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Hydrogen sulphide is a mediator of carrageenan-induced hindpaw oedema in the rat

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Hydrogen sulphide (H_2S) is a naturally occurring gas, with potent vasodilator activity. In this report, we identify a role for H_2S in carrageenan-induced hindpaw oedema in the rat. Intraplantar injection of carrageenan (150 μl, 2% (w v^{-1})) resulted in an increase in hindpaw H_2S synthesising enzyme activity and increased myeloperoxidase (MPO) activity. Pretreatment (i.p. 60 min before carrageenan) with DL-propargylglycine (PAG, 25–75 mg kg^{-1}), an inhibitor of the H_2S synthesising enzyme cystathionine-γ-lyase (CSE), significantly reduced carrageenan-induced hindpaw oedema in a dose-dependent manner (e.g. increase in hindpaw weight at 3 h, saline: 0.12 $\pm$ 0.017 g; carrageenan, 1.39 $\pm$ 0.037 g; PAG, 50 mg kg^{-1}, 1.11 $\pm$ 0.06 g, $n = 10$) and MPO activity (fold increase) in the hindpaw (saline: 1.0 $\pm$ 0.12; carrageenan, 2.92 $\pm$ 0.45 g; PAG, 50 mg kg^{-1}, 1.1 $\pm$ 0.22, $n = 10$); PAG (50 mg kg^{-1}) also inhibited H_2S synthesising enzyme activity (nmol μg DNA^{-1}) in the hindpaw in a dose-dependent manner (saline, 0.46 $\pm$ 0.05; carrageenan, 0.71 $\pm$ 0.08 g; PAG, 50 mg kg^{-1}, 0.17 $\pm$ 0.05, $n = 10$).
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Abbreviations: CSE, cystathionine-γ-lyase; H_2S, hydrogen sulphide; MPO, myeloperoxidase; PAG, DL-propargylglycine; NO, nitric oxide

Introduction

Gases such as nitric oxide (NO) and carbon monoxide (CO) play important roles both in normal physiology and in disease. In recent years, interest has been directed towards other naturally occurring gases such as hydrogen sulphide (H_2S), which has been shown to exhibit potent vasodilator activity both *in vitro* and *in vivo* most probably by opening smooth muscle K^+-ATP channels (Wang, 2002; Moore *et al*., 2003). Both cystathionine-γ-lyase (CSE) and cystathionine-β-synthetase (CBS) utilise L-cysteine as substrate to form H_2S. CSE appears to be the more important of the two enzymes in synthesising H_2S in the vasculature and heart (Wang, 2002; Moore *et al*., 2003). In animals, H_2S relaxes vascular smooth muscle *in vitro* and *in vivo* (Zhao *et al*., 2001), and recent reports suggest that changes in H_2S biosynthesis may occur in cardiovascular disease states such haemorrhagic (Mok *et al*., 2004) and septic shock (Hui *et al*., 2003) as well as essential (Yan *et al*., 2004) and pulmonary (Chunyu *et al*., 2003) hypertension. However, a role for H_2S as a mediator of inflammation has not yet been defined.

In this paper, we provide evidence that H_2S contributes to the hindpaw oedema induced by carrageenan injection. Intraplantar injection of carrageenan in the rat hindpaw resulted in an increase in the H_2S synthesising enzyme activity, while pretreatment with DL-propargylglycine (PAG), an irreversible inhibitor of CSE, significantly reduced inflammation in a dose-dependent manner in carrageenan-induced hindpaw oedema, as determined by a decrease in both hindpaw weight and MPO activity.

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Methods

Measurement of carrageenan-induced hindpaw oedema formation in the rat

All experiments were undertaken in accordance with local National University of Singapore regulations and European Community Council Directive 86/609 (OJL 358, 1, 12 December 1987) on the use of animals in the laboratory. Rats (male, Wistar, 110–160 g) were used in this study. Experiments were conducted essentially as described previously (Handy & Moore, 1998). Animals were accommodated in the Animal Housing Unit of this University for 3 days prior to use and thereafter acclimatised in the laboratory for 1 h prior to commencement of the experiment. Carrageenan (150 μl, 2% (w v^{-1})) or saline (0.9% (w v^{-1}), 150 μl) was administered intraplantar into one hindpaw. In some experiments, PAG (25–75 mg kg^{-1}) or vehicle (saline, 0.9% (w v^{-1}), 2 ml kg^{-1}) was injected i.p. 60 min before intraplantar carrageenan administration. To measure hindpaw oedema, animals were held firmly and a hindpaw (chosen at random) immersed into a beaker containing warm (37°C) water placed on a top-pan balance (Mettler Ltd, Japan). Oedema formation was monitored as the difference in hindpaw weight (g) before and after intraplantar carrageenan or saline injection. At the end of the experiment,

animals were killed by cervical dislocation, the injected hindpaw removed and frozen at −80°C until assayed as below.

Assay of tissue H_2S synthesis

H_2S synthesising activity was undertaken essentially as described elsewhere (Stipanuk & Beck, 1982). Briefly, hindpaws from animals treated as above were thawed and homogenised (1 : 20 Mettler) (Ultra-Turrax, U.S.A.) in 100 mM ice-cold potassium phosphate buffer (pH 7.4). The reaction mixture (total volume, 500 μl) contained L-cysteine (10 mM; 20 μl), pyridoxal 5′-phosphate (2 mM; 20 μl), and saline (30 μl). The reaction was performed in parafilmed eppendorf tubes and initiated by transferring the tubes from ice to a water bath at 37°C. 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 30 min thereafter using a 96-well microplate reader (Tecan Systems Inc., U.S.A.). The basal concentration of H_2S was determined in incubates in which trichlororacetic acid (10% (w v^{-1}), 250 μl) was added at zero time ($T=0$) prior to the addition of cysteine and incubation (37°C, 30 min). Basal concentration was subtracted from all measurements. 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 microgram DNA (which was determined spectrofluorometrically according to a previously described procedure; Labarca & Paigen, 1980).

Measurement of myeloperoxidase (MPO) activity

Neutrophil sequestration in the hindpaw was quantified by measuring tissue MPO activity (Bhatia *et al*., 1998). Briefly, hindpaws were thawed and homogenised in 20 mM phosphate buffer (pH 7.4), centrifuged (10,000 × *g*, 10 min, 4°C), and the resulting pellet resuspended in 50 mM phosphate buffer (pH 6.0) containing 0.5% (v v^{-1}) hexadecyltrimethylammonium bromide. The suspension was subjected to four cycles of freezing and thawing and further disrupted by sonication (40 s). Samples were then centrifuged (10,000 × *g*, 5 min, 4°C) and the supernatant used for the MPO assay. The reaction mixture consisted of tissue supernatant (50 μl), tetramethylbenzidine (1.6 mM), sodium phosphate buffer (80 mM, pH 5.4), and hydrogen peroxide (0.3 mM). The total incubation volume was 100 μl. Incubations were conducted at 37°C for 110 s, the reaction was then terminated with H_2SO_4 (50 μl, 0.18 M), and absorbance measured (450 nm). Tissue MPO activity was corrected for DNA concentration (Labarca & Paigen, 1980). Results are expressed as MPO activity per microgram DNA (fold increase over control).

Statistical analysis

Results indicate mean ± s.e.m., with the number of observations shown in parenthesis. Statistical significance of differences between groups of data was determined by one-way analysis of variance with *post-hoc* Tukey's test. A probability (P) value of less than 0.05 was taken to indicate statistical significance.

Drugs and chemicals

Unless indicated previously, all drugs and reagents were purchased from Sigma Ltd, St Louis, MO, U.S.A. PAG solutions were made fresh on the day of the experiment.

Results

Intraplantar carrageenan injection increases hindpaw H_2S synthesising enzyme activity: effect of pretreatment with PAG

Intraplantar injection of carrageenan in the rat hindpaw resulted in a significant increase (approx. 54%) in H_2S synthesising enzyme activity (from added L-cysteine) in this tissue. In contrast, intraplantar injection of saline did not significantly affect H_2S synthesising enzyme activity in this tissue (saline, 0.46 ± 0.05 nmol μg^{-1} DNA, c.f. 0.47 ± 0.06 nmol μg^{-1} DNA in untreated animals, $n=10$, $P>0.05$). Pretreatment of animals with the CSE inhibitor, PAG, prior to carrageenan injection reduced the increase in hindpaw H_2S synthesising enzyme activity in a dose-dependent manner. H_2S synthesising activity in the hindpaw was reduced to values below those detected in control (i.e. noncarageenan-injected) animals (Figure 1).

PAG pretreatment reduces carrageenan-induced hindpaw oedema and MPO activity in the hindpaw

As expected, hindpaw carrageenan injection results in oedema and an increase in neutrophil infiltration as determined by MPO activity (Figure 2). Pretreatment of animals with PAG, at doses that inhibit hindpaw H_2S synthesising enzyme activity, reduces carrageenan-induced hindpaw oedema and MPO activity in the hindpaw in a dose-dependent manner

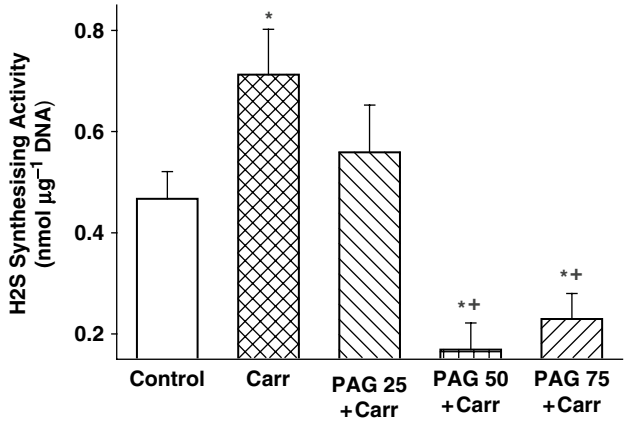

Figure 1 Effect of intraplantar carrageenan (Carr) injection on H_2S synthesising enzyme activity in the hindpaw and the effect of different doses of PAG on enzyme activity. Hindpaws were removed 3 h after carrageenan injection and assayed for enzyme activity. Results show mean ± s.e.m., $n=10$, $^*P<0.05$, c.f. control (administered intraplantar saline), $^{+}P<0.05$, c.f. carrageenan. Doses of PAG shown are mg kg^{-1} (i.p.).

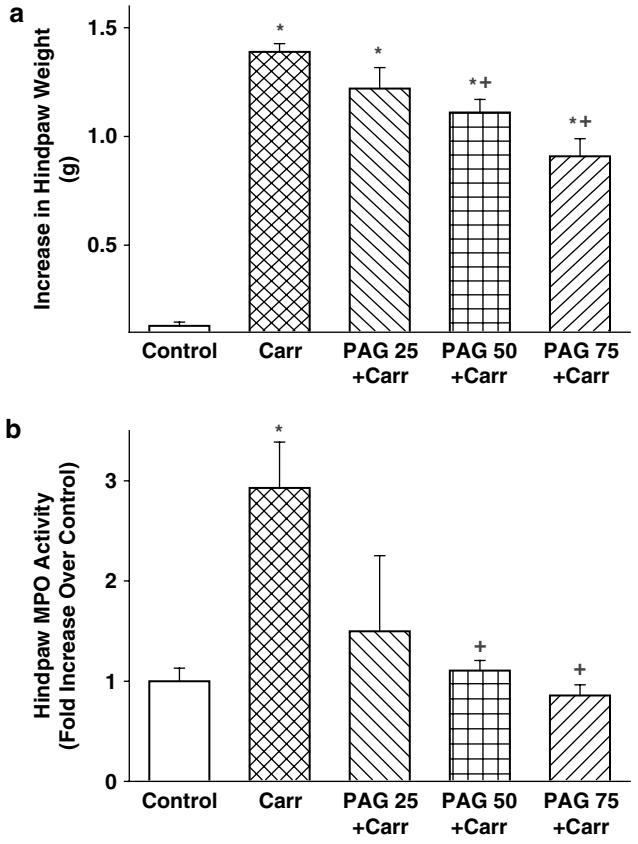

Figure 2 Effect of carrageenan (Carr) injection on hindpaw oedema at 3 h (panel a) and MPO activity (panel b) and the effect of different doses of PAG. Results show mean ± s.e.m., $n=10$, $^*P<0.05$, c.f. control, (administered intraplantar saline), $^+P<0.05$, c.f. carrageenan. Doses of PAG shown are mg kg^{-1} (i.p.).

(Figure 2). No increase in hindpaw weight was detected in control animals injected with intraplantar saline.

Discussion

In this study, we have identified H_2S as a novel mediator of inflammation in carrageenan-induced hindpaw oedema in the rat.

H_2S synthesising activity from added L-cysteine was detected in noninflamed hindpaw from animals administered i.p. saline. Furthermore, i.p. injection of carrageenan to trigger inflammatory oedema formation resulted in significantly augmented hindpaw H_2S synthesising activity. To the best of our knowledge, this is the first demonstration of H_2S synthesising activity in hindpaw and the first demonstration of increased H_2S synthesising activity in the inflamed hindpaw. The cellular origin of H_2S formation in hindpaw is not known. Neutrophils contain CSE (Glode *et al*., 1981), and as such, it is tempting to speculate that influx of these cells that occurs in the carrageenan-injected hindpaw (as evidenced by raised MPO activity) are responsible for the augmented H_2S synthesising activity seen in this study. However, the possibility that CSE in resident cells is upregulated during the inflammatory reaction cannot be discounted.

Pretreatment of rats with increasing doses of PAG produced dose-dependent inhibition of carrageenan-induced hindpaw oedema (assessed as increase in hindpaw weight) and neutrophil infiltration (assessed as increase in hindpaw MPO activity). PAG is an irreversible inhibitor of CSE, and when administered to rodents, it produces an almost complete inhibition of liver CSE enzyme activity (Uren *et al*., 1978). PAG is well absorbed and readily crosses biological membranes (Reed, 1995). We conclude therefore that PAG exhibits anti-inflammatory activity in this model by virtue of inhibiting hindpaw CSE activity and thus H_2S biosynthesis.

The present results point to a hitherto unknown proinflammatory effect of H_2S. H_2S has previously been shown to dilate large blood vessels such as rat aorta and portal vein and reduces blood pressure in the anaesthetised rat (Zhao *et al*., 2001). Moreover, a relaxant effect of H_2S on resistance arterioles has recently been reported (Cheng *et al*., 2004). As such, it is tempting to suggest that the proinflammatory effect of H_2S shown in the present work relates to its vasodilator activity. However, to the best of our knowledge, there are no reports in the literature on the effect of H_2S on the microcirculation or its possible role in inflammation.

The precise part played by H_2S in inflammation and its interaction with other proinflammatory mediators remains unclear. The possibility of an interaction with NO, which is known to play an important role in inflammation should perhaps be considered, especially since an interaction between H_2S and NO has already been postulated (Wang, 2002; Zhao & Wang, 2002; Moore *et al*., 2003). Conversion of L-cysteine to H_2S in rat aortic tissue is enhanced by treatment with sodium nitroprusside (SNP), an NO donor (Zhao *et al*., 2001). SNP also increases the expression of CSE in cultured vascular smooth muscle cells (Zhao *et al*., 2001). In one study, the vasorelaxant effect of SNP was reported to be enhanced by incubating rat aortic tissues with 30 μM NaHS (an H_2S donor) (Hosoki *et al*., 1997). Previous studies have pointed to a role of NO in this model of inflammation (Handy & Moore, 1998; Posadas *et al*., 2004). The possibility that NO and H_2S may work together to promote inflammation in this model therefore warrants further investigation.

Alternatively, H_2S may contribute to oedema formation in the carrageenan-injected rat hindpaw by an effect on sensory nerves (i.e. neurogenic inflammation). An important contribution of neurogenic inflammation in carrageenan-induced hindpaw oedema has been demonstrated in previous work (Amann *et al*., 2000). Furthermore, its has recently been shown that H_2S stimulates capsaicin-sensitive primary afferent neurons in the rat urinary bladder (Patacchini *et al*., 2004), pointing to a role for H_2S in the neurogenic inflammatory response in this tissue. Whether H_2S also promotes carrageenan-induced hindpaw oedema in the present experiments by a similar mechanism remain to be determined.

In conclusion, we demonstrate here that H_2S contributes to the hindpaw inflammation (oedema) induced by intraplantar carrageenan injection in the rat. The H_2S formed is likely to be synthesised by the action of CSE, since pretreatment with PAG, a CSE inhibitor, partially protected rats against inflammation induced by carrageenan. The precise molecular mechanism of the role of H_2S as an inflammatory mediator, and its potential interaction with other inflammatory mediators such as nitric oxide, which is also formed in large amounts in the carrageenan-inflamed hindpaw, will be the subject of future studies.

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