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Role of hydrogen sulphide in haemorrhagic shock in the rat: protective effect of inhibitors of hydrogen sulphide biosynthesis

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1 Haemorrhagic shock (60 min) in the anaesthetized rat resulted in a prolonged fall in the mean arterial blood pressure (MAP) and heart rate (HR).

2 Pre-treatment (30 min before shock) or post-treatment (60 min after shock) with inhibitors of cystathionine γ lyase (CSE; converts cysteine into hydrogen sulphide (H_2S)), dl-propargylglycine or β-cyanoalanine (50 mg kg^{-1}, i.v.), or glibenclamide (40 mg kg^{-1}, i.p.), produced a rapid, partial restoration in MAP and HR. Neither saline nor DMSO affected MAP or HR.

3 Plasma H_2S concentration was elevated 60 min after blood withdrawal ($37.5 \pm 1.3\,\mu$m, $n=18$ c.f. $28.9 \pm 1.4\,\mu$m, $n=15$, $P<0.05$).

4 The conversion of cysteine to H_2S by liver (but not kidney) homogenates prepared from animals killed 60 min after withdrawal of blood was significantly increased (52.1 ± 1.6 c.f. 39.8 ± 4.1 nmol mg protein^{-1}, $n=8$, $P<0.05$), as was liver CSE mRNA ($2.7\times$). Both PAG (IC_{50}, $55.0 \pm 3.2\,\mu$m) and BCA (IC_{50}, $6.5 \pm 1.2\,\mu$m) inhibited liver H_2S synthesizing activity *in vitro*.

5 Pre-treatment of animals with PAG or BCA (50 mg kg^{-1}, i.p.) but not glibenclamide (40 mg kg^{-1}, i.p., K_{ATP} channel inhibitor) abolished the rise in plasma H_2S in animals exposed to 60 min haemorrhagic shock and prevented the augmented biosynthesis of H_2S from cysteine in liver.

6 These results demonstrate that H_2S plays a role in haemorrhagic shock in the rat. CSE inhibitors may provide a novel approach to the treatment of haemorrhagic shock.
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Keywords: Hydrogen sulphide; haemorrhagic shock; DL-propargylglycine; β-cyanoalanine; glibenclamide

Abbreviations: BCA, β-cyanoalanine; b.p., base pairs; b.p.m., beats per minute; CBS, cystathionine β synthetase; cGMP, cyclic 3′5′ guanosine monophosphate; CSE, cystathionine γ lyase; DMSO, dimethylsulphoxide; HR, heart rate; H_2S, hydrogen sulphide; MAP, mean arterial blood pressure; NaHS, sodium hydrosulphide; NO, nitric oxide; PAG, DL-propargylglycine

Introduction

Haemorrhagic shock involves a complex interplay of vascular events, including a decrease in mean arterial blood pressure (MAP) associated with compensatory release of endogenous vasoconstrictors such as noradrenaline, angiotensin II and vasopressin (Darby & Watt, 1964). Vascular hyporeactivity to noradrenaline also occurs (e.g. Thiemermann *et al*., 1993). A number of previous studies have implicated excessive formation of nitric oxide (NO) produced by the activity of inducible nitric oxide synthase (iNOS) in the hypotension associated with haemorrhagic shock (Thiemermann *et al*., 1993; Ungureanu-Longrois *et al*., 1995).

Over recent years, there has been increased interest in the potential physiological and pathophysiological roles of hydrogen sulphide (H_2S) in the body (for reviews, see Moore *et al*., 2003; Wang, 2003). However, the role of this mediator in haemorrhagic shock has not been studied. It is now becoming increasingly clear that H_2S synthesis from cysteine occurs naturally in a range of mammalian tissues principally by the activity of two enzymes, cystathionine γ lyase (CSE) and cystathionine β synthetase (CBS), although alternative sources (e.g. by activity of cysteine aminotransferase and/or 3-mercaptosulphurtransferase; see Kamoun, 2004) cannot yet be discounted. Relatively high concentrations of H_2S (10–100 μM) have been detected in rat and human blood and in homogenates of brain, kidney and liver (Warenycz *et al*., 1989; Richardson *et al*., 2000; Zhao *et al*., 2001). Within the cardiovascular system, H_2S reduces blood pressure in the anaesthetized rat (Zhao *et al*., 2001) and causes dose-dependent relaxation of the isolated rat aorta (Zhao *et al*., 2001) and mesenteric vascular arterioles (Cheng *et al*., 2004), most probably by an effect on vascular smooth muscle K_{ATP} channels (Zhao & Wang, 2002). The vasorelaxant effect of H_2S is not restricted to mammals, as it occurs also in branchial arteries of the trout (Dombkowski *et al*., 2004). It should be noted that experiments in nonvascular smooth muscle do not rule out an additional K_{ATP}-independent mechanism (Teague *et al*., 2002).

Whether H_2S plays a role in the normal control of cardiovascular function is not yet known, although chronic

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treatment of rats with DL-propargylglycine (PAG, an irreversible 'suicide' inhibitor of CSE; Johnston *et al*., 1979) has recently been reported to cause an elevation in blood pressure in rats (Yan *et al*., 2004). Regardless of the potential physiological significance of H_2S in regulating blood vessel calibre, a number of publications have recently suggested that reduced biosynthesis of H_2S may contribute to the vasoconstriction associated with both pulmonary (Chunyu *et al*., 2003) and essential (Yan *et al*., 2004) hypertension, while excessive formation of this mediator may contribute to the hypotension associated with septic shock (Hui *et al*., 2003) in animal models. The possibility that deranged biosynthesis (or perhaps activity) of H_2S may be associated with cardiovascular disease therefore warrants further study.

Despite previous reports that glibenclamide (K_{ATP} channel inhibitor) provides partial restoration of blood pressure after haemorrhagic shock in the rat (Salzman *et al*., 1997), the role of H_2S (a putative endogenous ligand for K_{ATP} channels) in haemorrhagic shock has not previously been evaluated. Accordingly, we have now compared the effect of glibenclamide with that of PAG and β-cyanoalanine (BCA, a reversible inhibitor of CSE; Pfeffer & Ressler, 1967) on blood pressure of anaesthetized rats subjected to haemorrhagic shock, and monitored the effects of such treatments on plasma H_2S concentration, tissue H_2S formation from added cysteine and tissue CSE mRNA.

Methods

Haemorrhagic shock model in anaesthetized rats

All experiments were undertaken in accordance with the local National University of Singapore regulations and European Community Council Directive 86/609 (OJL 358, 1, December 12, 1987) on the use of animals in the laboratory.

The methods used were as described previously (Shirhan *et al*., 2004). Briefly, male Sprague–Dawley rats (300–350 g) were anaesthetized with a mixture of Hypnorm (1 ml; Jansen Pharmaceutica, Beerse, Belgium) containing fentanyl (0.315 mg), fluanisone (10 mg) and 1 ml of midazolam (Roche, Basel, Switzerland (5 ml of dormicum in 2 ml water)). Animals were injected with 0.3 ml $(100\,g)^{-1}$ (i.p.) of this mixture for induction and 0.1 ml $(100\,g)^{-1}$ (i.v.) for maintenance if required. The left femoral artery was exposed and cannulated for withdrawing blood and for monitoring the MAP and heart rate (HR) and by means of a pressure transducer connected to a PowerLab (AD Instruments, Australia) running Chart (v 5.1). The left femoral vein was also cannulated for injection of drugs or vehicle. At the end of the experiment, animals were killed by cervical dislocation

After an initial 30 min stabilizing period, haemorrhagic shock was induced by withdrawing blood (9–10 ml) in 2 min periods until the MAP decreased to approximately 40 mmHg. Control (sham-operated) animals underwent the same surgery but without withdrawal of blood. Two drug regimens were used in this study. PAG, BCA ($50\,mg\,kg^{-1}$) or vehicle (saline, 0.9% w v^{-1}, $1\,ml\,kg^{-1}$) were administered either 30 min before (pre-treatment) or 60 min after (post-treatment) blood withdrawal. Preliminary experiments revealed that dimethylsulphoxide (DMSO; $0.2\,ml\,kg^{-1}$) administered i.v. caused a rapid and substantial increase in MAP in control animals. Accordingly, glibenclamide ($40\,mg\,kg^{-1}$) or DMSO ($0.15\,ml\,kg^{-1}$) was administered i.p. 60 min after blood withdrawal according to a previously published protocol (Sorrentino *et al*., 1999). In some experiments, rats were administered both glibenclamide ($40\,mg\,kg^{-1}$ i.p.) plus BCA ($50\,mg\,kg^{-1}$, i.v.) 60 min after blood withdrawal. Following drug administration, MAP and HR were monitored continuously for a further 2 h. Blood samples (1 ml) for measurement of plasma H_2S concentration (as described below) were withdrawn from drug- and vehicle-injected animals both prior to and 60 min after blood withdrawal, and at 1 h and 2 h after drug or vehicle injection. Blood was centrifuged ($4000 \times g$, 5 min, room temperature) and plasma aspirated and stored at $-70^{\circ}C$ prior to assay for H_2S (as described below). At the end of the experiment, animals were killed by an overdose ($1\,ml\,kg^{-1}$, i.v.) of anaesthetic and liver, kidney and lung removed for *ex vivo* determination of H_2S biosynthesis (as described below).

Assay of tissue H_2S synthesis

Liver, kidney and lung H_2S-synthesizing activity was essentially as described elsewhere (Stipanuk & Beck, 1982). Briefly, 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 v v^{-1} (liver), 1 : 10 v v^{-1} (lung) and 1 : 20 v v^{-1} (kidney) were determined from preliminary experiments. The reaction mixture (total volume, 500 μl) contained L-cysteine (10 mM, 20 μl), pyridoxal 5′-phosphate (2 mM, 20 μl), saline (30 μl) and tissue homogenate (430 μl). 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 addition of trichloroacetic acid (10% w v^{-1}, 250 μl) to denature protein prior to addition of cysteine. After incubation for 30 min, zinc acetate (1% w v^{-1}, 250 μl) was added to trap evolved H_2S, followed by trichloroacetic acid (10% w v^{-1}, 250 μl). Subsequently, *N*,*N*-dimethyl-*p*-phenylenediamine sulphate (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.). In some experiments, PAG or BCA (0.1–2500 μM) were preincubated with liver homogenate (430 μl) on ice for 15 min before addition of cysteine (10 mM) and pyridoxal 5′-phosphate (2 mM), and then transferred to a water bath at 37°C. Control incubations contained an equal volume of water vehicle. The basal concentration of H_2S was determined in incubates in which trichloroacetic acid was added at zero time ($T = 0$) prior to addition of cysteine and incubation (37°C, 30 min). At the end of this period, trichloroacetic acid (10% w v^{-1}, 250 μl) 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 sodium hydrosulphide (NaHS; 3.12–250 μM) and results are expressed as nmol H_2S formed mg^{-1} protein (determined using the Bradford assay, Bio-Rad Ltd, U.S.A.).

Measurement of plasma H_2S

Aliquots (75–200 μl) of plasma were mixed with distilled water (100–450 μl; depending on volume of plasma used),

trichloroacetic acid (10% w v^{-1}, 300 μl), zinc acetate (1% w v^{-1}, 150 μl), *N*,*N*-dimethyl-*p*-phenylenediamine sulphate (20 μM, 100 μl) in 7.2 M HCl and $FeCl_3$ (30 μM, 133 μl) in 1.2 M HCl in 96-well plates as described above. The absorbance of the resulting solution (670 nm) was measured 15 min thereafter. As above, all samples were assayed in duplicate and H_2S was calculated against a calibration curve of NaHS (3.12–250 μM). Results show plasma H_2S concentration in μM.

Measurement of tissue CSE mRNA

Briefly, liver tissue (100 mg) was homogenized in 1 ml ice-cold TRIzol reagent (Invitrogen, U.S.A.) using a Polytron homogenizer (Heidolph, 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 top aqueous phase was transferred to an eppendorf tube and isoproponal (0.5 ml) added. After further incubation at room temperature (10 min), samples were re-centrifuged (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 μl) 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 stored at −80°C until required.

One-step RT–PCR method was employed in this study (QIAGEN® one-step RT–PCR kit, Qiagen Ltd, U.S.A.). Total RNA template(1 μg) was mixed with 5 × RT–PCR buffer (4 μl), dNTP mix (400 μM, 0.8 μl), 1.2 μl of each primer (0.6 μM), enzyme mix (0.8 μl, a mixture of omniscript, sensiscript reverse transcriptases and HotStar Taq DNA polymerase) and DEPC-treated water. The final volume was 20 μl. For the detection of CSE mRNA, the forward primer sequence used was 5′-CATGGATGAAGTGTATGGAGGC-3′, and the reverse primer sequence was 5′-CGGCAGCAGAGGTAA CAATCG-3′. The PCR product size was 445 bp. 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 primer-specific annealing and 72°C (30 s) for extension. The reaction without RNA template (none template control (NTC)) was also performed as a negative control. PCR products were analysed by 1.5% w v^{-1} agarose gel electrophoresis and imaged by the MultiGenius Bioimaging system (Syngene, U.K.). The band intensity was semi-quantified by densitometry using Gel analysis software (Syngene Ltd).

Drugs and chemicals

Unless noted previously, all drugs and reagents were purchased from Sigma Ltd, St Louis, MO, U.S.A.

Statistical analysis

Results indicate the mean ± s.e.m., with the number of observations shown in parenthesis. The statistical significance of differences between multiple groups of data was determined by one-way analysis of variance (ANOVA) with *post hoc* Tukey's test. The statistical significance of differences between two groups was determined by Student's *t*-test. In either case, a probability (*P*) value of less than 0.05 was taken to indicate statistical significance.

Results

Effect of PAG and BCA on MAP and HR in haemorrhagic shock

The MAP of sham-operated rats was 105.6 ± 4.4 mmHg ($n = 8$) at the end of the 30 min equilibration period and was not significantly altered 180 min thereafter (93.9 ± 3.6 mmHg, $n = 8$, $P > 0.05$). In animals subjected to haemorrhagic shock, withdrawal of blood (to an initial approx. MAP of 40 mmHg) resulted in consistently lowered MAP for up to 180 min (48.1 ± 4.1 mmHg, $n = 8$) (Figure 1a). In these animals, an i.v. injection of saline did not affect MAP or HR. No significant change in HR was detected in sham-operated controls (e.g. 312.2 ± 5.3 b.p.m. at the start of the experiment c.f. 301 ± 6.5 b.p.m. at 180 min, $n = 8$, $P > 0.05$). HR in animals subjected to haemorrhagic shock reduced gradually over the period of the experiment from 315.3 ± 3.8 b.p.m. immediately prior to blood withdrawal to 260.6 ± 2.2 b.p.m. at 180 min, $n = 8$, $P < 0.05$ (Figure 1b).

Preliminary experiments were carried out to determine the effect of PAG or BCA (50 mg kg^{-1}, i.v.) on MAP and HR of control (i.e. nonshocked) animals. Treatment of rats with either drug failed to affect MAP (Figure 1c) or HR (e.g. PAG 295.0 ± 12.5 b.p.m. and BCA 290 ± 9.4 b.p.m., both measured after 180 min, c.f. 309.2 ± 10.1 b.p.m. at the start of the experiment, $n = 8$, $P > 0.05$).

In contrast, either pre-treatment (i.e. 30 min before blood withdrawal) or post-treatment (i.e. 60 min after blood withdrawal) of rats with PAG or BCA (50 mg kg^{-1}, i.v.) resulted in an increase in MAP (Figures 2 and 3). Post-treatment with either drug resulted in a relatively rapid (within 2–5 min) rise in MAP, which peaked and was then well maintained over the following 120 min observation period. Similarly, both pre-treatment and post-treatment of rats with PAG or BCA restored the decline in HR, which follows blood withdrawal. Thus, HR (measured at 180 min after withdrawal of blood) of animals administered PAG pre-treatment/post-treatment was 302.6 ± 3.1 and 301.5 ± 3.0 b.p.m., respectively (both $n = 8$; $P < 0.05$ c.f. nontreated, shocked animals measured at the same time, 260.6 ± 2.2, $n = 8$). Corresponding HR for pre-treatment/post-treatment with BCA was 301.3 ± 3.1 and 309.9 ± 4.2 b.p.m. (both $n = 8$, $P < 0.05$).

Effect of glibenclamide (administered alone and in combination with BCA) on MAP and HR in haemorrhagic shock

Administration of glibenclamide (40 mg kg^{-1}, i.p.) 60 min after withdrawal of blood (i.e. post-treatment) also caused an increase in MAP (Figure 4a). The rise in MAP was slower in onset (peaking after approx. 15 min) than following PAG or BCA injection, but again was well maintained over 180 min. Glibenclamide administration also increased HR in shocked animals (304.0 ± 8.5, $n = 8$, $P < 0.05$, 180 min). In control experiments, injection of vehicle (DMSO, 0.15 ml kg^{-1}, i.p.)

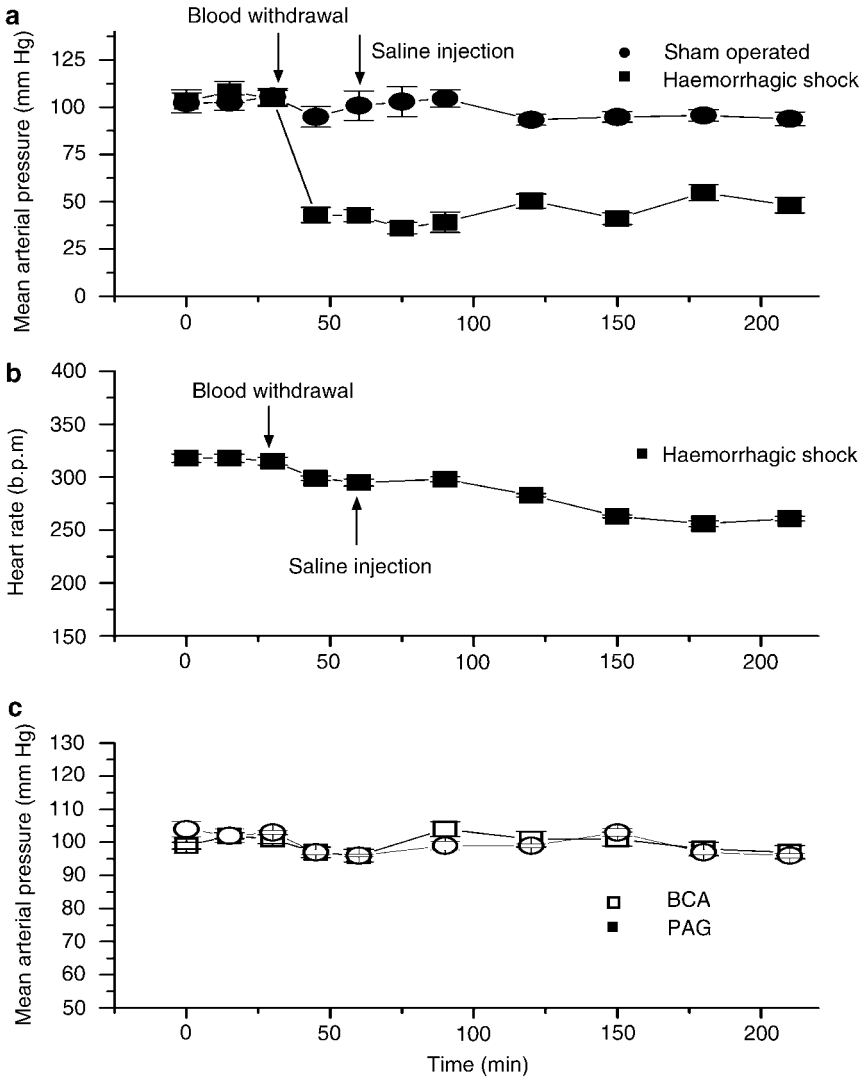

Figure 1 Effect of haemorrhagic shock on (a) MAP and (b) HR in anaesthetized rats. Arrows indicate timing of blood withdrawal and saline (0.9% w v^{-1} NaCl, 1 ml kg^{-1}) injection (60 min after withdrawal of blood). (c) Effect of PAG and BCA (50 mg kg^{-1}, i.v.) in control (i.e. unshocked) anaesthetized rats. Results show MAP in mmHg, and are mean ± s.e.m., $n = 8$.

did not affect MAP (Figure 4a) or HR (data not shown) in shocked animals over the same time period. Glibenclamide, at this dose and route of administration, did not affect MAP (Figure 4b) or HR (data not shown) in control (i.e. unshocked rats). Furthermore, combination treatment with both glibenclamide (40 mg kg^{-1}, i.p.) and BCA (50 mg kg^{-1}, i.v.) given 60 min after blood withdrawal in shocked rats also increased MAP in these animals (Figure 5). However, combination treatment in this way proved to be no more effective in raising MAP after haemorrhagic shock than with either drug given alone.

Effect of PAG, BCA and glibenclamide on plasma H_2S concentration in haemorrhagic shock

Plasma H_2S concentration prior to withdrawal of blood in anaesthetized rats was 28.9 ± 1.4 μM ($n = 15$). At 60 min after withdrawal of blood, plasma concentration of H_2S was increased by approximately 30% to 37.5 ± 1.3 μM ($n = 18$, $P < 0.05$). Thereafter, plasma H_2S concentration declined to control values (e.g. 33.4 ± 1.6 μM at 120 min and 33.2 ± 1.0 μM at 180 min, both $n = 6$–14, $P > 0.05$ c.f. prior to shock). Pretreatment of animals (30 min before blood withdrawal) with PAG (17.0 ± 1.2 μM, $n = 8$, $P < 0.05$) or BCA (13.3 ± 0.8 μM, $n = 8$, $P < 0.05$), but not glibenclamide (40 mg kg^{-1}, i.p., 37.2 ± 4.8 μM, $n = 8$, $P > 0.05$), abolished the rise in plasma H_2S concentration, which occurred 60 min after withdrawal of blood. Indeed, PAG and BCA administration reduced plasma H_2S concentrations below that observed in nonshocked animals by 41.1 and 53.9%, respectively.

Effect of PAG and BCA on liver, kidney and lung H_2S biosynthesis in haemorrhagic shock

Incubation of rat liver and kidney (but not lung) homogenates with cysteine (3 mM) and pyridoxal 5′-phosphate (2 mM) resulted in the formation of significant amounts of H_2S, as determined spectrophotometrically at the end of 30 min incubation (Figure 6a). In control experiments in which trichloroacetic acid (10% v v^{-1}, 250 μl) was added to the

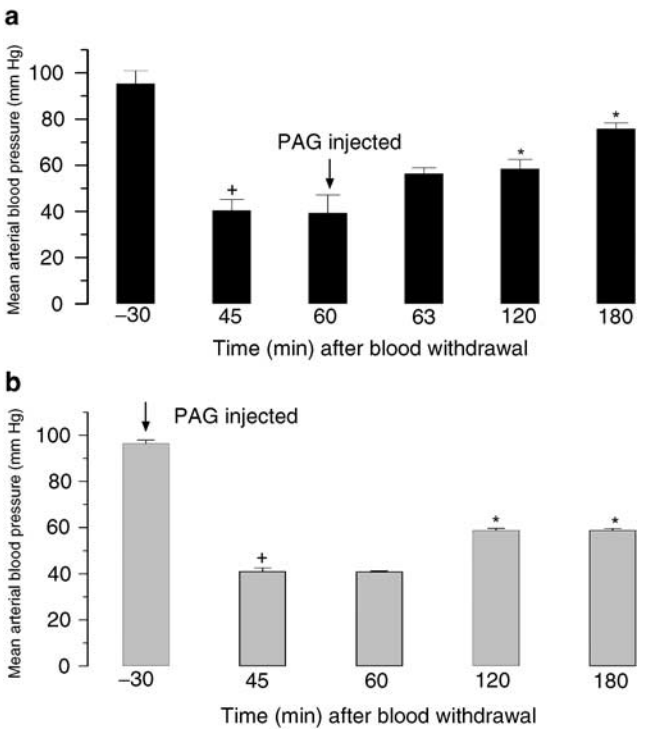

Figure 2 Time-dependent effect of pre-treatment (a; 30 min before blood withdrawal of blood) and post-treatment (b; 60 min after withdrawal of blood) with PAG (50 mg kg^{-1}, i.v.) on MAP in anaesthetized rats subjected to haemorrhagic shock. Arrows indicate timing of drug injection. Results show MAP in mmHg, and are mean ± s.e.m., $n=8$, $^{+}P<0.05$ c.f. control MAP (−30 min), $^{*}P<0.05$ c.f. haemorrhagic shock measured at 45 min.

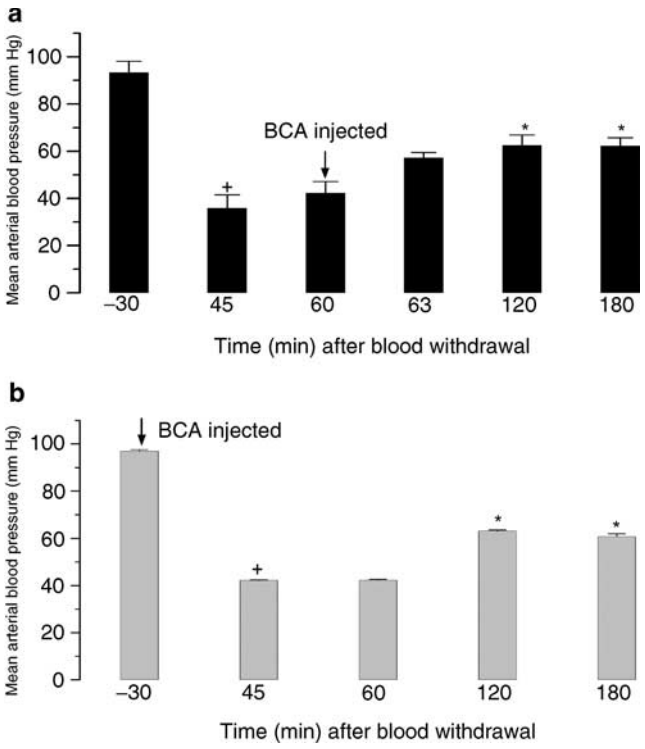

Figure 3 Time-dependent effect of pre-treatment (a; 30 min before blood withdrawal of blood) and post-treatment (b; 60 min after withdrawal of blood) with BCA (50 mg kg^{-1}, i.v.) on MAP in anaesthetized rats subjected to haemorrhagic shock. Arrows indicate timing of drug injection. Results show MAP in mmHg, and are mean ± s.e.m., $n=8$, $^{+}P<0.05$ c.f. control MAP (−30 min), $^{*}P<0.05$ c.f. haemorrhagic shock measured at 45 min.

incubate prior to cysteine, the concentrations of H_2S detected were at or close to the limit of detection of the assay ($<1.5\,\mu$M). The formation of H_2S in homogenates prepared from liver (but not kidney or lung) from animals subjected to haemorrhagic shock (60 min after withdrawal of blood) was significantly greater ($P<0.05$) than in homogenates prepared from animals prior to shock (Figure 6a).

Pre-treatment of animals (i.e. 30 min before blood withdrawal) with either PAG or BCA (50 mg kg^{-1}, i.v.) significantly reduced liver H_2S biosynthesis in shocked (60 min after blood withdrawal) animals. PAG but not BCA administration also significantly reduced kidney H_2S biosynthesis at this time point (Figure 6b).

In separate experiments, both PAG and BCA produced concentration-related inhibition of rat liver (nonshocked) formation of H_2S from added cysteine with IC_{50} values of $55.0\pm3.2\,\mu$M ($n=7$) and $6.5\pm1.3\,\mu$M ($n=5$), respectively (Figure 7).

Effect of haemorrhagic shock on liver CSE mRNA

Rat liver homogenates from nonshocked animals contained detectable amounts of mRNA for CSE (Figure 7). Haemorrhagic shock (60 min after blood withdrawal) significantly increased liver CSE mRNA in the present experiments (Figure 8).

Discussion

The present experiments provide the first evidence for a role of endogenous H_2S in haemorrhagic shock in the rat. We show here that both PAG and BCA (inhibitors of CSE) produce partial restoration of the lowered MAP and HR after blood withdrawal by inhibition of endogenous H_2S biosynthesis. We also confirm the results from a previous study (Salzman *et al.*, 1997) in which the K_{ATP} channel inhibitor, glibenclamide, partially restored MAP in haemorrhagic shock.

Role of H_2S in haemorrhagic shock – biochemical studies

Blood withdrawal in anaesthetized rats caused 60 min thereafter a significant increase in plasma H_2S concentration coupled with evidence of augmented H_2S-synthesizing activity in the liver. Both effects are relatively short-lived in that plasma H_2S concentration and liver H_2S-synthesizing activity returned to control values within 120 min after withdrawal of blood. Liver CSE mRNA was also significantly increased 60 min after withdrawal of blood.

The identity of the cells/tissues responsible for synthesizing H_2S following withdrawal of blood is not known. Since rat liver contains large amounts of CSE, it is possible that the increased H_2S-synthesizing activity, which we have detected in this organ after haemorrhagic shock, may account for the observed rise in plasma levels of this mediator. However, the

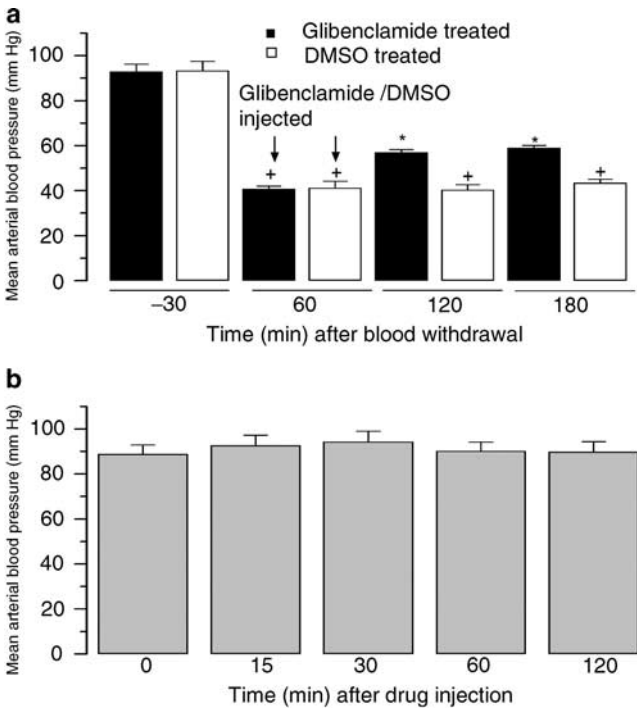

Figure 4 (a) Time-dependent effect of post-treatment (60 min after withdrawal of blood) with glibenclamide (40 mg kg^{-1}, i.p.) or DMSO vehicle (0.15 ml kg^{-1}, i.p.) on MAP in anaesthetized rats subjected to haemorrhagic shock. Arrows indicates timing of drug or vehicle injection. Results show MAP in mmHg, and are mean ± s.e.m., $n=8$, $^{+}P<0.05$ c.f. control MAP (−30 min), $^{*}P<0.05$ c.f. haemorrhagic shock measured at 60 min. (b) Time-dependent effect of glibenclamide (40 mg kg^{-1}, i.p.) on MAP in control (i.e. unshocked) anaesthetized rats. Results show MAP in mmHg and are mean ± s.e.m., $n=8$.

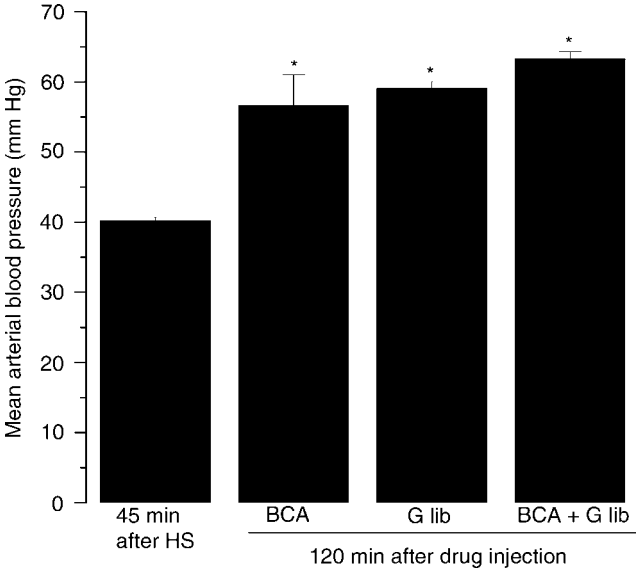

Figure 5 Effect of post-treatment (60 min after withdrawal of blood) with BCA (50 mg kg^{-1}, i.v.), glibenclamide (40 mg kg^{-1}, i.p.) or both BCA and glibenclamide (at doses stated) on MAP of shocked rats determined 120 min after withdrawal of blood. Results show MAP in mmHg, and are mean ± s.e.m., $n=8$, $^{*}P<0.05$ c.f. MAP of shocked animals measured at 45 min.

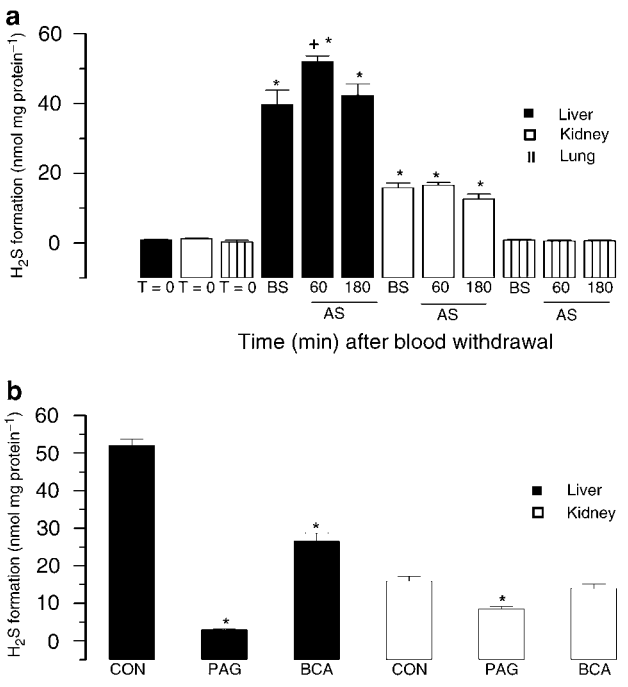

Figure 6 (a) Formation of H_2S from cysteine (10 mM) in the presence of pyridoxal 5′ phosphate (1 mm) following incubation (37°C, 30 min). Homogenates were prepared from livers removed from anaesthetized animals prior to (BS) or 60 or 120 min after haemorrhagic shock (AS). $T=0$ shows the concentration of H_2S in control liver incubations in which the reaction was stopped at zero time by addition of 10% w v^{-1} trichloroacetic acid (250 μl) prior to addition of cysteine (10 mM) and incubation (37°C, 30 min), as described above. (b) Formation of H_2S from cysteine (10 mM) in the presence of pyridoxal 5′ phosphate (1 mm) following incubation (37°C, 30 min) in animals pre-treated with either PAG or BCA (50 mg kg^{-1}, i.v.). Results show H_2S formation as nmol formed mg protein^{-1}, and are mean ± s.e.m., $n=8$, $^{*}P<0.05$ c.f. $T=0$ controls, $^{+}P<0.05$ c.f. H_2S formation before haemorrhagic shock (BS).

participation of other cells types cannot be excluded. Indeed, CSE present either in vascular smooth muscle or endothelial cells may also contribute to the rise in plasma H_2S following shock. Clearly, further studies are required to identify the cell types involved in H_2S biosynthesis following haemorrhagic shock.

Although liver CSE mRNS is elevated after haemorrhagic shock (implying upregulation), it is also possible that the enzyme activity of liver CSE is increased following withdrawal of blood. Little is known of the factors that control CSE activity either *in vitro* or *in vivo*. However, since sodium nitroprusside (NO donor) administration in anaesthetized rats reportedly increases H_2S formation in various tissues by a cGMP-dependent mechanism (Zhao *et al.*, 2003), it is conceivable that NO, formed in large quantities during haemorrhagic shock (e.g. Shirhan *et al.*, 2004), may 'switch on' liver H_2S biosynthesis. The possibility of 'crosstalk' between NO and H_2S has been raised previously and reviewed by Moore *et al.* (2003) and Wang (2003).

Role of H_2S in haemorrhagic shock – use of CSE inhibitors

Administration of either PAG or BCA provided partial restoration of MAP and HR in shocked animals. Complete

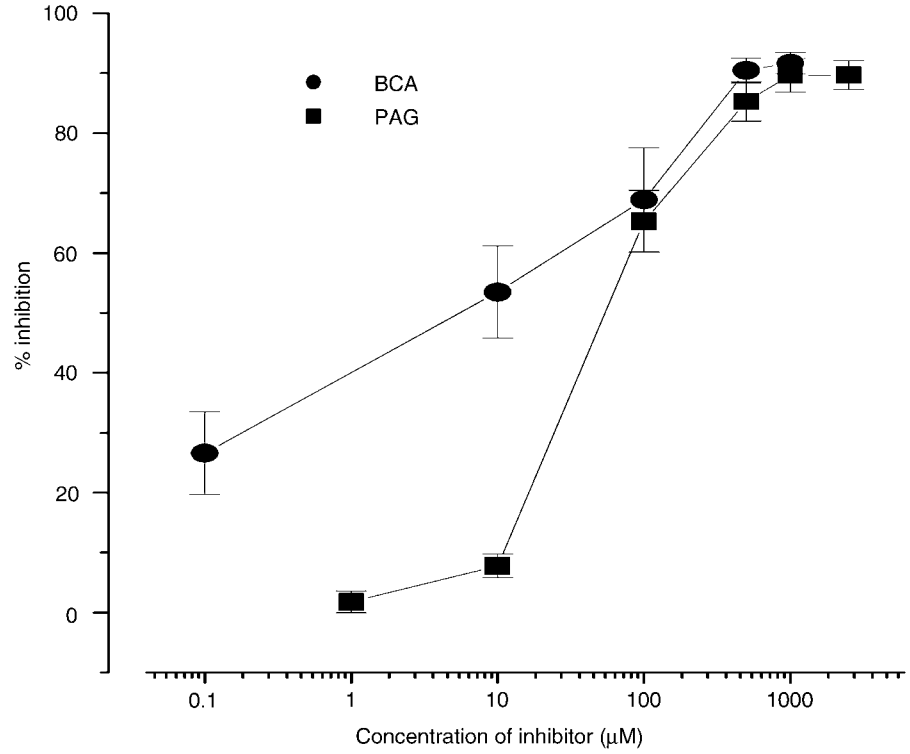

Figure 7 Inhibition of rat liver H_2S synthesis by PAG and BCA *in vitro*. Results show % inhibition of H_2S formation (c.f. incubates containing an equivalent of water vehicle), and are mean ± s.e.m., $n = 5$–6.

reversal of MAP and HR was not achieved in these experiments, implying that factors other than H_2S are involved in the hypotension of haemorrhagic shock, thereby testifying to the multifactorial nature of the condition. It should be noted that pre-treatment or post-treatment with saline did not affect cardiovascular parameters, indicating that simple fluid replacement in these animals does not explain the beneficial effect of PAG and BCA on the cardiovascular system.

PAG is an irreversible (covalent) CSE inhibitor, while the effect of BCA is reversible (Pfeffer & Ressler, 1967; Washtien *et al*., 1977; Johnston *et al*., 1979). PAG does not inhibit rat liver or kidney CBS activity *in vitro* (Stipanuk & Beck, 1982). Accordingly, the actions of PAG observed in the present study are unlikely to be due to inhibition of CBS enzyme activity. Both PAG and BCA block CSE by binding to the pyridoxal 5′-phosphate site, and as such the possibility that these drugs may inhibit other enzymes that require this cofactor should be considered. A number of enzymes with amino-acid substrates (e.g. aminotransferases) are pyridoxal 5′-phosphate dependent (for a review, see Christen & Mehta, 2001), and therefore potential targets for these inhibitors. While such enzymes are unlikely to play a part in the present results, it is important to correlate the pharmacological activity of CSE inhibitors with effects on the cysteine : H_2S system.

To this end, we undertook additional experiments to examine the effect of PAG and BCA on H_2S biosynthesis both *in vitro* and *ex vivo* following drug administration. Both drugs inhibited H_2S formation from cysteine by liver homogenates *in vitro*. This effect was concentration related and achieved at relatively low concentrations (IC50 s of 55 and 6.5 μM, respectively). A similar inhibitory effect of PAG on rat liver CSE activity *in vitro* has been reported previously (Abeles & Walsh, 1973; Washtien *et al*., 1977). The observation in the present work that high concentrations (1 mM) of PAG and BCA cause >90% inhibition of H_2S formation suggests that CSE is the predominant H_2S-synthesizing enzyme in rat liver. This finding accords well with previous studies in which large amounts of this enzyme have been detected in liver hepatocytes (for a review, see Ishii *et al*., 2004), but are contrary to a recent report in which liver H_2S formation from added cysteine was attributed largely to CBS (Zhao *et al*., 2003).

Interestingly, BCA was considerably more potent (approx. 8.5 times) than PAG as an inhibitor of H_2S biosynthesis *in vitro* and, even though only used at a single dose, was more effective in terms of reducing plasma H_2S levels after haemorrhagic shock. In contrast, liver from BCA-pre-treated animals exhibited less inhibition of H_2S formation than liver from PAG-treated animals. Similarly, PAG (but not BCA) inhibited kidney H_2S synthesis *ex vivo*. Conceivably, BCA (reversible inhibitor) is less effective than PAG (irreversible inhibitor) in this assay due to dissociation from the enzyme during preparation of homogenates. It should be noted that, on a mol for mol basis, animals received similar amounts of both PAG (442 μmol kg^{-1}) and BCA (438 μmol kg^{-1}).

There is currently a dearth of information in the literature concerning the pharmacokinetic profile of either drug following parenteral administration. In one study, PAG administered i.p. achieved peak maximal serum concentrations within 1–2 h and was thereafter rapidly excreted in the urine (Zhang *et al*., 1994). In a second study, i.p. injection of PAG in the rat resulted in an almost complete inhibition of liver CSE (without any effect on CBS) activity within 120 min (Kodama *et al*., 1984). In the present experiments, PAG and BCA were administered i.v. and hence a more rapid distribution might

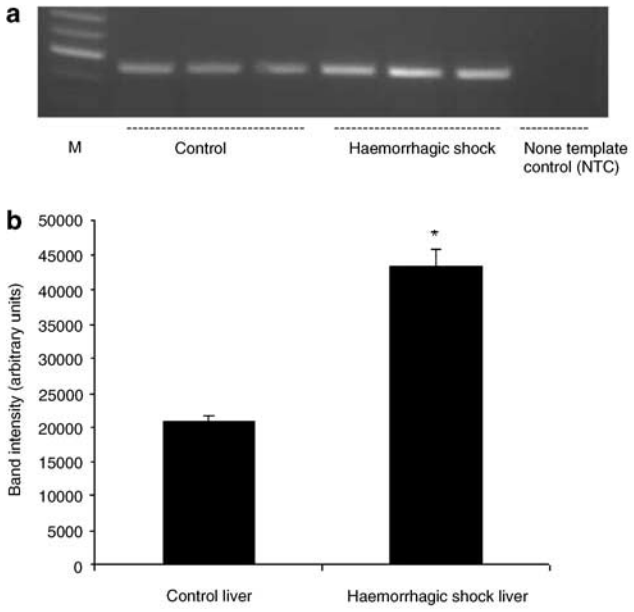

Figure 8 (a) Representative blots from three livers, showing the presence of CSE (445 b.p., 29 cycles). M shows molecular weight markers. (b) Quantitation of blots shown in (a). Data indicate the relative intensities in arbitrary units, and are mean ± s.e.m., $n = 3$, $^*P < 0.05$.

be expected. Further work to elucidate the time course and effective plasma/tissue concentrations of these inhibitors after i.v./i.p. administration would be useful. However, we did observe that administration of PAG and BCA to rats 30 min prior to blood withdrawal resulted in both abolition of the haemorrhagic shock-induced rise in plasma H_2S and a significant (and in the case of PAG an almost complete) inhibition of liver H_2S biosynthesis from cysteine as measured *ex vivo*, determined 60 min thereafter. Accordingly, we conclude that both the dose administered and the time course(s) of injection used for each drug were appropriate.

Role of H_2S in haemorrhagic shock – use of glibenclamide

We also noted that administration of glibenclamide (but not DMSO vehicle) partially restored the MAP in animals subjected to haemorrhagic shock. At the dose used, no change in MAP or HR was apparent in control, that is, nonshocked animals. This result confirms data from a previous study (Salzman *et al*., 1997). As noted in the Introduction, H_2S has been reported to dilate blood vessels by opening K_{ATP} channels. Whether glibenclamide restores MAP in shocked rats by blocking the effect of endogenously released H_2S has not been directly addressed in this work, but should be considered a possibility. That glibenclamide administration, unlike PAG and BCA, did not affect the shock-induced rise in plasma H_2S suggests that an effect on H_2S biosynthesis can be excluded. With this in mind, we examined the effect of combined treatment of shocked rats with both glibenclamide and BCA. Interestingly, no further beneficial effect on MAP could be detected. It is conceivable that these drugs were used at maximally effective concentrations and as such no additive effect was possible.

Intriguingly, glibenclamide also partially reversed the hypotension associated with lipopolysaccharide injection in anaesthetized rats (Gardiner *et al*., 1999; Sorrentino *et al*., 1999) and caused reduction (i.e. vasoconstriction) in renal, mesenteric and hindquarters conductances in these animals (Gardiner *et al*., 1999). Endotoxic shock is associated with augmented H_2S biosynthesis (Hui *et al*., 2003) and glibenclamide may again be acting to inhibit the effect of endogenously released H_2S. However, it should also be noted that NO has recently been suggested to activate K_{ATP} channels in mesenteric resistance arteries in LPS-treated rats (Wu *et al*., 2004) and thus an interaction between glibenclamide and NO cannot be discounted.

In conclusion, we show here that inhibitors of H_2S biosynthesis mimic the ability of glibenclamide to bring about partial restoration of MAP and HR after haemorrhagic shock in the anaesthetized rat. Accordingly, we propose that at least part of the hypotension associated with haemorrhagic shock in the rat is due to the release of endogenous H_2S. Since H_2S is a vasodilator, it seems likely that overproduction of H_2S during haemorrhagic shock contributes to the hypotension observed. However, inhibition of cardiac H_2S biosynthesis might also be expected to improve cardiac output (and hence raise blood pressure) as a negative inotropic effect of injected H_2S has recently been noted in anaesthetized rats (Geng *et al*., 2004). The finding that pre-treatment of animals with CSE inhibitors increased MAP only 60 min after withdrawal of blood suggests that H_2S does not play a part in the immediate (i.e. within a few minutes) response to haemorrhagic shock, but is involved in the later stages (i.e. 60 min and onwards).

Whether inhibition of H_2S biosynthesis (possibly combined with concurrent antagonism of the K_{ATP} channel) represents a novel approach to the treatment of haemorrhagic shock in the clinic remains to be determined.

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