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Inhibition of endogenous hydrogen sulfide formation reduces the organ injury caused by endotoxemia

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1   Hydrogen sulfide (H_2S) is a naturally occurring gaseous transmitter, which may play important roles in normal physiology and disease. Here, we investigated the role of H_2S in the organ injury caused by severe endotoxemia in the rat.

2   Male Wistar rats were subjected to acute endotoxemia (*Escherichia coli* lipopolysaccharide (LPS) 6 mg kg^{-1} intravenously (i.v.) for 6 h) and treated with vehicle (saline, 1 ml kg^{-1} i.v.) or DL-propargylglycine (PAG, 10–100 mg kg^{-1} i.v.), an inhibitor of the H_2S-synthesizing enzyme cystathionine-γ-lyase (CSE). PAG was administered either 30 min prior to or 60 min after the induction of endotoxemia.

3   Endotoxemia resulted in circulatory failure (hypotension and tachycardia) and an increase in serum levels of alanine aminotransferase and aspartate aminotransferase (markers for hepatic injury), lipase (indicator of pancreatic injury) and creatine kinase (indicator of neuromuscular injury). In the liver, endotoxemia induced a significant increase in the myeloperoxidase (MPO) activity, and in the expression and activity of the H_2S-synthesizing enzymes CSE and cystathionine-β-synthase.

4   Administration of PAG either prior to or after the injection of LPS dose-dependently reduced the hepatocellular, pancreatic and neuromuscular injury caused by endotoxemia, but not the circulatory failure. Pretreatment of rats with PAG abolished the LPS-induced increase in the MPO activity and in the formation of H_2S and in the liver.

5   These findings support the view that an enhanced formation of H_2S contributes to the pathophysiology of the organ injury in endotoxemia. We propose that inhibition of H_2S synthesis may be a useful therapeutic strategy against the organ injury associated with sepsis and shock.
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Introduction

Gaseous transmitters, such as nitric oxide and carbon monoxide (CO), play important roles both in physiology and in disease. In recent years, another naturally occurring gas, hydrogen sulfide (H_2S), has been found to be of importance (Moore *et al.*, 2003). Cystathionine-γ-lyase (CSE) and cystathionine-β-synthase (CBS) are key enzymes of the trans-sulfuration pathway, 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. Although other enzymes can catalyze the production of H_2S, CBS seems to be the main H_2S-forming enzyme in the central nervous system, whereas CSE is the main enzyme forming H_2S in the cardiovascular system (Moore *et al.*, 2003).

H_2S dilates blood vessels *in vivo* and *in vitro* probably by opening vascular smooth muscle K$^+$-ATP channels (Zhao *et al.*, 2001; Moore *et al.*, 2003; Cheng *et al.*, 2004). 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). However, the role of H_2S in the regulation of blood pressure or vascular perfusion remains unclear. In a rat model of hypoxic pulmonary hypertension, a reduction in the expression and activity of CSE was associated with a decrease in plasma H_2S concentration in the lung tissue (Chunyu *et al.*, 2003). Notably, parenteral H_2S administration in these animals opposed the rise in pulmonary arterial pressure and partially prevented the pulmonary vascular remodelling. Therefore, H_2S deficiency may contribute to the pathophysiology of this disease (Moore *et al.*, 2003). It has also been found that exogenous supply of an inhibitor of H_2S

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synthesis decreased the plasma H_2S content and worsened hypoxic pulmonary hypertension (Qingyou *et al.*, 2004). At the same time, plasma CO level and the expressions of hemioxygenase (HO)-1 protein and mRNA in pulmonary arteries were decreased. These results suggested that H_2S could play a regulatory role in the pathogenesis of hypoxic pulmonary hypertension through upregulating CO/HO pathway. The possibility that excess H_2S contributes to the hypotension associated with either septic (cecal ligation and puncture) or endotoxic shock in the rat has been suggested (Hui *et al.*, 2003). In that study, the systemic inflammation resulted in increased H_2S concentrations in the aorta, pulmonary, mesenteric and tail arteries when compared with controls, suggesting that overproduction of vasodilatory H_2S may play a role in the vascular consequences of septic and endotoxic shock. In hemorrhagic shock, inhibitors of H_2S synthesis were recently shown to reduce partially the hypotensive response associated with hemorrhage (Mok *et al.*, 2004). Hemorrhage also increased the synthesis of H_2S in the liver (Mok *et al.*, 2004).

The role of H_2S in inflammation is beginning to emerge. A recent report demonstrated that endogenous H_2S plays a role in the pathophysiology of cerulein-induced pancreatitis (Bhatia *et al.*, 2005b). Severe acute pancreatitis was associated with lung injury characterized by neutrophil accumulation, which was attenuated by treatment with an irreversible inhibitor of H_2S-synthesizing enzyme CSE, DL-propargylglycine (PAG) (Hosoki *et al.*, 1997; Teague *et al.*, 2002; Cheng *et al.*, 2004; Mok *et al.*, 2004). Another recent study provided evidence of the role of H_2S in inflammation, by showing that inhibition of CSE reduced the hindpaw edema induced by carrageenan injection (Bhatia *et al.*, 2005a). Hence, the present study was designed to investigate the effects of endogenous H_2S on the organ injury caused by acute severe endotoxemia in the rat, by using PAG, an inhibitor of H_2S-synthesizing enzyme CSE.

Methods

Surgical procedure and quantification of organ injury

This study was carried out on 54 male Wistar rats (Tuck, Rayleigh, Essex, U.K.) weighing 220–320 g, receiving a standard diet and water *ad libitum*. The investigation was performed in accordance with the Home Office *Guidance on the Operation of the Animals (Scientific Procedures) Act 1986*, published by HMSO, London. All animals were anesthetized with thiopentone sodium (Intraval®, 120 mg kg⁻¹ intraperitoneally (i.p.)), and anesthesia was maintained by supplementary injections of thiopentone sodium (approximately 1–2 mg kg⁻¹ h⁻¹ intravenously (i.v.)) as required. The general surgical procedures were performed as described previously (Millar & Thiemermann, 2002). Upon completion of the surgical procedure, mean arterial pressure (MAP) and heart rate (HR) were allowed to stabilize for 15 min. At 6 h after the induction of endotoxemia, 1.5 ml of blood was collected into a serum gel S/1.3 tube (Sarstedt, Germany) from a catheter placed in the carotid artery, and values were examined as previously described for indices of liver injury (Baue, 1993; Hewett *et al.*, 1993), pancreatic injury (Ruetten & Thiemermann, 1997) and neuromuscular injury (Ruetten *et al.*, 1996). All serum samples were analyzed within 24 h by a contract

laboratory for veterinary clinical chemistry (Vetlab Services, Sussex, U.K.).

Experimental design

Animals were assigned to five experimental groups:

(1) *Sham Control*: Rats were treated with saline (vehicle for PAG, 1 ml kg⁻¹ i.v., $n = 9$) without causing endotoxemia (received saline rather than endotoxin).
(2) *Sham PAG*: Rats were treated with PAG (50 mg kg⁻¹ i.v., $n = 6$) without causing endotoxemia. The dose of PAG was chosen based on recent report showing inhibition of H_2S formation in the rat liver at this dose (Mok *et al.*, 2004).
(3) *Lipopolysaccharide (LPS) Control*: Rats were treated with saline (1 ml kg⁻¹ i.v., $n = 9$) at 30 min before they were subjected to endotoxemia: *Escherichia coli* LPS (6 mg kg⁻¹ i.v., serotype 0127:B8) was given slowly over 10 min.
(4) *LPS PAG pretreatment with 10 mg kg⁻¹ (LPS PAG 10 Pre)*: Rats were treated with PAG 10 mg kg⁻¹ i.v. ($n = 8$) at 30 min before they were subjected to endotoxemia.
(5) *LPS PAG pretreatment with 50 mg kg⁻¹ (LPS PAG 50 Pre)*: Rats were treated with PAG 50 mg kg⁻¹ i.v. ($n = 9$) at 30 min before they were subjected to endotoxemia.
(6) *LPS PAG post-treatment with 50 mg kg⁻¹ (LPS PAG 50 Post)*: Rats were treated with PAG 50 mg kg⁻¹ i.v. ($n = 9$) at 60 min after they were subjected to endotoxemia.
(7) *LPS PAG pretreatment with 100 mg kg⁻¹ (LPS PAG 100 Pre)*: Rats were treated with PAG 100 mg kg⁻¹ i.v. ($n = 4$) at 30 min before they were subjected to endotoxemia.

Measurement of myeloperoxidase (MPO) activity

Neutrophil sequestration in the liver was quantified by measuring tissue MPO activity (Bhatia *et al.*, 1998; 2000). Liver samples were thawed, homogenized in 20 mM phosphate buffer (pH 7.4), centrifuged ($10,000 \times g$, 10 min, 4°C) and the resulting pellet resuspended in 50 mM phosphate buffer (pH 6.0) containing 0.5% (w v⁻¹) hexadecyltrimethylammonium bromide (Sigma-Aldrich Corp., St Louis, MO, U.S.A.). The suspension was subjected to four cycles of freezing and thawing and further disrupted by sonication (40 s). The sample was then centrifuged ($10,000 \times g$, 5 min, 4°C) and the supernatant used for the MPO assay. The reaction mixture consisted of the supernatant (50 μl), 1.6 mM tetramethylbenzidine (Sigma), 80 mM sodium phosphate buffer (pH 5.4) and 0.3 mM hydrogen peroxide (reagent volume: 50 μl). This mixture was incubated at 37°C for 110 s, the reaction terminated with 50 μl of 0.18 M H_2SO_4 and the absorbance measured at 450 nm. This absorbance was then corrected for the DNA content of the tissue sample and results are expressed as enzyme activity.

Assay of liver H_2S synthesis

Liver H_2S-synthesizing activity was determined essentially as described elsewhere (Stipanuk & Beck, 1982). Briefly, liver tissue from animals treated as above was thawed and homogenized (Ultra-Turrax) in 100 mM ice-cold potassium phosphate buffer (pH 7.4). Optimal (w v⁻¹) ratios of 1 : 20 were determined from preliminary experiments. The reaction

mixture (total volume, $500\,\mu l$) contained L-cysteine ($10\,mM$; $20\,\mu l$), pyridoxal 5′-phosphate ($2\,mM$; $20\,\mu l$), saline ($30\,\mu l$) and tissue homogenate ($430\,\mu l$). The reaction was 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^{-1}), $250\,\mu l$) to denature protein prior to the addition of cysteine. After incubation for $30\,min$, zinc acetate (1% (w v^{-1}), $250\,\mu l$) was added to trap evolved H_2S followed by trichloroacetic acid (10% (w v^{-1}), $250\,\mu l$). Subsequently, *N*,*N*-dimethyl-*p*-phenylenediamine sulfate ($20\,\mu M$; $133\,\mu l$) in $7.2\,M$ HCl and FeCl$_3$ ($30\,\mu M$; $133\,\mu l$) 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^{-1}), $250\,\mu l$) was added and H_2S generated assayed spectrophotometrically as described above. All samples were assayed in duplicate. The H_2S concentration of each sample was calculated against a calibration curve of NaHS ($3.12-250\,\mu M$) and results are expressed as nmol H_2S formed mg^{-1} protein (determined using the Bradford assay, Bio-Rad Ltd, Hercules, CA, U.S.A.).

Reverse transcription–polymerase chain reaction (RT–PCR) analysis of liver CSE and CBS mRNA

Expression of CSE and CBS in tissues was determined essentially as described previously (Mok *et al.*, 2004). Briefly, liver tissue ($100\,mg$) was homogenized in $1\,ml$ ice-cold TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.) using a Polytron homogenizer (Heidolph Ltd, Schwabach, Germany) and thereafter incubated for $10\,min$ at room temperature. Samples were mixed with chloroform ($0.2\,ml$), vigorously shaken and incubated at room temperature for $3\,min$ followed by centrifugation ($12,000 \times g$, $4°C$, $15\,min$). The upper aqueous phase was transferred to an eppendorf tube and isopropanol ($0.5\,ml$) added. After further incubation at room temperature ($10\,min$), samples were recentrifuged ($12,000 \times g$, $4°C$, $10\,min$) and the resulting RNA pellet was washed with 75% (v v^{-1}) ethanol ($1.5\,ml$) and centrifuged again ($7500 \times g$, $4°C$, $5\,min$). Supernatants were discarded and the RNA pellets were air-dried ($5-10\,min$), dissolved in diethyl pyrocarbonate (DEPC)-treated water ($50-100\,\mu l$) and incubated ($55-60°C$) for $10\,min$. The concentration of isolated nuclei acids was determined spectrophotometrically by measuring the absorbance at $260\,nm$. All samples were thereafter stored at $-80°C$ until required.

One-step RT–PCR method was employed in this study (QIAGEN® Onestep RT–PCR kit, Qiagen Ltd, Valencia, CA, U.S.A.). Total RNA template ($1\,\mu g$) was mixed with $5 \times$ RT–PCR buffer ($4\,\mu l$), dNTP mix ($400\,\mu M$, $0.8\,\mu l$), $1.2\,\mu l$ of each primer ($0.6\,\mu M$), enzyme mix ($0.8\,\mu l$, a mixture of omniscript, sensiscript reverse transcriptases and HotStar *Taq* DNA polymerase) and DEPC-treated water. The final volume was $20\,\mu l$. For the detection of CSE mRNA, the forward primer sequence used was 5′-CATGGATGAAGTGTATGGAGGC-3′, and the reverse primer sequence was 5′-CGGCAGCA GAGGTAACAATCG-3′. For detection of CBS, the forward

primer sequence used was 5′-GAGCGAGCCGGAACCTT GAAGC-3′, and the reverse primer sequence was 5′-CACC TATCCACCACCGCCCTGTC-3′. The PCR product size was $445\,bp$ (CSE) and $579\,bp$ (CBS). RT–PCR was performed at $50°C$ for $30\,min$ and at $95°C$ for $15\,min$ for reverse transcription followed by 29 cycles of PCR reaction consisting of $94°C$ ($30\,s$) for denaturation, $58°C$ ($30\,s$) for CSE primer-specific annealing and $60°C$ ($30\,s$) for CBS primer-specific annealing, and $72°C$ ($30\,s$) for extension. PCR products were analyzed by 15% (w v^1) agarose gel electrophoresis and imaged by Multi-Genius Bioimaging system (Syngene, Cambridge, U.K.). The band intensity was semiquantified by densitometry using gel analysis software (Syngene Ltd, Frederick, MD, U.S.A.) using glyceraldehyde-3-phosphate dehydrogenase as a marker gene.

Materials

PAG and *E. coli* LPS (serotype 0127:B8) were obtained from Sigma-Aldrich Company Ltd (Poole, Dorset, U.K.). Thiopentone sodium (Intraval Sodium®) was purchased from Rhône Mérieux Ltd (Harlow, Essex, U.K.). All stock solutions were prepared in nonpyrogenic saline (0.9% NaCl; Baxter Healthcare Ltd, Thetford, Norfolk, U.K.).

Statistical evaluation

All data are presented as means $\pm$ s.e.m. of *n* observations, where *n* represents the number of animals or blood samples studied. For repeated measurements, a two-way analysis of variance (ANOVA) was performed. Data without repeated measurements was analyzed by one-way ANOVA, followed by a Dunnett's test for multiple comparisons. A *P*-value of less than 0.05 was considered to be statistically significant.

Results

Circulatory failure caused by endotoxemia

Baseline values of MAP and HR were similar in all of the animal groups studied and ranged from 122 ± 6 to $128 \pm 5\,mmHg$ (Figure 1a) and from 399 ± 15 to 437 ± 11 beats min^{-1} (Figure 1b), respectively. In animals without endotoxemia (sham-operated animals), injection of PAG did not result in any significant alterations in MAP or HR compared to Sham Control (Figure 1).

Injection of LPS ($6\,mg\,kg^{-1}$ i.v. over $10\,min$) resulted in a modest biphasic fall in MAP from $125 \pm 6\,mmHg$ (baseline) to $75 \pm 6\,mmHg$ at $360\,min$ (Figure 1a, $P < 0.05$). Pretreatment of rats with PAG at the doses of $10-50\,mg\,kg^{-1}$ did not affect the fall in MAP caused by endotoxemia (Figure 1a). An increase in HR was observed in rats subjected to endotoxemia and treated with vehicle (Figure 1b, $P < 0.05$). The observed increase in HR was not affected by PAG at the doses of $10-50\,mg\,kg^{-1}$ (Figure 1b). A higher dose of $100\,mg\,kg^{-1}$ of PAG proved lethal ($n = 4$, mortality rate 100% between 3 and $6\,h$).

Inhibition of H2S formation reduces organ injury caused by endotoxemia

When compared to the rats treated with vehicle rather than LPS (Sham Control), endotoxemia resulted in significant rises
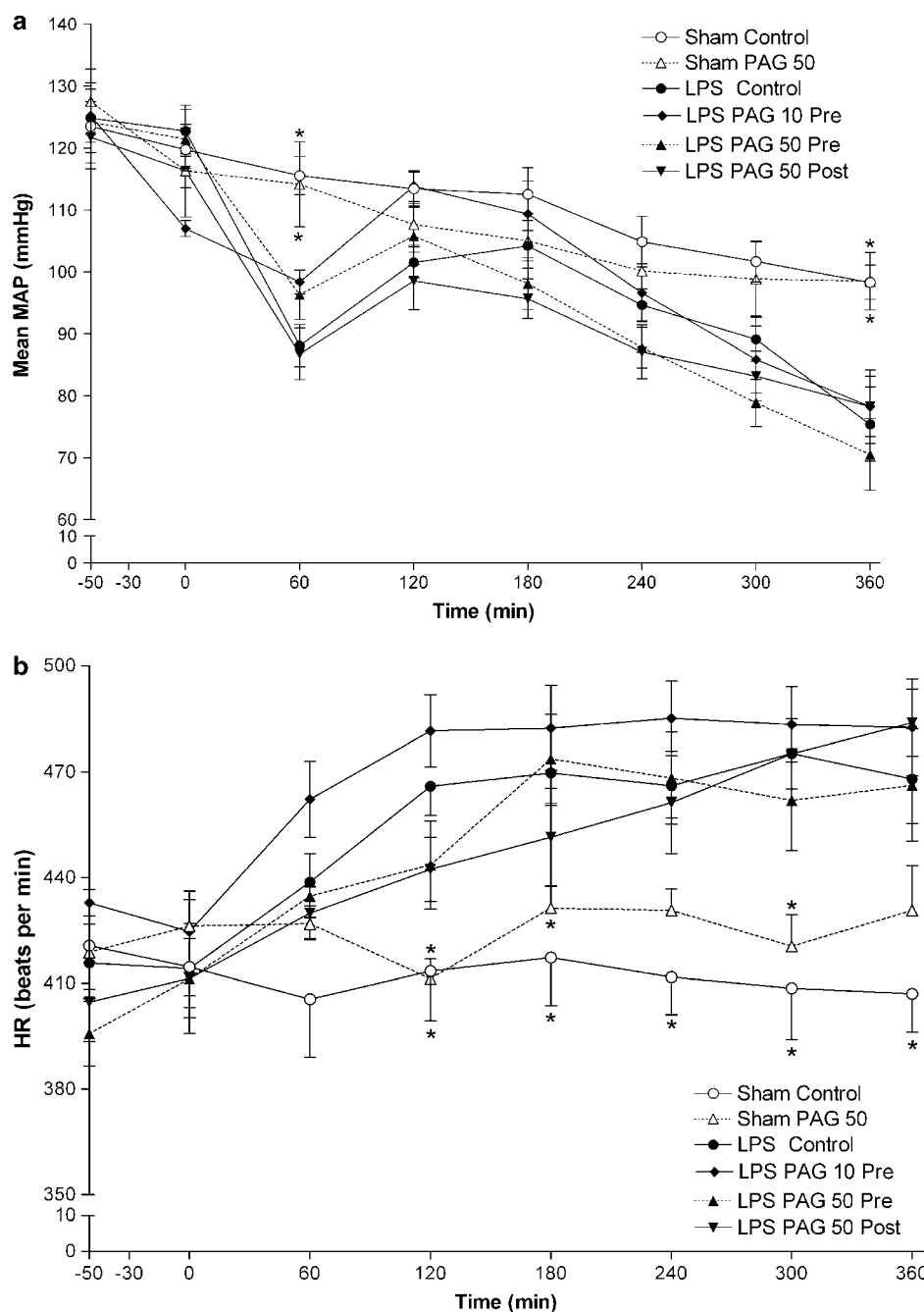
Figure 1 Alterations in (a) mean arterial blood pressure (MAP) and (b) heart rate (HR) in rats subjected to the surgical procedure and pretreated with either saline (Sham Control, $n = 9$) or DL-propargylglycine (Sham PAG, $n = 6$). Rats subjected to endotoxemia (LPS 6 mg kg^{-1} i.v.) were pretreated with either saline (LPS Control, $n = 9$) or PAG. PAG was administered either 30 min prior to the induction of endotoxemia at 10 mg kg^{-1} (LPS PAG 10 Pre, $n = 8$) or 50 mg kg^{-1} (LPS PAG 50 Pre, $n = 9$), or 60 min after the induction of endotoxemia at 50 mg kg^{-1} (LPS PAG 50 Post, $n = 9$). *$P < 0.05$ when compared with LPS Control.

in the serum levels of alanine transaminase (ALT) and aspartate transaminase (AST) (liver injury, Figure 2a and b, $P < 0.05$), creatine kinase (CK) (neuromuscular injury, Figure 2c, $P < 0.05$) and lipase (pancreatic injury, Figure 2d, $P < 0.05$).

Pre- or post-treatment with 50 mg kg^{-1} of PAG (LPS PAG 50 Pre or LPS PAG 50 Post, respectively) of rats subjected to endotoxemia attenuated the rises in the serum levels of ALT and AST and, hence, the liver injury caused by endotoxemia

(Figure 2a and b, $P < 0.05$). In addition, PAG at 50 mg kg^{-1} also attenuated the rises in the serum levels of CK (Figure 2c, $P < 0.05$) and lipase (Figure 2d, $P < 0.05$), and hence, respectively, the neuromuscular and pancreatic injury caused by endotoxemia. Pretreatment with a lower dose of PAG (10 mg kg^{-1}) (LPS PAG 10 Pre) reduced the liver injury (measured as serum ALT levels). The serum levels of CK and lipase were also reduced by the lower dose of PAG, but these effects were not statistically significant. In sham-operated
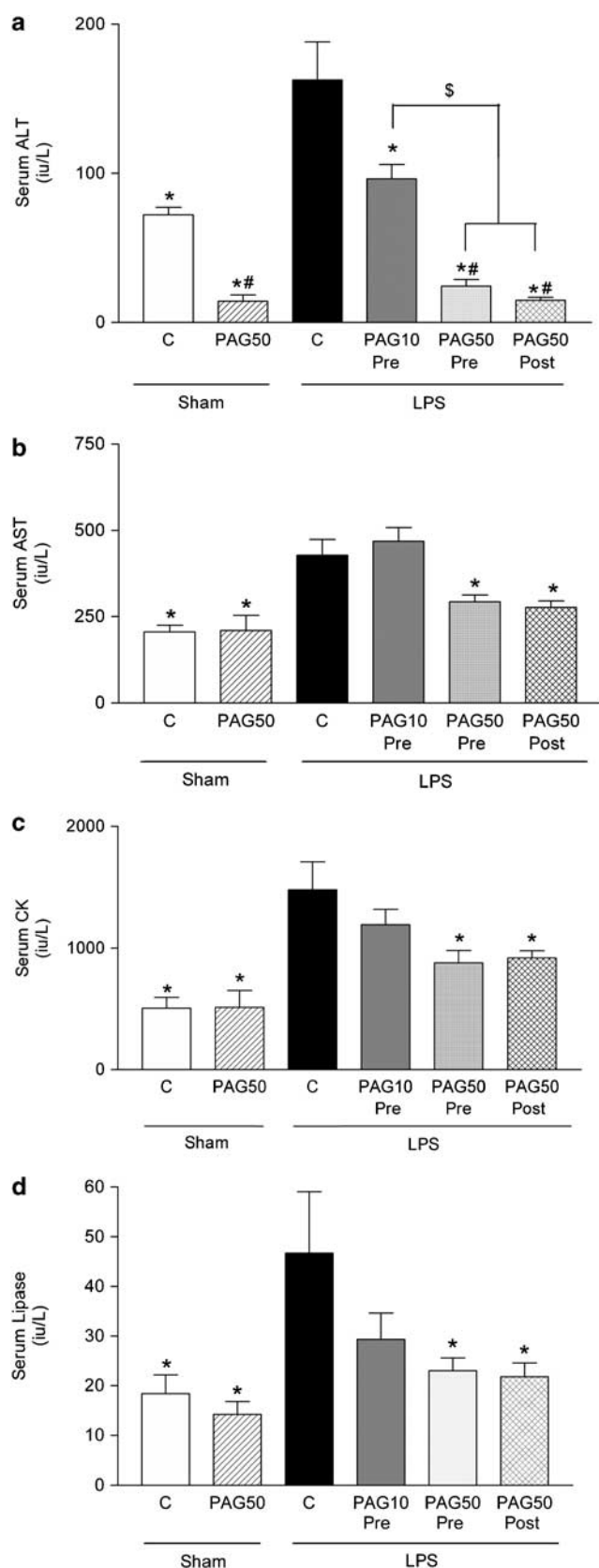
animals, administration of PAG at $50 \, \mathrm{mg \, kg^{-1}}$ resulted in a reduction in the serum level of ALT when compared to sham-operated animals treated with saline (Figure 2a, $P < 0.05$).

Endotoxemia increases MPO activity in the rat liver: effect of PAG

When compared to the sham-operated rats (Sham Control), endotoxemia (LPS Control) resulted in significant increases in the MPO activity in the liver, indicating increased leukocyte infiltration (Figure 3, $P < 0.05$). Pretreatment of rats with PAG at $50 \, \mathrm{mg \, kg^{-1}}$ (LPS PAG 50 Pre) significantly reduced the MPO activity in the liver.

Endotoxemia increases H₂S-synthesizing activity in the rat liver: effect of PAG

When compared to the sham-operated rats (Sham Control), endotoxemia (LPS Control) resulted in significant increases in the expression of H₂S-forming enzymes CSE and CBS, as evidenced by RT–PCR of RNA extracted from the liver (Figure 4, $P < 0.05$). In addition, incubation of rat liver homogenates with cysteine resulted in significantly increased formation of H₂S in endotoxemic rats when compared to the
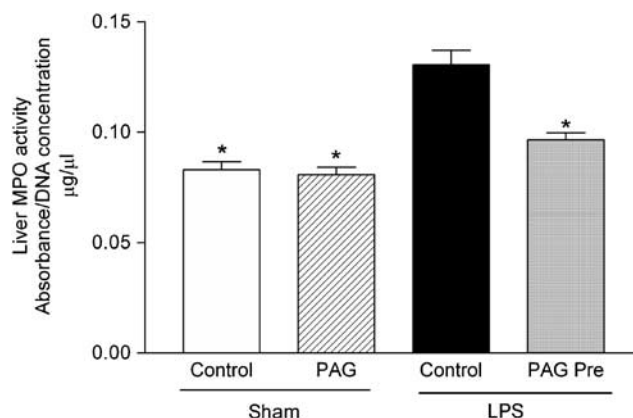

Figure 3 Myeloperoxidase (MPO) activity in the liver of rats subjected to the surgical procedure and pretreated with either saline (Sham Control) or DL-propargylglycine (Sham PAG). Rats subjected to endotoxemia (LPS $6 \, \mathrm{mg \, kg^{-1}}$ i.v.) were pretreated with either saline (LPS Control) or PAG 30 min prior to (LPS PAG Pre) the induction of endotoxemia. $N = 6$ for each group. *$P < 0.05$ when compared with LPS Control.

Figure 2 Alterations in the serum levels of (a) alanine aminotransferase (ALT) and (b) aspartate aminotransferase (AST), (c) creatine kinase (CK) and (d) lipase in rats subjected to the surgical procedure and pretreated with either saline (Sham Control, $n = 9$) or DL-propargylglycine (Sham PAG, $n = 6$). Rats subjected to endotoxemia (LPS $6 \, \mathrm{mg \, kg^{-1}}$ i.v.) were treated with either saline (LPS Control, $n = 9$) or PAG. PAG was administered either 30 min prior to the induction of endotoxemia at $10 \, \mathrm{mg \, kg^{-1}}$ (LPS PAG 10 Pre, $n = 8$) or $50 \, \mathrm{mg \, kg^{-1}}$ (LPS PAG 50 Pre, $n = 9$), or 60 min after the induction of endotoxemia at $50 \, \mathrm{mg \, kg^{-1}}$ (LPS PAG 50 Post, $n = 9$). *$P < 0.05$ when compared with LPS Control, #$P < 0.05$ when compared with Sham Control, $P < 0.05$ when compared with LPS PAG 10 Pre.

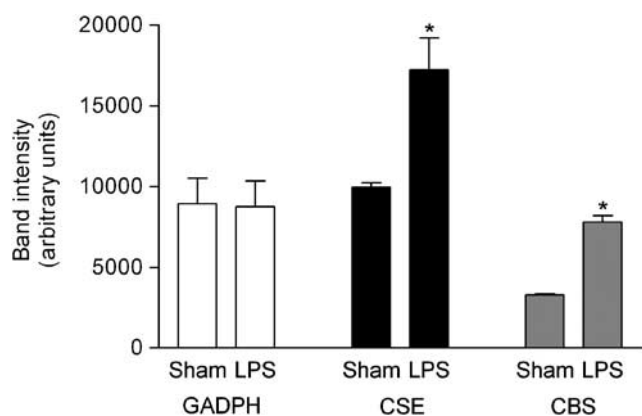**Figure 4** CSE and CBS mRNA expression in the liver of rats subjected to the surgical procedure only (Sham) or endotoxemia (LPS). $N = 6$ for each group. $*P < 0.05$ when compared with respective Sham.
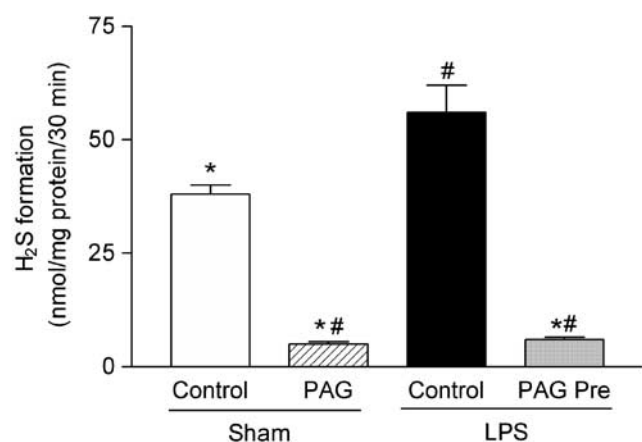

Figure 5 H₂S formation in the liver of rats subjected to the surgical procedure and pretreated with either saline (Sham Control) or DL-propargylglycine (Sham PAG). Rats subjected to endotoxemia (LPS 6 mg kg^{-1} i.v.) were pretreated with either saline (LPS Control) or PAG 30 min prior to (LPS PAG Pre) the induction of endotoxemia. $N = 6$ for each group. $*P < 0.05$ when compared with LPS Control, $^{\#}P < 0.05$ when compared with Sham Control.

sham-operated animals (Figure 5, $P < 0.05$). Treatment of rats with PAG abolished the H₂S synthesis in the rat liver.

Discussion

Severe sepsis and septic shock are important causes of death in intensive care units. The patient outcome correlates with the progression of shock to multiple organ failure and the number of organs failing (Baue, 1999). We demonstrate here that endotoxemia caused multiple organ injury, including a substantial increase in the serum concentrations of ALT and AST, indicating hepatocellular injury. Endotoxemia was also associated with an increase in the serum lipase, indicative of pancreatic injury, and an increase in serum CK, indicative of neuromuscular (brain tissue, skeletal or cardiac muscle tissue) injury. Here, we report that pretreatment with an inhibitor of H₂S synthesis, PAG, attenuated the liver injury, neuromus-

cular injury and pancreatic injury caused by acute severe endotoxemia in the rat.

It has been suggested that many therapeutic approaches for septic shock have failed, as in experimental settings they have been given prophylactically rather than therapeutically. Therefore, we also investigated whether employing a post-treatment regimen, that is, administering PAG 60 min after the induction of endotoxemia, would afford protection against the associated organ injury. We found that the development of organ injury caused by acute severe endotoxemia was indeed attenuated by the therapeutic treatment with the inhibitor of H₂S synthesis, PAG. This broader therapeutic window interestingly suggests a possibility for the use of H₂S synthesis inhibitors in the treatment of systemic inflammatory response and its complications. Indeed, it was recently reported that therapeutic administration of PAG reduces the severity of cerulein-induced pancreatitis and affords protection against the associated lung injury in mice (Bhatia *et al.*, 2005b).

As leukocyte recruitment plays a pivotal role in the pathogenesis of organ injury caused by LPS (Jaeschke *et al.*, 1991; Tanaka *et al.*, 1993; Klintman *et al.*, 2002), we investigated whether the protection afforded by PAG against the organ injury and dysfunction caused by endotoxemia was associated with a reduction in the leukocyte recruitment. We report here that pretreatment with PAG of rats subjected to endotoxemia indeed reduced the MPO activity in the liver, indicating attenuated neutrophil infiltration in this organ. In accordance with our finding, it was reported (while this manuscript was under evaluation) that pretreatment with PAG of mice subjected to endotoxemia reduced the LPS-induced increase in the MPO activity and lung and liver tissue damage (Li *et al.*, 2005).

H₂S is a potent vasodilator *in vitro* and *in vivo* (Kimura, 2002; Wang, 2002). In the present study, however, inhibition of H₂S synthesis by PAG at doses ranging from 10 to 50 mg kg^{-1} elicited no hemodynamic effects. This is in contrast with a previous study, which suggested that overproduction of H₂S might play a role in the vascular consequences of sepsis or endotoxemia in the rat (Hui *et al.*, 2003). In addition, another recent paper reported that inhibitors of H₂S synthesis partially reduced the hypotensive response associated with severe hemorrhage (Mok *et al.*, 2004). Nevertheless, as the circulatory failure induced by endotoxemia was not attenuated by PAG in the present study despite the dose-dependent reduction in organ injury and dysfunction, favorable hemodynamic effects are unlikely to have contributed to the protection of the organs afforded by the inhibition of H₂S synthesis. However, the effects of H₂S and inhibition of H₂S synthesis in the microvasculature still warrant further investigation.

The protection against the organ injury afforded by PAG was most pronounced in the liver. Therefore, we investigated the expression and activity of the H₂S-synthesizing enzymes CSE and CBS specifically in this organ. We found that the expression and the activity of these enzymes in the rat liver were significantly increased by endotoxemia. In agreement with this finding, increased H₂S synthesis particularly in the liver has also been observed subsequent to severe hemorrhage in a rat model of hemorrhagic shock (Mok *et al.*, 2004). The formation of H₂S in the liver was completely abolished by the treatment of rats with PAG. As PAG elicits a complete and irreversible inactivation of CSE activity *in vitro* (Johnston

et al., 1979), and a near complete inhibition of liver CSE enzyme activity in rodents (Uren *et al.*, 1978), our finding supports the role of CSE as the enzyme mainly responsible for H$_2$S formation outside the central nervous system, where, in turn, CBS is considered the main H$_2$S-forming enzyme (Abe & Kimura, 1996; Chen *et al.*, 1999; Zhao *et al.*, 2003). Interestingly, our finding that inhibition of H$_2$S synthesis lowered ALT (a specific marker for hepatic parenchymal injury) levels in sham-operated animals indicates basal H$_2$S production in the liver. This finding is in accordance with a previous report, which suggested that the liver may be the major site in the body for H$_2$S production, perhaps even responsible for maintaining H$_2$S concentration in the circulation (Zhao *et al.*, 2003).

In conclusion, our results support the view that H$_2$S contributes to the organ injury caused by acute severe endotoxemia. It is likely that the H$_2$S is formed by CSE, since treatment with PAG, an inhibitor of this enzyme, afforded protection against the multiple organ injury caused by LPS. We propose that inhibitors of H$_2$S synthesis may be useful in the therapy of the organ injury associated with sepsis and shock.

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