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Endogenous hydrogen sulfide formation mediates the liver damage in endotoxemic rats

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ABSTRACT

Background: Hydrogen sulfide (H_2S) is a naturally occurring gaseous transmitter and may play important roles in normal physiology and liver disease.
Aims: To investigate the relationships between the formation of liver H_2S and liver damage in endotoxemic rats caused by lipopolysaccharide (LPS).
Methods: Male SD rats were sacrificed to acute endotoxemia and pretreated with H_2S donor sodium hydrogen sulfide (NaHS) or H_2S inhibitor DL-propargylglycine (PAG). Liver H_2S concentration, liver cystathionine-γ-lyase (CSE) mRNA, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) level, liver histopathological alteration in different time after treatment were determined.
Results: Endotoxemia resulted in an increase in serum levels of ALT and AST. In the liver, endotoxemia induced a significant increase in the H_2S concentration, and in the expression of the H_2S-synthesizing enzymes CSE. Pretreatment with NaHS promoted the increase the liver H_2S concentration and aggravated the LPS-induced liver damage, However, administration of PAG abolished the increase the liver H_2S concentration and reduced the liver injury caused by endotoxemia.
Conclusions: These findings support the view that an enhanced formation of H_2S contributes to the liver injury in endotoxemia. We propose that inhibition of H_2S synthesis may be a useful the rapeutic strategy against the liver injury associated with endotoxemia.

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1. Introduction

For hundreds of years, hydrogen sulfide (H_2S) has been considered as a toxic environmental pollutant with the smell of rotten eggs. However, recent studies have shown that H_2S is not only a chemical hazard in certain industrial manufacturing, but it can also mediate various inflammation and damage (Chen et al., 2007; Wagner et al., 2009). As a gaseous transmitter, like nitric oxide (NO) and carbon monoxide (CO), H_2S has been shown the third gaseous transmitter and plays important roles, both in normal physiology conditions and in the process/progress of several diseases (Moore et al., 2003).

It is now apparent that endogenous H_2S is synthesized naturally in the body from L-cysteine mainly by the activity of two key enzymes, cystathionine-γ-lyase (CSE) and cystathionine-β-synthetase (CBS), which interconverts L-methionine and L-cysteine, but can also use L-cysteine as an alternative substrate to form H_2S. Both enzymes are pyridoxal phosphate dependent and are expressed in a range of mammalian cells and tissues. Of theses, CSE is most highly concentrated in the vasculature and is thus the focus of attention in this work (Chen et al., 2007; Moore et al., 2003).

Recently, attention has been focused on the possible role of H_2S in inflammation. H_2S dilates blood vessels in vivo and in vitro probably by opening vascular smooth muscle K^+-ATP channels (Wagner et al., 2009; Zhao et al., 2001). Some evidence has also been published of the ability of the K^+-ATP channel blocker glibenclamide to inhibit the hypotensive response to a H_2S donor sodium hydrogen sulfide (NaHS) (Geng et al., 2004). Previous study has provided evidence of a role for H_2S as a proinflammatory mediator in various animal models of hemorrhagic (Mok and Moore, 2008) and endotoxic (Collin et al., 2005; Li et al., 2005) shock as well as in caerulein-induced hindpaw edema in the rat (Moore et al., 2003). In endotoxemic rat models, the relationship between H_2S and the lung and myocardial damage has been elucidated (Huang et al., 2008, 2009; Sivarajah et al., 2006; Xian et al., 2007), but data about the role of H_2S in liver damage in endotoxemic rat are scarce.

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Therefore, the present study was designed to investigate the effects of endogenous H_2S on the liver damage caused by acute severe endotoxemia induced by *Escherichia coli* lipopolysaccharide (LPS) in the rat, by using NaHS (H_2S) and DL-propargylglycine (PAG), an inhibitor of CSE.

2. Materials and methods

2.1. LPS-induced endotoxemia model

96 SPF male Sprague–Dawley (SD) rats weighing 140–160 g were obtained from Kunming medical college. The rats were randomly divided into four groups: normal control group (I group): 24 rats were injected i.v. through tail with 0.9% saline (5 ml /kg); LPS group (II group):24 rats were injected i.v. with *E. coli* LPS (5 mg/kg, serotype O111:B4) (Sigma–Aldrich Corp., St Louis, MO, U.S.A.); LPS + NaHS group (III group): 24 rats were administered NaHS (Sigma) 28 mg/kg i.v. at 10 min before they were subjected to endotoxemia; LPS + PAG group (IV group): 24 rats were treated with PAG (Sigma) 50 mg/kg i.v. at 30 min before they were subjected to endotoxemia. All group rats were killed at 3, 4, 8, 12 h after accordingly injection, and blood removed by cardiac puncture into heparinized (50 units/ml) tubes. Liver was also removed for biochemical and histological assays as described below. All animals were received human care and that study protocols complied with the institutions guidelines.

2.2. Assay of liver H_2S concentration

H_2S concentration in liver homogenate was measured essentially as described elsewhere (Li et al., 2005; Anuar et al., 2006). Briefly, liver tissue from rats was thawed and homogenized in 100 mM ice-cold potassium phosphate buffer (pH 7.4). Optimal w/v ratios of 1:20 were determined from preliminary experiments (data not shown). The reaction mixture (total volume, 500 μl) contained L-cysteine (10 mM; 20 μl), pyridoxal 5′-phosphate (10 mM; 20 μl), Saline (30 μl) and tissue homogenate (430 μl). The reaction performed in parafilmed eppendorf tubes and initiated by transferring the tubes from ice to a water bath at 37 °C. In some experiments, the enzymatic reaction was stopped immediately by the addition of trichloroacetic acid (10% w/v, 250 μl) to denature protein prior to the addition of cysteine. After incubation for 30 min, zinc acetate (1% w/v, 250 μl) was added to trap evolved H_2S followed by trichloroacetic acid (10% w/v, 250 μl). Subsequently, N,N-dimethyl-p-phenyle-nediamine sulfate (20 μM; 133 μl) in 7.2 M HCl and $FeCl_3$ (30 μM; 133 μl) in 1.2 M HCl were added and the absorbance of the resulting solution (670 nm) measured 15 min thereafter using a 96-well microplate reader (Tecan Systems Inc., San Jose, CA, U.S.A.). The basal concentration of H_2S was determined in incubates in which trichloroacetic acid was added at zero time ($T = 0$) prior to the addition of cysteine and incubation (37 °C, 30 min). At the end of this period, trichloroacetic acid (10% w/v, 250 μl) was added and H_2S generated assayed by spectrophotomet as described above. All samples were assayed in duplicate. The H_2S concentration of each sample was calculated against a calibration curve of NaHS (3.12–250 μM) and results are expressed as nmol H_2S formed per milligram soluble protein (determined using the Bradford assay, Bio-Rad Ltd, Hercules, CA, U.S.A.).

2.3. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of liver CSE mRNA

Expression of CSE in liver was determined essentially as described previously (Mok et al., 2004), Briefly, 100 mg liver samples were homogenized in 1 ml ice-cold TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.) and total RNA was extracted and treated with RNase-free DNase (DNase I; Amersham Pharmacia Biotech Sollentuna, Sweden). Total RNA concentration and purity were determined by electrophoresis on an ethidium bromide-stained 0.8% agarose gel followed by UV illumination and measurement of absorbance at wavelengths of 260 and 280 nm. Reverse transcription-polymerase chain reaction (RT-PCR) was performed with QIAGEN® OneStep RT-PCR kit. Each reaction contained 0.5 μg total RNA as template, 4 μl of 5 × RT-PCR buffer, 0.8 μl of dNTP mix (400 μM), 1.2 μl of each primer (0.6 μM), 0.8 μl of enzyme mix (contains Omniscript™ Reverse Transcriptase, Sensiscript™ Reverse Transcriptase and HotStarTaq® DNA Polymerase) and RNase-free water in a final volume of 20 μl. Rat GAPDH served as an internal control gene. The RT-PCR profile was one cycle of cDNA synthesis at 50 °C for 30 min and one cycle of initial PCR activation at 95 °C for 15 min, followed by 33 cycles (CSE) or 20 cycles (GAPDH) of denaturation at 94 °C for 30 s, annealing at 61 °C (CSE) or 60 °C (GAPDH) for 45 s, and extension at 72 °C for 30 s (CSE) or 35 s (GAPDH) followed by one cycle of final extension at 72 °C for 10 min. After RT-PCR, aliquots of the RT-PCR products were electrophoresed through 1.2% agarose gels (Bio-Rad) containing 0.5 μg/ml of ethidium bromide (Bio-Rad) and gels were visualized under UV light and photographed. Semiquantitative analysis of bolts was obtained using Gel analysis software (Syngene, UK). The primer sequences of CSE and GAPDH were as follows: CSE (f) 5′-CTT TGG CTC TGG GTG CTG AT-3′; CSE (r) 5′-ATG CTG AGG GTG AGA CGG TAG-3′; GAPDH (f) 5′-CAG CAA TGC ATC CTG CAC-3′; GAPDH (r) 5′-GAG TTG CTG TTG AAG TCA CAG G-3′. The RT-PCR product size for CSEand GAPDH was 308 and 468 bp.

2.4. Assay of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) level

Blood samples were allowed to clot, and the sera were isolated by centrifugation at 1000 rpm for 10 min and kept at −20 °C before determination. Sera ALT and AST were determined using commercial kits by Nanjing Jiancheng Bioengineering Institute. These activities from spectrophotometry at wavelengths of 340 nm are expressed as an international unit (U/L).

2.5. Histological studies

Liver specimens, sized approximately $1.0 \times 0.5 \times 0.3$ cm^3 were processed under a light microscopy. The specimens were fixed in 10% formaldehyde for 12–24 h, embedded in paraffin, sectioned for 5 μm in thickness, stained with hematoxylin and eosin (HE), and viewed by light microcopy at 100× magnification.

2.6. Statistical analysis

Data were expressed as mean ± SD. Statistical significance of differences between groups were determined by ANOVA followed by Student's *t* test. *P* value of less than 0.05 was considered statistically significance.

3. Results

3.1. Endotoxemia increases H_2S-synthesizing activity and effect of PAG and NaHS in the rat liver

Incubation of liver homogenates with cysteine and pyridoxal 5′-phosphate resulted in the formation of significant amounts of H_2S as determined spectrophotometrically. When compared to the normal control rats (I group), endotoxemia (II group) caused

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