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Research Paper

Hydrogen sulfide protects from intestinal ischaemia–reperfusion injury in rats

Hao Liu[a], Xiao-Bin Bai[a], Song Shi[a] and Yong-Xiao Cao[b]

[a]Department of General Surgery, the First Affiliated Hospital and [b]Department of Pharmacology, Xi'an Jiaotong University College of Medicine, Xi'an, Shaanxi, P.R. China

Abstract

Objectives Hydrogen sulfide (H_2S) is an endogenously gaseous mediator, regulating many pathophysiological functions in mammalian cells. H_2S has been shown to inhibit myocardial ischaemia–reperfusion (I/R) injury. However, little is known about whether H_2S could modulate intestinal I/R injury. This study aimed to investigate the effect of H_2S on intestinal I/R injury and potential mechanism(s) underlying the action of H_2S in regulating the development of intestinal I/R injury in rats.

Methods Following surgical induction of intestinal I/R injury for 1 h, groups of Sprague-Dawley rats were treated with, or without, tetramethylpyrazine (8 mg/kg), or sodium hydrosulfide (NaHS, an H_2S donor at 7 or 14 μmol/kg) 30 min after occlusion. All rats were sacrificed immediately after the reperfusion. Their intestinal injury, together with that of sham-control rats, was histologically examined and their sera and intestinal malondialdehyde (MDA), superoxide dismutase (SOD), peroxidase (GSH-Px) activities were characterized by biochemical analysis.

Key findings The results showed that NaHS significantly reduced intestinal I/R injury and the levels of sera and intestinal MDA activity, and dramatically increased the levels of serum and intestinal SOD and GSH-Px activity.

Conclusions The results suggest that H_2S protects from intestinal I/R injury in rats, which is associated with increase in the activity of antioxidant enzymes.

Keywords antioxidant enzymes; hydrogen sulfide; intestinal ischaemia–reperfusion

Introduction

Ischaemia–reperfusion (I/R) injury is a leading cause of high morbidity and mortality in patients with surgical trauma.[1] Among the internal organs, the intestine is probably the most sensitive to I/R injury.[2] Intestinal I/R injury is considered to be a major clinical challenge as it often occurs in patients suffering from coagulopathies, mechanical obstruction and severe trauma, and in patients who have experienced cardiac surgery and liver or intestinal transplantation. The injury can progress into multiple organ dysfunction syndrome.[3] Although great efforts have been made to develop new drugs for treatment of intestinal I/R injury in animal models there is none showing clear promise at the clinic. Therefore, discovery and development of new therapeutic agents for intervention of intestinal I/R injury will be of great significance.

Hydrogen sulfide (H_2S), like nitric oxide and carbon monoxide, is an endogenously gaseous mediator, regulating the process of many physiological and pathological functions, such as blood pressure, neurotransmission and inflammation, in mammalian tissues.[4,5] Recently, a large body of evidence has demonstrated that H_2S protects from I/R injury in various tissues. Recent studies have shown that exogenous H_2S or treatment with an H_2S donor, sodium hydrosulfide (NaHS), protects against irreversible myocardial I/R injury in rats and mice.[6,7] These protective effects may be associated with a decrease in the mitochondrial respiration during reperfusion, thereby reducing oxidant generation and subsequent myocardial cell apoptosis. Furthermore, S-diclofenac, a new H_2S-releasing derivative of diclofenac, shows strong anti-ischaemic activity in the I/R rabbit model and can increase the formation of reduced glutathione (GSH) and directly activate the K_{ATP} channels.[8] However, little is known about whether H_2S could regulate intestinal I/R injury.

To address these questions, we examined the impact of treatment with NaHS on intestinal tissue injury in a rat model of intestinal I/R injury and attempted to determine the mechanism underlying the action of NaHS treatment in regulating intestinal I/R injury by measuring the levels of malondialdehyde (MDA), a marker of radical injury, and the

Correspondence: Prof. Yong-Xiao Cao, Department of Pharmacology, Xi'an Jiaotong University College of Medicine, Xi'an, Shaanxi, 710061, P.R. China.
E-mail: yxy@xjtu.edu.cn

activity of antioxidant enzymes superoxidase dismutase (SOD) and glutathione peroxidase (GSH-Px). We found that treatment with NaHS significantly reduced the severity of intestinal I/R injury and MDA levels, and increased the levels of SOD and GSH-Px activity in rats. Our results suggest that H_2S protects from intestinal I/R injury by increasing the levels of antioxidant enzymes.

Materials and Methods

Reagents

NaHS was purchased from Sigma Co. Ltd (MO, US) and dissolved in 0.9% saline for administration. Injectable tetramethylpyrazine was from Xi'an Pharmaceutical Ltd (Xi'an, China).

Animals

Male Sprague-Dawley rats, 200–240 g, were obtained from the Animal Center of Xi'an Jiaotong University College of Medicine (Xi'an, China). All animal experiments were conducted in accordance with international ethical guidelines and the experimental protocols for using rats have been reviewed and approved by the Animal Ethics Committee at Xi'an Jiaotong University.

Experimental design and intestinal I/R model

A total of 40 rats were randomly divided into five groups ($n = 8$ for each group): the sham-control group (A); the I/R group (B); the tetramethylpyrazine positive control group (C; tetramethylpyrazine possesses a protective effect on intestinal I/R injury);[9–12] the 7 μmol/kg NaHS group (D); and the 14 μmol/kg NaHS group (E).[13,14] All of the rats were subjected to the same surgical procedure to establish a rat model of intestinal I/R injury as described previously,[15] except for the sham control group that received the surgery, but not occlusion. Briefly, experimental rats were fasted overnight and were anaesthetized intraperitoneally with chloral hydrate (10 mg/kg). The right groin of each rat was opened and the femoral artery was cannulated with a polyethylene (PE)-50 catheter attached to an injection tube for administration of medicine or collection of blood. The abdominal cavity was opened with a midline abdominal incision and the superior mesenteric artery was isolated meticulously at its origin; the accompanying vein remained intact. After stabilization for 10 min, the superior mesenteric artery was occluded with an atraumatic microvascular clamp for 1 h and subsequently it was reperfused for 1 h.[16–20] The C group of rats was treated with 8 mg/kg tetramethylpyrazine while the D and E groups of rats were treated with NaHS at 7 μmol/kg or 14 μmol/kg, respectively, via the femoral artery 30 min before reperfusion.[10] The control and intestinal I/R groups of rats received an equal volume of saline. All rats were sacrificed immediately after the reperfusion. Intestinal tissues and blood samples were obtained from individual rats.

Histopathological examination of intestines

A segment of ileum, 5 cm long, near the ileum–caecum from individual rats was fixed with 4% paraformaldehyde for 24 h, and transferred to 30% sucrose at 4°C overnight. After being embedded in OCT, the ileum sections at 10 μm were prepared and stained with haematoxylin and eosin for examination under a light microscope. Intestinal injury was evaluated by a pathologist in a blind fashion according to the criteria described by Chiu et al.[21] and graded from 0 to 5.[21,22] The grades are: grade 0, normal mucosa; grade 1, formation of subepithelial detachments at the tip of the villi with capillary congestion; grade 2, subepithelial detachments exerted a moderate amount of upward push on the mucosa epithelium; grade 3, large subepithelial detachments exerted a massive amount of upward push on the mucosa epithelium along the villi and few denuded villus tips were observed; grade 4, the villi were denuded to the level of lamina propria and dilated capillaries; grade 5, presence of ulceration, disintegration of lamina propria and haemorrhage.

Biochemical analysis

Individual sera were prepared by centrifugation. Approximately 100 mg of ileum tissue from near the ileum–caecum from individual rats was homogenized in 2 ml Kreb's solution and the supernatants were obtained by centrifugation. The contents of MDA, SOD and GSH-Px in sera and intestinal tissue supernatants were measured using the enzyme-specific activity detection kits from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China), according to the manufacturer's instructions.

Statistical analysis

Data were presented as mean ± SD, and differences were analysed by one-way analysis of variance using the SPSS 11.5 statistical software package (SPSS, IL, US). $P < 0.05$ was considered statistically significant.

Results

NaHS reduced the severity of intestinal I/R injury

We firstly evaluated the effect of H_2S on intestinal I/R injury in rats. Haematoxylin and eosin staining was carried out to determine the histological changes in the ileum tissue. Histological evaluation was performed according to the Chiu scoring method. In comparison with the sham-operated group (Figure 1a), serious intestinal injury was observed in the I/R group of rats (Figure 1b), shown by scattered haemorrhage and ulceration on intestinal mucosa, dilated epithelial cells, broken villi, moderate lifting of the epithelial layer, neutrophil infiltration under the epithelial mucosa and expansion of capillaries and lymphatic capillaries. In contrast, treatment with tetramethylpyrazine reduced the severity of intestinal I/R injury, shown by the characteristics of fewer dilated epithelial cells and neutrophil infiltration (Figure 1c),[12] consistent with previous reports.[23] Treatment of rats with 7 μmol/kg NaHS resulted in a remarkable reduction in the severity of intestinal I/R injury, demonstrated by intact villi, little neutrophil infiltration and little expansion of capillaries (Figure 1d). According to the Chiu scoring system, the injury score in the sham group was 0.6 ± 0.04, and the injury score in the I/R group was increased to 3.8 ± 0.7 ($P < 0.01$). It was determined that

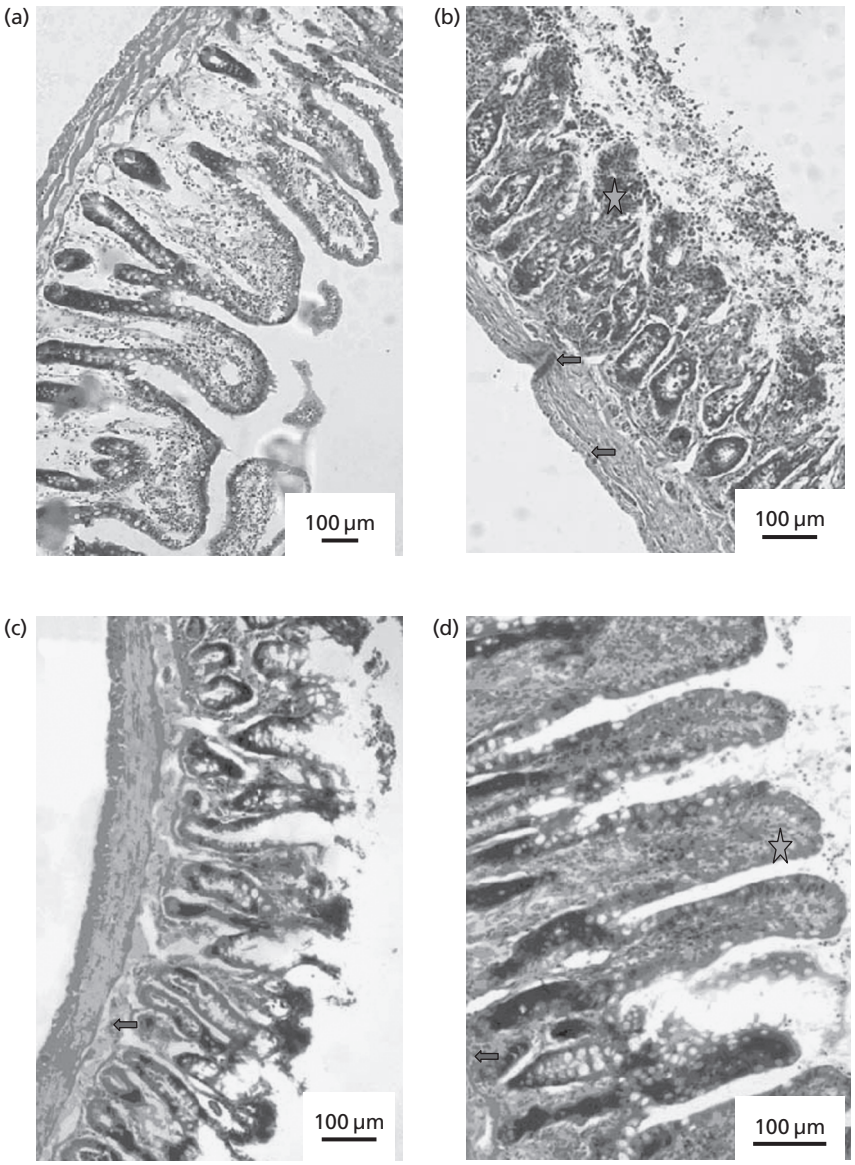

Figure 1 Effect of NaHS on intestinal I/R injury in rats. Intestine tissues were dissected from individual rats and stained by haematoxylin and eosin. Data shown are representatives of ileum sections from each group. Compared with the control sham-operated group (a), serious intestinal injury was observed in the I/R group (b), as shown by scattered haemorrhage and ulceration on intestinal mucosa, dilated epithelial cells, broken villi, moderate lifting of the epithelial layer (star), neutrophil infiltration under the epithelial mucosa and expansion of capillaries and lymphatic capillaries (arrows). Treatment with 8 mg/kg tetramethylpyrazine (c), reduced the severity of intestinal I/R injury by the characteristics of fewer dilated epithelial cells and neutrophil infiltration (arrow). Treatment with 7 mol/kg NaHS (d), resulted in a remarkable reduction in the severity of intestinal I/R injury, demonstrated by intact villi (star), little neutrophil infiltration and little expansion of capillaries (arrow).

NaHS 7 μmol/kg and 14 μmol/kg significantly prevented the mucosal injury caused by I/R (NaHS 7 μmol/kg, I/R 2.6 ± 0.4; NaHS 14 μmol/kg, I/R 2.3 ± 0.3 vs I/R 3.8 ± 0.7; $P < 0.01$, respectively), as did tetramethylpyrazine (3.1 ± 0.6 vs I/R 3.8 ± 0.7; $P < 0.05$). These observations demonstrated that treatment with NaHS effectively mitigated the severity of intestinal I/R injury and its therapeutic effect appeared to be better than that of treatment with tetramethylpyrazine. Therefore, H_2S was likely to protect from the I/R intestinal injury *in vivo*.

NaHS inhibited the levels of serum and intestinal MDA

Oxidative stress and the subsequent over-production of free radical species have been thought to contribute to the pathogenesis of I/R.[24] To understand the mechanism underlying the action of H_2S in regulating the intestinal I/R injury, we characterized the levels of serum and intestinal MDA, one of the markers of free radical species-related injury[25] in the rats with intestinal I/R injury. As expected, the levels of serum and

Table 1 The effect of H_2S on the levels of serum and intestinal MDA in rats

Group	Dose	Serum MDA (nmol/ml)	Intestinal MDA (nmol/mg protein)
Sham	N.A.	6.9 ± 1.5**	10.6 ± 1.7**
I/R model	N.A.	14.9 ± 2.8	17.8 ± 1.6
Tetramethylpyrazine	8 mg/kg	10.2 ± 2.4**	16.2 ± 2.1
NaHS	7 μmol/kg	9.8 ± 2.0**	15.4 ± 1.9*
NaHS	14 μmol/kg	8.4 ± 1.8**	15.1 ± 1.8**

I/R, ischaemia–reperfusion; MDA, malondialdehyde; N.A., not applicable. Data are presented as mean ± SD of each group of rats, $n = 8$ per group. *$P < 0.05$, **$P < 0.01$ vs the I/R group.

intestinal MDA were significantly elevated in the I/R group of rats, as compared with the controls (Table 1). Treatment with tetramethylpyrazine at 8 mg/kg greatly reduced the levels of serum MDA, but not intestinal MDA, as compared with the levels in the I/R group of rats. Treatment with 7 μmol/kg NaHS significantly decreased the levels of serum and intestinal MDA by 34% and 14%, respectively. Treatment with an increased dose of NaHS had a trend of further decreasing serum MDA. However there was no significant difference between the low and high dose of NaHS, or between high NaHS and tetramethylpyrazine. The results suggest that H_2S appeared to be a potent antioxidant regulator, inhibiting the I/R-related intestinal injury in rats.

NaHS increased the levels of serum and intestinal SOD and GSH-Px

Next, we tested whether treatment with NaHS could modulate the levels of SOD and GSH-Px, two of the most important antioxidant enzymes, in sera and intestine tissues of rats with I/R injury. We found that following the induction of intestinal I/R injury, the levels of serum and intestinal SOD in the I/R group were dramatically lower than those in sham-control group, while treatment with tetramethylpyrazine reversed the effect of the I/R injury by increasing the levels of serum and intestinal SOD significantly (Table 2). Treatment with NaHS increased the levels of both serum and intestinal SOD, with a dose-dependent trend, and its antioxidant activity was significantly greater than that with tetramethylpyrazine.

Table 2 The effect of H_2S on the levels of serum and intestinal SOD activity in rats

Group	Dose	Serum SOD (U/ml)	Intestinal SOD (U/ml)
Sham	N.A.	106 ± 10**	319 ± 14**
I/R model	N.A.	54 ± 15	245 ± 15
Tetramethylpyrazine	8 mg/kg	75 ± 7*	276 ± 11**
NaHS	7 μmol/kg	86 ± 10**$^{\#}$	290 ± 15**$^{\#}$
NaHS	14 μmol/kg	95 ± 9**$^{\#\#}$	302 ± 13**$^{\#\#}$

I/R, ischaemia–reperfusion; N.A., not applicable; SOD, superoxide dismutase.
Data are presented as mean ± SD of each group of rats, $n = 8$ per group. *$P < 0.05$, **$P < 0.01$ vs the I/R group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs tetramethylpyrazine.

Table 3 The effect of H_2S on the levels of serum and intestinal GSH-Px activity in rats

Group	Dose	Serum GSH-Px (U)	Intestinal GSH-Px (U)
Sham	N.A.	880 ± 86**	1143 ± 139**
I/R model	N.A.	612 ± 88	687 ± 100
Tetramethylpyrazine	8 mg/kg	698 ± 71*	807 ± 118*
NaHS	7 μmol/kg	704 ± 62*	902 ± 123**
NaHS	14 μmol/kg	752 ± 67**	998 ± 121**$^{\#}$

GSH-Px, glutathione peroxidase; I/R, ischaemia–reperfusion; N.A., not applicable.
Data are presented as mean ± SD of each group of rats, $n = 8$ per group. *$P < 0.05$, **$P < 0.01$ vs I/R model; $^{\#}P < 0.01$ vs tetramethylpyrazine.

Similarly, while intestinal I/R injury reduced the levels of GSH-Px, treatment with tetramethylpyrazine mitigated the reduction in serum and intestinal GSH-Px activity induced by I/R injury. Importantly, treatment with 14 μmol/kg NaHS significantly increased the levels of GSH-Px activity from 612 ± 88 U to 752 ± 67 U in sera and 687 ± 100 to 998 ± 121 U in intestinal tissue, which was a greater effect than that of treatment with tetramethylpyrazine (Table 3). Together, the elevated levels of serum and intestinal SOD and GSH-Px activity by NaHS should contribute to the protection by H_2S against intestinal I/R injury in rats.

Discussion

H_2S is the latest gas to be recognized as an important endogenous mediator and is biosynthesized from the amino acid cysteine by the action of cystathionine β-synthase (CBS) with serine, which is controlled by the intracellular concentration of calcium and regulated by S-adenosylmethionine. To investigate the effect of H_2S, NaHS was used as an H_2S donor to treat rats for the following reasons: (1) NaHS dissociates to Na^+ and HS^- in solution, and then HS^- associates with H^+ and produces H_2S;[26] (2) NaHS relaxes the ileum and colon of rats and rabbits.[27]

Similar to NO, H_2S is being implicated both as an agent preventing tissue damage and inflammation and as an agent causing tissue damage and inflammation. H_2S, similarly to NO and CO, probably acts as a neuromodulator and an intracellular messenger, and exhibits complex biological effects such as contraction versus relaxation of blood vessels, apoptotic versus anti-apoptotic, neurodegenerative versus neuroprotective, pro-inflammatory versus anti-inflammatory and hyperalgesic versus analgesic activity in many mammalian and non-mammalian systems.[28] Possible physiological functions of H_2S include potentiating long-term potentials through activation of the NMDA receptors, regulating the redox status, maintaining the excitatory/inhibitory balance in neurotransmission and inhibiting oxidative damage through scavenging free radicals and reactive species.[29] In neurons, H_2S stimulates the production of cAMP probably by direct activation of adenylyl cyclase and thus activates cAMP-dependent processes. Rat gastrointestinal mucosa could synthesize H_2S. In the context of the digestive system, roles for H_2S in the maintenance of mucosal integrity, regulation of blood flow and modulation of

inflammatory reactions are emerging.[30] Apart from these relaxant effects, it seems that H_2S may have also exerted antinociceptive effects because NaHS reduced the pain induced by colorectal distention in healthy rats and in rats with colitis.[26]

In this study, we examined whether H_2S could modulate intestinal I/R injury and determined the potential mechanism(s) underlying the action of H_2S in regulating intestinal I/R injury in rats. We found that following the induction of intestinal I/R injury, massive intestinal destruction with scattered haemorrhage and intestinal mucosal ulceration, dilated epithelial cells, broken villi and epithelial layer, neutrophil infiltration under the epithelial mucosa and blood and lymphatic capillary expansion occurred in the rats, consistent with previous reports.[31,32] In contrast, NaHS, a widely-recognized H_2S donor, significantly reduced the severity of epithelial and mucosal damage, as did tetramethylpyrazine, an important alkaloid from Rhizoma Chuanxiong. Many investigations have shown that tetramethylpyrazine has a protective effect on intestinal I/R injury in rats and rabbits,[9–12] which is consistent with our results. Also, the anti-I/R activity of NaHS was more potent than that of tetramethylpyrazine, demonstrating that H_2S can protect against the intestinal I/R injury in rats.

The intestinal I/R injury is a complex pathophysiological process, involving many factors, such as oxidative-stress-related free radical species and pro-inflammatory cytokines. During the process of intestinal I/R injury, the I/R activates neutrophils and damages the mucosal epithelia, which induces the production and release of reactive oxygen species (ROS) and cytotoxic factors into the extra-cellular matrix, triggering the inflammatory cascades of the radical-induced I/R injury.[2] As a result, the ROS-related inflammation promotes lipid peroxidation and aggravates oxidative-stress-associated tissue injury, increasing the formation of MDA. Subsequently, oxidative stress and higher levels of ROS production stimulate pathophysiological responses, which over-exhaust the antioxidant enzymes, SOD and GSH-Px, reducing the levels of SOD and GSH-Px in the body. We found that induction of intestinal I/R injury promoted higher levels of serum and intestinal MDA in the rats, in accordance with previous reports.[31,32] Treatment with tetramethylpyrazine reduced the levels of serum MDA, while treatment with NaHS greatly inhibited the formation of both serum and intestinal MDA. Importantly, treatment with NaHS significantly increased the levels of serum and intestinal SOD and GSH-Px. The significantly higher levels of antioxidant and free radical scavengers, SOD and GHS-Px, promoted by H_2S should contribute to the protective effect of H_2S by increasing the metabolism of superoxide, and cysteine transport, and neutralizing hydrogen peroxides and lipoperoxides.[8] Indeed, H_2S has been demonstrated to increase serum GSH by up-regulating cysteine transport in a model of I/R and GSH can act either as an electron donor to neutralize hydrogen peroxides and lipoperoxides or as a direct free radical scavenger.[33] Importantly, increased levels of GSH-Px have been shown to protect against myocardial I/R injury.[34] Apparently, H_2S inhibits the intestinal I/R injury by up-regulating the activity of antioxidant SOD and GSH-Px, which in turn scavenges the I/R-related ROS, mitigating the intestinal I/R injury in rats.

Compared with NO and CO, the study of the biology of H_2S is still in its infancy. Roles for H_2S have been identified but not yet completely defined. Nevertheless, these controversies will likely only be clarified when more highly selective inhibitors of H_2S synthesis and simpler methods for quantification of H_2S production become available.

Conclusions

Our data provide the first evidence to demonstrate that H_2S can protect against intestinal I/R injury in rats and that its therapeutic effects may be mediated by increasing the activity of antioxidant SOD and GSH-Px and reducing oxidative stress-related mucosal injury. Our findings may provide a basis for the design of new therapy for intervention in intestinal I/R injury.

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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