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Hydrogen Sulfide Attenuates Carbon Tetrachloride-Induced Hepatotoxicity, Liver Cirrhosis and Portal Hypertension in Rats

Gang Tan1, Shangha Pan1, Jie Li3, Xuesong Dong1, Kai Kang2, Mingyan Zhao2, Xian Jiang1, Jagat R. Kanwar4, Haiquan Qiao1, Hongchi Jiang1, Xueying Sun1,5*

1 Department of General Surgery, The Hepatoplastic Surgery Center, The First Affiliated Hospital of Harbin Medical University, Harbin, China, 2 Department of ICU, The First Affiliated Hospital of Harbin Medical University, Harbin, China, 3 Department of Hepatobiliary Surgery, Affiliated Qianfoshan Hospital of Shandong University, Jinan, China, 4 Laboratory of Immunology and Molecular Biomedical Research, Centre for Biotechnology and Interdisciplinary Biosciences, Institute for Technology and Research Innovation, Deakin University, Geelong, Victoria, Australia, 5 Department of Molecular Medicine and Pathology, Faculty of Medical and Health Sciences, The University of Auckland, Auckland, New Zealand

Abstract

Background: Hydrogen sulfide (H2S) displays vasodilative, anti-oxidative, anti-inflammatory and cytoprotective activities. Impaired production of H2S contributes to the increased intrahepatic resistance in cirrhotic livers. The study aimed to investigate the roles of H2S in carbon tetrachloride (CCl4)-induced hepatotoxicity, cirrhosis and portal hypertension.

Methods and Findings: Sodium hydrosulfide (NaHS), a donor of H2S, and DL-propargylglycine (PAG), an irreversible inhibitor of cystathionine γ-lyase (CSE), were applied to the rats to investigate the effects of H2S on CCl4-induced acute hepatotoxicity, cirrhosis and portal hypertension by measuring serum levels of H2S, hepatic H2S production and CSE expression, liver function, activity of cytochrome P450 (CYP) 2E1, oxidative and inflammatory parameters, liver fibrosis and portal pressure. CCl4 significantly reduced serum levels of H2S, hepatic H2S production and CSE expression. NaHS attenuated CCl4-induced acute hepatotoxicity by supplementing exogenous H2S, which displayed anti-oxidative activities and inhibited the CYP2E1 activity. NaHS protected liver function, attenuated liver fibrosis, inhibited inflammation, and reduced the portal pressure, evidenced by the alterations of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), hyaluronic acid (HA), albumin, tumor necrosis factor (TNF-α), interleukin (IL)-1β, IL-6 and soluble intercellular adhesion molecule (ICAM)-1, liver histology, hepatic hydroxyproline content and α-smooth muscle actin (SMA) expression. PAG showed opposing effects to NaHS on most of the above parameters.

Conclusions: Exogenous H2S attenuates CCl4-induced hepatotoxicity, liver cirrhosis and portal hypertension by its multiple functions including anti-oxidation, anti-inflammation, cytoprotection and anti-fibrosis, indicating that targeting H2S may present a promising approach, particularly for its prophylactic effects, against liver cirrhosis and portal hypertension.


Introduction

Hydrogen sulfide (H2S) has displayed many physiological and pathological activities [1]. Administration of H2S limited myocardial infarct size caused by ischemia reperfusion injury [IRI] [2,3], suppressed development of gastric ulcer [4], alleviated hypoxic pulmonary hypertension [5], attenuated neuronal injury [6], and prevented the development of hypertension [7]. An H2S-releasing molecule, GYY4137, protected endotoxic shock by decreasing the production of proinflammatory cytokines including tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) [8]. The multiple actions of H2S mainly involve inhibiting oxidative stress [9], production of lipid peroxidation and inflammatory factors [4], and activating ATP sensitive potassium (KATP) channels [10]. H2S has also shown regulatory effects on hepatic physiology and pathology [11]. H2S is endogenously produced in mammalian tissues by cystathionine γ-lyase (CSE) and cystathionine β-synthase (CBS) [1], but CBS accounts for only 3% of H2S production in livers [12]. CSE-derived H2S contributes to hepatic arterial buffer response, mediates vasorelaxation of the hepatic artery via activation of KATP channels [13], and modulates biliary bicarbonate excretion [14]. H2S has exhibited anti-inflammatory and cytoprotective activities against hepatic IRI [15], and protected acetaminophen-induced hepatotoxicity in mice [16]. Diallyl trisulfide, an H2S-releasing chemical, protected from carbon tetrachloride (CCl4)-induced liver injury [17,18].

The reduction of H2S production and CSE expression is related to the development of increased intrahepatic resistance and portal
hypertension in a rat model of liver cirrhosis induced by CCl₄ [19], and H₂S counteracts the impaired vasodilation and hepatic stellate cell contraction, which contribute to the dynamic component of portal hypertension [20]. These two studies have focused on the regulatory effects of H₂S in already established portal hypertension mainly by its vasodilative activities via activating K₅₀P channels. As mentioned in one report [20], liver fibrosis represents the main causative factor in portal hypertension in cirrhosis, but the role of H₂S in the development of cirrhosis remains unclear. CCl₄ has been widely used to induce liver injury and cirrhosis in animal models, as it is rapidly metabolized into the trichloromethyl radical by cytochrome P450 (CYP) 2E1, and the reactive intermediate attacks membrane lipids, resulting in the formation of lipid peroxide molecules and necrosis of hepatocytes [21]. Given that H₂S displays anti-oxidative, anti-inflammatory and cytoprotective activities, an anti-fibrotic effect against pulmonary fibrosis [22], and a regulatory effect on hypoxic pulmonary hypertension [23] and portal hypertension [19,20], we hypothesized that H₂S might have a protective effect against CCl₄-induced acute hepatotoxicity, and the resulting liver cirrhosis and portal hypertension.

Methods

Ethics Statement

All the procedures and care administered to the animals have been approved by the institutional ethic committee, under a permit of animal use (SYXK20020009) in the First Affiliated Hospital of Harbin Medical University, compliance with the Experimental Animal Regulations by the National Science and Technology Commission, China.

Animals

Male Wistar rats (weighing 210-230 g) were supplied by the Animal Research Center at the First Affiliated Hospital of Harbin Medical University, Harbin, China. They were housed in the animal facility with a 12-h-light/ -dark cycle and the temperature was maintained at 22-23°C.

Acute hepatotoxicity experiment

The rats received phenobarbital sodium (0.35 g/L) in drinking water for 3 days, followed by a single i.p. injection of CCl₄ (Nanjing Chem. Ltd., Nanjing, China) diluted in equal volume of paraffin oil at a dose of 3 ml/kg body weight. Then the rats were randomly assigned to 3 groups (each had 6 rats): control, NaHS and PAG, receiving an i.p. injection of 1 ml of physiological saline, NaHS (Sigma-Aldrich) solution (14 μmol/kg body weight), or PAG (Sigma-Aldrich) solution (50 mg/kg body weight), respectively. The injection was repeated every 6 h. The rats were killed 48 h after CCl₄ administration. Six untreated rats served as healthy controls. Blood was collected via cardiac puncture, and serum was prepared by centrifugation at 2000 × g for 10 min, and stored at -80°C. Livers were divided into two parts, one fixed in 10% buffered formalin, the other stored at -80°C.

Induction of liver cirrhosis

Liver cirrhosis was induced by CCl₄ as described previously [20,21]. Briefly, rats received phenobarbital sodium (0.35 g/L) in drinking water for 3 days, followed by i.p. injections of 1 ml/kg body weight of CCl₄ diluted in an equal volume of paraffin oil twice a week for 12 weeks.

Prevention experiment

Thirty-six rats receiving i.p. injections of CCl₄ as above were randomly assigned into 3 groups (each had 12 rats): saline, NaHS and PAG, receiving i.p. injections of 1 ml of saline, NaHS solution (10 μmol/kg body weight) and PAG solution (30 mg/kg body weight), respectively, every two days for 12 weeks, starting on the same day of CCl₄ administration. 300 μl of blood samples were collected by tail tipping at indicated time points. At completion of the experiments, the rats were weighed, and the portal pressure measured as described below, and 300 μl of blood sample was collected from the portal vein. The spleen was weighed as % of bodyweight and used as a parameter for portal hypertension. Blood was collected via cardiac puncture, and then the liver and spleen were collected. Serum and liver samples were prepared and stored as above.

Measurement of portal pressure

The methodology has been described previously [24]. Briefly, under anesthesia by an i.p. injection of sodium pentobarbital (50 mg/kg body weight), rats underwent laparotomy, and a PE-tube (23G, 0.6 × 30 mm) was inserted over the ileocolic vein and advanced toward the confluence of the portal and splenic veins. This cannula was used to monitor portal pressure for 5 min by a Medlab-Ulg4Cs bio-signal processing system (Nanjing Medease Science and Technology co., Ltd, Nanjing, China).

Treatment experiment

Rats with liver cirrhosis induced by 12-week administration of CCl₄ as above were randomly assigned to 3 groups (each had 6 rats): saline, NaHS and PAG, receiving daily injection of 1 ml of saline, NaHS solution (10 μmol/kg) and PAG solution (30 mg/kg), respectively, for 5 days. At completion of the experiments, the portal pressure measured, and then serum and liver samples were prepared.

Measuring H₂S production in livers and H₂S concentration in sera

The methods have been described previously [15,25]. All samples were assayed in triplicate and H₂S was calculated against a calibration curve of NaHS (0.122–250 μmol/L). The H₂S producing activity was expressed as μmol of H₂S formed/g tissues.

RT-PCR analysis

The analysis of CES mRNA by RT-PCR has been described previously [15]. A pair of primers 5'-GAC CTC ATT AGT CGG CTT CTT TTC -3' and 5'-CAG TTT TGC GTA TGC TCC GTA ATG -3' was used to generate a 618 bp product of CES mRNA. A 497 bp PCR product was amplified from glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA as an internal control, with a pair of primers: 5'-GAAGGTTAGACGGAGT-3' and 5'-TGAAACCATTAGCACCCTC-3'. PCR products underwent a 2% agarose gel electrophoresis, and the density of each band was evaluated using the gel image analyzer (UV ChemiDOC, USA). The relative density of mRNA levels was calculated as the following formula: band density/ GAPDH band density.

Measurement of serum parameters

The serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), hyaluronic acid (HA) and albumin were measured with an auto-biochemical analyzer (Toshiba, Japan). The serum levels of TNF-α, IL-6, interleukin-1β (IL-1β) and soluble intercellular adhesion molecule-1 (ICAM-1) were measured with ELISA kits (BPB Biomedicals, Inc., USA).

Measurement of CYP2E1 activity in livers

The CYP2E1 activity was assayed using p-nitrophenol (Sigma-Aldrich) as a substrate as described previously [26].
Measurement of hepatic malondialdehyde (MDA) and glutathione (GSH)

Liver tissue was homogenized in a buffer containing 9 volumes of 0.15 mol/L KCl-1.0 mmol/L EDTA to obtain 1:10 (w/v) homogenates. Homogenates were then centrifuged at 10,000 g (4°C) for 30 min to collect the supernatant for determining the concentrations of MDA, GSH and total protein. MDA was evaluated by the thioribarbituric acid reactive substances (TBARS) method [27]. The final concentration of MDA was expressed as μmol/g protein. GSH were measured using commercially available kits (Jiancheng Institute of Biotechnology, Nanjing, China) and expressed as nmol/mg protein. Protein concentration of liver homogenate was determined by the Bradford method, using bovine serum albumin as a standard [28].

Measurement of hepatic hydroxyproline

Hepatic hydroxyproline was measured as described previously [29].

Histological analysis

Formalin-fixed liver specimens were embedded in paraffin, sectioned, stained with hematoxylin and eosin (HE) or Masson, and examined under light microscopy. Quantifying liver fibrosis was performed by measuring blue pixels of the images taken from Masson-stained sections. Ten photographs (200 × magnification) were randomly taken from each liver at fixed exposure time and conditions. The pictures were saved as JPEG [joint photographic experts group]. The histogram function in Adobe Photoshop CS4 was used to count the blue pixels, and the numbers of red pixels were recorded for each picture.

Western Blot analysis

The methodology has been described previously [15]. The Abs against CSE or α-smooth muscle actin (α-SMA) (Santa Cruz Biotechnology, Inc. Santa Cruz, CA) were used. The levels of proteins were normalized with respect to band density of β-actin, as an internal control.

Statistics

Results were expressed as mean values ± standard deviation (SD). A one-way analysis of variance (ANOVA) followed by the post-hoc Dunnett’s test was used for evaluating statistical significance (SPSS 17.0). A value of P<0.05 was considered significant.

Results

H₂S protects livers from CCl₄-induced acute toxicity

Administration of NaHS significantly attenuated, while PAG further raised, the elevated serum levels of ALT and AST by CCl₄ (Table 1). The impaired liver function by CCl₄ and the protective effects of H₂S were supported by the histological alterations (Figure S1). The liver sections from healthy control rats showed normal histology (Figure S1A), CCl₄-treated livers had focal midzonal necrosis and ballooning degeneration (Figure S1B). NaHS attenuated the necrosis and vacuolization (Figure S1C), while PAG further aggravated the hepatotoxicity and caused more inflammatory cells infiltration (Figure S1D). NaHS also significantly reduced the activity of CYP2E1, the major enzyme to metabolize CCl₄, in livers from CCl₄-treated rats, compared with saline, while either CCl₄ or PAG had no effects on its activity (Table 1). CCl₄-treated rats had a significant higher level of hepatic MDA (a marker of lipid peroxidation) and a significantly lower level of hepatic GSH, compared to healthy controls. NaHS significantly attenuated the increase of hepatic MDA, and restored hepatic GSH, but PAG only had slight effects on hepatic MDA and GSH, in CCl₄-treated rats, compared with saline (Table 1).

Serum levels of H₂S, production of H₂S and CSE expression in the acute experiment

The serum levels of H₂S in CCl₄ + saline-treated rats were significantly lower than that in healthy controls (P<0.05) (Fig. 1A), supported by that CCl₄ significantly (P<0.05) reduced hepatic H₂S producing activity (Fig. 1B). Hepatic expressions of CSE mRNA (Fig. 1C and D) and protein (Fig. 1E and F) were significantly lower than that in healthy rats. NaHS significantly (P<0.001) increased the levels of H₂S (Fig. 1A), but had no effect on hepatic H₂S producing activity (Fig. 1B) and the expression of CSE mRNA (Fig. 1C and D) and protein (Fig. 1E and F) in CCl₄-treated rats, compared with saline. However, PAG significantly (both P<0.05) reduced the levels of H₂S (Fig. 1A) and H₂S producing activity (Fig. 1B), but had no effect on the expression of CSE mRNA (Fig. 1C and D) and protein (Fig. 1E and F) in CCl₄-treated rats, compared with saline.

H₂S production in cirrhotic rats induced by CCl₄

Among the rats in the prevention experiment, one from the CCl₄ + saline group and two from the CCl₄ + PAG group died.
before completion of the experiment, and were excluded from the study. Blood samples were collected at indicated time points to measure the serum levels of H2S in rats. The healthy rats had a stable serum level of H2S, while the H2S levels in CCl4-induced cirrhotic rats receiving injection of saline declined at week 4 after CCl4 administration, and continued dropping as the H2S levels were even lower at week 8 and 12 than that at week 4 (Fig. 2A). However, NaHS significantly (P<0.05) elevated the serum level of H2S at week 4 after CCl4 administration in CCl4-induced cirrhotic rats, compared with saline, and serum H2S was maintained at a higher level at week 8 and 12 (Fig. 2A). Administration of PAG resulted in significantly (All P<0.05) lower serum levels of H2S in
CCl4-induced cirrhotic rats, than saline, at all the three time points (Fig. 2A). The portal serum levels of H2S at the completion of experiment was significantly (P<0.05) lower in CCl4-induced cirrhotic rats receiving injection of saline than the healthy controls (Fig. 2B). However, NaHS significantly (P<0.05) elevated, but PAG did not significantly (P>0.05) decrease, the portal serum levels of H2S, in CCl4-induced cirrhotic rats, compared with saline (Fig. 2B). We further examined the H2S producing activity (Fig. 2C) in livers from CCl4-induced cirrhotic rat receiving injections of saline, NaHS or PAG, and the similar results were obtained as...
H₂S attenuates the impaired liver function and elevated inflammatory factors in cirrhotic rats

Cirrhotic rats induced by 12-week administration of CCl₄ had significantly higher levels of ALT, AST, HA and lower level of albumin, than healthy controls (Table 2). However, simultaneous administration of NaHS resulted in significantly lower levels of ALT and slight reduction of albumin level, in CCl₄-induced cirrhotic rats, compared with saline (Fig. 2D and E). PAG significantly (P<0.05) higher level of albumin, than healthy controls (Table 2). However, simultaneous administration of NaHS resulted in significantly lower levels of all the four cytokines including TNF-α, IL-1β and IL-6, and soluble ICAM-1 (Table 2), which have been shown to play important roles in development of cirrhosis. However, simultaneous administration of NaHS resulted in significantly lower levels of all the four parameters in CCl₄-induced cirrhotic rats than saline, while PAG led to significantly higher levels of TNF-α and soluble ICAM, and slight increase of IL-1β and IL-6, in CCl₄-induced cirrhotic rats, compared with saline (Table 2).

H₂S attenuates CCl₄-induced liver cirrhosis and portal hypertension

The Masson-stained liver sections from healthy controls had almost no collagen fibers of blue color (Fig. 3A), but those from CCl₄-induced cirrhotic rats injected with saline had abundant and widespread fibers of blue color (Fig. 3B). However, the liver sections from CCl₄-induced cirrhotic rats injected with NaHS had fewer fibers (Fig. 3C), while those from PAG-injected cirrhotic rats (Fig. 3D) had even more fibers, compared with those from saline-injected cirrhotic rats. The numbers of blue pixels in Masson-stained liver sections were measured to quantify collagen fibers. CCl₄ highly significantly (P<0.001) increased the number of blue pixels in liver sections (Fig. 3E). However, administration of NaHS resulted in a significant (P<0.05) lower number of blue pixels, but PAG, a significant (P<0.05) higher number of blue pixels, in CCl₄-induced cirrhotic rats, compared with saline (Fig. 3E). We further measured hydroxyproline, a marker of fibrosis, and found that CCl₄-treated rats had significantly (P<0.001) higher hepatic hydroxyproline content than the healthy controls. However, administration of NaHS significantly (P<0.05) reduced, but PAG did not significantly (P>0.05) increase, the hepatic hydroxyproline contents in CCl₄-induced cirrhotic rats, compared with saline (Fig. 3F). Next, we detected hepatic expression of α-SMA, another marker of liver fibrosis, and found that the CCl₄-treated rats had significantly higher hepatic expression of α-SMA, compared to the healthy controls. However, administration of NaHS significantly (P<0.05) downregulated, but PAG did not significantly (P>0.05) increase, hepatic expression of α-SMA in CCl₄-induced cirrhotic rats, compared with saline (Fig. 2D and E).

The spleen weight and portal pressure were used as parameters for portal hypertension. The spleen weight of CCl₄-treated rats was highly significantly (P<0.001) higher than that of healthy controls (Fig. 3G). However, administration of neither NaHS nor PAG resulted in significant difference in spleen weight of CCl₄-induced cirrhotic rats, compared with saline (Fig. 3G). CCl₄-induced cirrhotic rats had a significantly (P<0.001) higher portal pressure than the healthy controls (Fig. 3H). However, administration of NaHS resulted in a significant (P<0.05) lower level of portal pressure, while PAG, a significant (P<0.05) higher level of portal pressure, in CCl₄-induced cirrhotic rats, than saline (Fig. 3H).

The therapeutic effect of H₂S on liver cirrhosis and portal hypertension

Given that H₂S displayed a preventive effect against CCl₄-induced liver cirrhosis and portal hypertension, we next investigated whether H₂S could have a similar therapeutic effect in already established liver cirrhosis. Liver cirrhosis was induced in rats by 12-week administration of CCl₄ as above, and then saline, NaHS or PAG was administered to the cirrhotic rats, respectively, for 5 days. Administration of NaHS significantly (Both P<0.05) elevated the levels of H₂S in sera from the peripheral blood (Fig. 4A) and portal vein (Fig. 4B), but again had no effect on H₂S production activity in livers (Fig. 4C). PAG significantly (P<0.05) reduced the levels of H₂S in sera from the systemic circulation (Fig. 4A), marginally significantly (P=0.07) reduced the levels of H₂S in the portal vein (Fig. 4B), and significantly (P<0.05) reduced the H₂S production in livers (Fig. 4C). NaHS or PAG had no significantly influence on the histological alterations of livers (Data not shown), and the hepatic hydroxyproline in NaHS or PAG-treated rats were not significant different from the saline-
Figure 3. Histology of liver cirrhosis, hepatic hydroxyproline, spleen weight and portal pressure in the prevention experiment.

Representative illustrations (200 × magnification) of Masson-stained liver sections were taken from healthy controls (A), or rats treated with CCl₄ + saline (B), CCl₄ + NaHS (C) or CCl₄ + PAG (D), for 12 weeks. (E) The numbers of blue pixels of each image of the above Masson-stained liver sections were counted, and the average number of blue pixels for each liver was calculated. (F) The level of hydroxyproline in livers taken from the above rats was measured. (G) Each spleen was weighed as % of bodyweight. (H) The portal pressure of each was measured. Data were expressed mean ± SD. n, number of samples. Compared to the healthy controls, a significant difference is denoted by “*”, and a highly significant difference, by “**” (P<0.001). Compared to saline + CCl₄-treated rats, a significant increase is denoted by “#”, and a significant reduction is denoted by “†”.

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injected rats (Fig. 4D). However, administration of NaHS significantly (P < 0.05) reduced the portal pressure, while PAG significantly (P < 0.05) elevated the portal pressure, compared with saline (Fig. 4E), but their effects on spleen weight were not significant, compared with saline (Fig. 4F).

Discussion

CCl₄ has been widely used as a chemical model to induce hepatotoxicity, liver cirrhosis and portal hypertension, since it is metabolized into trichloromethyl radical, leading to increased lipid peroxidation, depletion of GSH, impaired hepatic anti-oxidant activity and necrosis of hepatocytes [21,30]. The present study has demonstrated that CCl₄ downregulated the expression of CSE, the major enzyme accounting for 97% of H₂S production in livers [1,12,19], resulting in decreased hepatic H₂S producing activity. Administration of NaHS, a donor of H₂S, attenuated CCl₄-induced acute hepatotoxicity, evidenced by the reduction of serum levels of AST and ALT, and attenuation of histopathological alterations, in accordance with the previous reports on its cytoprotective effects against myocardial IRI [2], endotoxin-induced cardiac injury [31], hepatic IRI [15], acetaminophen-induced hepatotoxicity [16], or gastric mucosa damage caused by stress [4], nonsteroidal anti-inflammatory drugs [32] or oxidative stress [33]. The protective effects of H₂S may rely on its anti-oxidative activity by reducing the production of lipid peroxides, as NaHS inhibited the activity of CYP2E1, one of the major enzymes metabolizing CCl₄ [21]. The vasodilative activity of H₂S may also contribute this protective activity, as H₂S-induced vasorelaxation [34] can improve microcirculation of livers, which help livers to get rid of excessive lipid peroxides. In addition, though not investigated herein, the anti-apoptotic activity of H₂S as demonstrated against hepatic IRI [15], may also contribute to this action.

The effects of CCl₄ on H₂S production were shown to be accumulative as the serum levels of H₂S declined in a time-dependent course. NaHS had no effect on either hepatic CSE expression or H₂S producing activity, and led to a relatively higher but stable level of H₂S in sera, implying that the body has the ability to adjust endogenous production of H₂S or get rid of excessive H₂S, which has been recognized as a “toxic gas” in environmental pollution [35]. In both the acute and prevention experiments, PAG, an irreversible inhibitor of CSE, significantly suppressed the H₂S producing activity and reduced the H₂S levels, thus showing opposite effects to NaHS on liver function, lipid peroxides, and liver fibrosis, but had no impact on CSE expression, in accordance with previous reports [3,15].

The present study has for the first time demonstrated the antifibrotic activity of H₂S against liver cirrhosis, evidenced by reduced number of collagenous fibers in livers, and hepatic hydroxyproline content and expression of α-SMA. Besides the direct mechanism on fibrogenesis, the protective effect of H₂S on hepatic injury also contributes to its anti-fibrotic activity. In addition, NaHS inhibited the production of inflammatory factors including TNF-α, IL-1β, IL-6, soluble ICAM-1. The anti-inflammatory effect, particular inhibition of IL-6 production by H₂S, can contribute to its anti-fibrotic activity, as IL-6 is...
upregulated in cirrhotic livers and contributes to the fibrogenic process in an autocrine/paracrine manner [36].

The regulation of sinusoidal resistance depends on the contraction of hepatic stellate cells (HSCs) around sinusoidal endothelial cells [19]. It has been demonstrated that H2S is an autocrine mediator involved in regulating HSCs contraction and maintaining portal venous pressure by targeting KATP channels [19]. H2S can counteract the impaired vasodilation and HSC maintaining portal venous pressure by targeting KATP channels [36].

H2S may present a potentiom effect, particularly for its prophylactic effects, against liver cirrhosis and portal hypertension. Preclinical trials by applying some promising H2S-releasing chemicals have already been launched though at their infancy. For instance, diallyl trisulfide, a stable H2S donor and organic polysulfide compound [37], has been shown to protect from carbon tetrachloride (CCl4)-induced liver injury [17,18]. However, caution should be taken as H2S has been recognized as a “toxic gas” [35], and more preclinical trials are required before H2S-releasing agents reach the clinics for use in preventing liver cirrhosis and portal hypertension.

**Supporting Information**

**Figure S1** Histology of hepatotoxicity induced by CCl4. Representative illustrations (200 × magnification) of HE-stained liver sections were taken from healthy Wistar rats (A), or CCl4-treated rats receiving administration of saline (B), NaHS (C) or PAG (D).

**Author Contributions**

Conceived and designed the experiments: GT XS JL HJ. Performed the experiments: GT SP XD KK MZ HQ XJ. Analyzed the data: JL HQ XJ XS. Contributed reagents/materials/analysis tools: JL JK XS. Wrote the paper: XS GT SP JK HJ.

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