigated in further experiments. Both vehicle- and H₂S-donor-treated mice had significantly higher liver transaminases as a consequence of I/R injury, compared with sham group. Sham values for AST were 58.4 ± 12.8 U/l, and ALT values in sham-operated controls were 38.3 ± 9.4 U/l.
H$_2$S increased hepatic tissue GSH-to-GSSG ratio. The GSH-to-GSSG ratio (Fig. 2A) was calculated for sham and vehicle- and H$_2$S-treated mice ($n = 6$ in each group). There was a significant decrease ($P < 0.001$) in the GSH-to-GSSG ratio in both the vehicle and H$_2$S groups as consequence of I/R injury compared with sham. However, H$_2$S attenuated the I/R-induced reduction significantly ($P < 0.05$) when compared with the vehicle-treated mice at 5 and 24 h of reperfusion. Additionally, similar trend was observed at 1 h of reperfusion. Specifically, the ratios in the H$_2$S-treated mice were 18.1, 35.8, and 63.3% higher than the vehicle-treated mice at 1, 5, and 24 h of reperfusion, respectively.

Trx-1 expression was preserved by H$_2$S after I/R injury. Western blot analysis for protein expression of Trx-1 (Fig. 2B) revealed that Trx-1 levels decreased significantly ($P < 0.05$) in the vehicle group at 5 and 24 h of reperfusion compared with the sham group. Conversely, Trx-1 levels in the H$_2$S-treated group ($1.0$ mg/kg) remained at levels similar to those of the sham group at both 5 and 24 h of reperfusion. The mean expression of Trx-1 was higher at both 5 and 24 h of reperfusion in H$_2$S group compared with vehicle-treated mice, but a statistical significance was only observed for at the 24 h time point ($P < 0.01$).

H$_2$S attenuated oxidative stress. Lipid peroxidation was used as measure of hepatic oxidative stress after I/R injury. LPO levels (Fig. 3) were measured for sham and vehicle- and H$_2$S-treated mice ($n = 8$ in each group) after I/R injury. The vehicle group exhibited significantly higher levels of lipid hydroperoxides at 1 h postreperfusion and 5 h postreperfusion compared with sham-operated controls, as a consequence of I/R injury. Conversely, H$_2$S-treated mice displayed LPO levels similar to sham at both 1 and 5 h. H$_2$S-treated mice had significant reductions in lipid peroxidation by 36% at 1 h ($P < 0.05$) and 52% at 5 h ($P < 0.001$) reperfusion compared with vehicle.

HSP-90 mediated protective effects of H$_2$S. After I/R injury, the hepatic HSP-90 protein levels in the vehicle-treated mice remained at levels similar to those observed in the sham mice (Fig. 4, A and B). Conversely, the administration of H$_2$S significantly increased the expression of HSP-90 compared with the levels observed in the sham ($P < 0.01$) and vehicle-treated mice ($P < 0.05$) at both 5 and 24 h of reperfusion.

H$_2$S increased mitochondrial Bcl-2 levels and inhibited apoptosis. To gain insights into apoptotic signaling, we evaluated the protein expression of the antiapoptotic protein Bcl-2 at 5 and 24 h after reperfusion (Figs. 4, C and D). I/R injury led
to a significant reduction in Bcl-2 expression in both the vehicle- and H2S (P < 0.001)-treated groups compared with the sham group. However, Bcl-2 protein levels were significantly (P < 0.05) higher in H2S-treated mice compared with vehicle-treated mice at 5 h reperfusion. However, at 24 h of reperfusion, Bcl-2 was not increased in the H2S-treated group.

Western blot analysis of the hepatic tissue lysate (Fig. 5, A and B) obtained after 60 min of ischemia and 24 h of reperfusion revealed a significant (P < 0.01) increase in the expression of cleaved caspase-3 in the vehicle group compared with the sham group. In contrast, the expression of cleaved caspase-3 in the H2S-treated group only increased slightly.

DISCUSSION

In the current study, administration of H2S before reperfusion significantly attenuated I/R injury in the liver. The conferred hepatoprotection was characterized by a significant attenuation of serum transaminases level and lipid peroxidation by an upregulation of antioxidant and antiapoptotic signaling.

I/R injury involves the activation of the inflammatory cascade characterized by leukocyte infiltration as well as generation of reactive oxygen and nitrogen species (21, 38). H2S possesses a number of signaling actions that are likely to attenuate the pathological aspects of I/R injury. Recently, Zanardo et. al. (45) observed that H2S inhibits aspirin-induced leukocyte adherence in mesenteric venules and subsequent diapedesis suggesting that H2S is a potent anti-inflammatory agent. H2S also directly scavenges of reactive oxygen and nitrogen species preventing tissue damage (36, 40). Furthermore, H2S reduces the protein carbonyl formation after burn and smoke injury, indicative of overall antioxidant effects (7).

GSH is a potent intracellular antioxidant, and the GSH-to-GSSG ratio corresponds to the capacity of a cell to attenuate oxidative stress (9). Thioredoxin like glutathione is a regulator of cellular oxidation/reduction (redox) status and has been shown to protect against oxidative stress (41, 46) and to inhibit apoptosis (41). Moreover, mice over expressing the human thioredoxin gene (hTrx tg) are resistant to renal I/R injury (22). In our study, we observed that H2S preserved the GSH levels,
and Trx-1 expression after hepatic I/R injury, suggesting that H₂S maintains the intracellular antioxidant capacity of hepatocytes. I/R injury involves oxidative damage not only to proteins and nucleic acids but also to lipids. Stress-induced lipid peroxidation has been associated with development of atherosclerosis, neurodegeneration, and even carcinogenesis (13). In the current study, H₂S significantly attenuated lipid peroxidation suggesting its protective effects against oxidative stress.

Apoptosis plays a significant role in the pathophysiology of I/R injury (12, 20). Previously, Rose et al. (32) demonstrated that H₂S abolished β-phenylethyl isothiocyanate induced apoptotic cell death in the human adenocarcinoma cell line HCT116, suggesting its potential to be a potent inhibitor of apoptosis. We found that the protein expression of HSP-90 was elevated in H₂S-treated mice. HSP-90 is known to attenuate the injury caused by stress by maintaining the conformation of proteins, refolding proteins damaged by stressful stimuli, and rendering cells more resistant to apoptosis. HSP-90, the most abundant heat shock protein present in eukaryotic cells, is constitutively expressed but is induced under conditions of stress like I/R injury (15). HSP-90 interacts with a variety of intracellular pathways to evade apoptosis and promote cell survival, including the phosphatidylinositol 3-kinase-AKT pathway, IGF-1, and inositol hexakisphosphate kinase-2 (2, 42). Additionally HSP-90 is known to mediate the upregulation of Bcl-2 expression induced by VEGF further contributing to apoptosis inhibition (4). Bcl-2 is an antiapoptotic protein vital for the integrity of the mitochondrial membrane, as it prevents the release of proapoptotic protein such as cytochrome c (33). Rat liver transfected with human Bcl-2 gene (hBcl-2) has been shown to be resistant to I/R injury (43). In the present study, H₂S therapy increased the protein expression of HSP-90 and Bcl-2 after I/R injury and also inhibited apoptosis, as revealed by decreased levels of cleaved caspase-3 in H₂S-treated mice. H₂S has previously been shown to protect the ischemic myocardium by preserving mitochondrial function (6). Therefore, it is possible that H₂S mediates preservation of mitochondrial function after I/R injury by upregulating Bcl-2 expression.

H₂S is a potent reversible inhibitor of cytochrome c oxidase and has been shown to inhibit mitochondrial respiration in a dose-dependent manner (6). It is possible that suprapharmacological concentrations of H₂S depress mitochondrial respiration irreversibly, affecting ATP synthesis and contributing further to I/R injury. However, to assess a complete side effect profile of H₂S is beyond the scope of current study. Pharmacological preconditioning with H₂S has been shown to protect the heart from I/R injury, and endogenous H₂S has been suggested to play a role in ischemic preconditioning (1, 17). Further studies are required to assess role of H₂S preconditioning in hepatic I/R injury. In the current study, H₂S has been shown to be protective in nondiabetic animals but its role in conditions with enhanced oxidative stress including diabetes and obesity remains unknown. Clearly, additional studies are warranted to further our understanding regarding the efficacy of H₂S under these pathological conditions.

In summary, our findings demonstrate that H₂S significantly attenuates hepatic I/R injury via preservation of intracellular redox balance and by inhibition of apoptosis during the evolution of I/R injury. These results suggest that H₂S is a promising therapeutic agent to protect against hepatic I/R injury.

REFERENCES

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