British Journal of Pharmacology (2006) 147, 966–974
www.nature.com/bjp

Nitric oxide-releasing flurbiprofen reduces formation of proinflammatory hydrogen sulfide in lipopolysaccharide-treated rat

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1 The biosynthesis of both nitric oxide (NO) and hydrogen sulfide (H_2S) is increased in lipopolysaccharide (LPS)-injected mice and rats but their interaction in these models is not known. In this study we examined the effect of the NO donor, nitroflurbiprofen (and the parent molecule flurbiprofen) on NO and H_2S metabolism in tissues from LPS-pretreated rats.

2 Administration of LPS ($10\,mg\,kg^{-1}$, i.p.; 6 h) resulted in an increase ($P<0.05$) in plasma TNF-α, IL-1β and nitrate/nitrite (NO_x) concentrations, liver H_2S synthesis (from added cysteine), CSE mRNA, inducible nitric oxide synthase (iNOS), myeloperoxidase (MPO) activity (marker for neutrophil infiltration) and nuclear factor-kappa B (NF-κB) activation.

3 Nitroflurbiprofen (3–$30\,mg\,kg^{-1}$, i.p.) administration resulted in a dose-dependent inhibition of the LPS-mediated increase in plasma TNF-α, IL-1β and NO_x concentration, liver H_2S synthesis (55.00 ± 0.95 nmole mg protein^{-1}, c.f. 62.38 ± 0.47 nmole mg protein^{-1}, $n=5$, $P<0.05$), CSE mRNA, iNOS, MPO activity and NF-κB activation.

4 Flurbiprofen ($21\,mg\,kg^{-1}$, i.p.) was without effect.

5 These results show for the first time that nitroflurbiprofen downregulates the biosynthesis of proinflammatory H_2S and suggest that such an effect may contribute to the augmented anti-inflammatory activity of this compound.

6 These data also highlight the existence of 'crosstalk' between NO and H_2S in this model of endotoxic shock.

British Journal of Pharmacology (2006) **147,** 966–974. doi:10.1038/sj.bjp.0706696;
published online 20 February 2006

Keywords: Nitric oxide; nitroflurbiprofen; flurbiprofen; hydrogen sulphide; lipopolysaccharide-induced endotoxic shock; interleukin-1β; tumour necrosis factor-α cystathionine γ lyase

Abbreviations: CBS, cystathionine β synthetase; CSE, cystathionine γ lyase; FLU, flurbiprofen; H_2S, hydrogen sulfide; IL-1β, interleukin-1β; LPS, lipopolysaccharide; MPO, myeloperoxidase; NO, nitric oxide; NOF, nitroflurbiprofen; NO-NSAID, NO-releasing nonsteroidal anti-inflammatory drug; NO_x, nitrate/nitrite; TNF-α, tumour necrosis factor-α; VEH, vehicle

Introduction

Until relatively recently hydrogen sulphide (H_2S) was considered as a toxic environmental pollutant of little or no physiological significance. However, it is now clear that H_2S is synthesised naturally in a range of mammalian and non-mammalian tissues by two pyridoxal phosphate-dependent enzymes (cystathionine γ lyase (CSE) and cystathione β synthetase (CBS)) acting on cysteine as substrate and most likely plays a part in physiological and pathophysiological processes (Moore *et al*., 2003).

Recently, attention has been focused on the possible role of H_2S in inflammation. Interestingly, the ability of inhaled H_2S (following accidental environmental exposure) to trigger pulmonary inflammation (e.g. damage to pulmonary epithelial cells, alveolar swelling, oedema etc.) was noted several decades ago and is one of the major factors in the toxic response to this gas (Milby & Baselt, 1999; Knight & Presnell, 2005). Rats exposed (4 h) to an atmosphere of H_2S ($615\,mg\,m^{-3}$) also showed histological evidence of pulmonary oedema and fibrinocellular alveolitis (Lopez *et al*., 1988). In stark contrast, exposure to H_2S baths (balnaeotherapy) has been widely employed in some parts of the world as a treatment for arthritis (Grabski *et al*., 2004) although the mechanism(s) involved have not been determined. Over the last year, work in this and other laboratories has provided evidence of a role for H_2S as a proinflammatory mediator in various animal models of haemorrhagic (Mok *et al*., 2004) and endotoxic (Collin *et al*., 2005; Li *et al*., 2005) shock as well as in caerulein-induced pancreatitis (Bhatia *et al*., 2005b) and carrageenan-induced hindpaw oedema in the rat (Bhatia *et al*., 2005a). In each of these disease models, tissue CSE expression is upregulated leading to enhanced H_2S biosynthesis and plasma levels while CSE inhibitors (e.g. DL-propargylglycine) have been shown to exhibit pronounced anti-inflammatory activity. In addition, H_2S administration to rats reportedly causes neurogenic inflammation in the airways which is mediated by activation of vanilloid 1 receptors (Trevisani *et al*., 2005).

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Numerous analogies exist between H_2S and nitric oxide (NO). Both are cell permeable gases under physiological conditions and are synthesised in a range of mammalian cell types and tissues from simple amino acids (cysteine and arginine, respectively). In addition, both gases occur naturally in the bloodstream (H_2S concentration in human plasma, approximately, 50 μM Richardson *et al*., 2000; NO detected as its stable breakdown products, nitrate and nitrite). Furthermore H_2S and NO exhibit vasodilator activity *in vitro* and *in vivo* albeit by different mechanisms (for H_2S most probably opening of smooth muscle K-ATP channels (Zhao *et al*., 2001) and for NO activation of soluble guanylate cyclase) and the cellular synthesis of each mediator is enhanced in a variety of inflammatory disorders most probably following the induction of CSE and inducible NO synthase (iNOS) in inflammatory and other cells (Evans, 1995). While induction of iNOS most probably occurs by several mechanisms notably activation of the intracellular nuclear factor-kappa B (NF-κB) transduction pathway, the mechanism(s) by which CSE expression is upregulated either *in vitro* or *in vivo* is not known.

Intriguingly, recent studies have suggested that NO may work in concert (i.e. 'crosstalk') with H_2S. A number of potential 'crossover' points between H_2S and NO have been reported. For example, H_2S stabilises NO in solution (Sorensen *et al*., 1980) and augments NO release from *S*-nitrosothiols (Roediger & Babidge, 2000). Furthermore, NO acts on cultured smooth muscle cells to increase both H_2S production and CSE expression (Zhao *et al*., 2001). Both NO and H_2S bind to, and are consequently quenched by, haemoglobin (Searcy & Lee, 1998). In addition, H_2S scavenges peroxynitrite (Whiteman *et al*., 2004). Finally, the vasorelaxant effect of NO is variously augmented or inhibited (Zhao *et al*., 2003) by the presence of H_2S. While the precise nature of this 'crosstalk' remains unclear it seems reasonable to suggest that the overall biological effect of H_2S on smooth muscle (and other systems) *in vivo* may well be inextricably linked with the synthesis/activity of NO.

With these observations in mind we decided to investigate the interaction between H_2S and NO in an animal model of systemic inflammation (*Escherichia coli* lipopolysaccharide (LPS)-induced endotoxic shock). For these experiments, the NO-releasing nonsteroidal anti-inflammatory drug (NO-NSAID), nitroflurbiprofen was used.

Methods

LPS-induced endotoxic shock model

The 'Principles of laboratory animal care' (NIH publication no. 85-23, revised 1985; http://grants1.nih.gov/grants/olaw/references/phspol.htm) were followed in these experiments which were approved by the animal ethics committee of National University of Singapore.

Conscious rats (male, Sprague–Dawley, 250–280 g) were injected i.p. with either saline (2.5 ml kg^{-1}, sham), *E. coli* LPS (10 mg kg^{-1}, LPS treated) or LPS pretreatment with either equivalent volume (2 ml kg^{-1}, i.p.) of vehicle (ethanol : olive oil, 5:95 v v^{-1}), flurbiprofen (21 mg kg^{-1}, i.p., the mol for mol of the highest dose of nitroflurbiprofen used in this study) or nitroflurbiprofen (3–30 mg kg^{-1}, i.p.). After 6 h, rats were terminally anaesthetized (1 ml kg^{-1}, i.p.) with a mixture of hypnorm-containing fentanyl, fluanisone and 1 ml of midazolam and blood (approx. 5 ml, 50 U ml^{-1} heparin) was obtained by cardiac puncture prior to assay for plasma nitrate/nitrite (NO_x), TNF-α and IL-1β. Tissues (liver and kidney) were also removed and kept in cryotubes that were frozen in liquid nitrogen and stored at -80°C. They were later assayed for protein iNOS expression, H_2S synthesizing ability, CSE mRNA expression, myeloperoxidase (MPO) activity and NF-κB activation.

Measurement of plasma NO_x, TNF-α and IL-1β

Aliquots (80 μl) of plasma were incubated (37°C, 30 minNO_x, final incubation volume of 200 μl) with nitrite reductase (10 mU) in the presence of NADPH (100 μmol l^{-1}) to reduce nitrate to nitrite and then centrifuged (14,000 r.p.m., 25 min, 40°C). The resulting supernatant as well as sodium nitrite standard (100 μl; 5–35 μmol l^{-1}) were added, in duplicate, to 96-well microtitre plates. Thereafter, Greiss reagent (0.2% w v^{-1} *N*-1-napthyl ethylenediamine dihydrochloride and 2% w v^{-1} sulfanilamide in 5% v v^{-1} H_3PO_4) was added to the above mixture in a ratio of 1 : 1 (v v^{-1}) and incubated for 10 min at room temperature after which absorbance was determined at 550 nm in a 96-well microplate reader (Tecan Systems, Inc., San Jose, CA, U.S.A.). The concentration of nitrite (indicative of NO_x in the original samples) was calculated from a standard curve of $NaNO_2$ (5–35 μmol l^{-1}) and expressed as μmol l^{-1} nitrite. TNF-α and IL-1β were measured in plasma aliquots (75 and 50 μl, respectively) by ELISA using commercially available kits according to the manufacturer's instructions. Results are shown as pg ml^{-1} of each cytokine in plasma.

Detection of liver tissue iNOS expression by Western blot analysis

Tissues (0.15 g) were homogenized in 4 ml of ice cold homogenization buffer (250 mM sucrose, 20 mM Tris, 1 mM DTT). The homogenates were centrifuged at 4°C, 9000 × *g* for 30 min. Supernatants were collected and centrifuged at 4°C, 14,000 × *g* for 30 min. Protease Inhibitor Cocktail (F. Hoffmann-La Roche Ltd, Grenzacherstrasse, Basel, Switzerland) was added to the final supernatants. Western blotting conditions were conducted essentially as described (Whiteman *et al*., 2004; 2005) using an enhanced chemiluminescence detection kit (Amersham Biosciences, Amersham, Buckinghamshire, U.K.) followed by analysis using a Kodak Image Analyser (IS440CF, NEN Life Science, Boston, MA, U.S.A.) and captured images analysed using Kodak digital science 1D image analysis software. Protein concentration was determined using a commercial kit (Dc protein assay, Bio-Rad Ltd, Hercules, CA, U.S.A.) and 30 μg total protein analysed for each assays.

Assay of tissue H_2S synthesis

Liver and kidney tissues from animals treated as above were thawed and homogenized in 100 mM ice-cold potassium phosphate buffer (pH 7.4). Optimal w v^{-1} ratios of 1 : 20 v v^{-1} 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 reactions were 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 tricholoroacetic acid. 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., 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 addition of cysteine and incubation (37°C, 30 min). At the end of this period, trichloroacetic acid 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, Hercules, CA, U.S.A.).

Measurement of liver and kidney tissue CSE mRNA

Tissue (100 mg) was homogenized in 1 ml ice-cold TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.) 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 × *g*, 4°C, 15 min). The top aqueous phase was transferred to an eppendorf tube and isopropanol (0.5 ml) was added. After further incubation at room temperature (10 min), samples were centrifuged (12,000 × *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 × *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 (20–80 μl) and incubated (55–60°C) for 10 min. The concentration of isolated nucleic 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 Inc., Valencia, CA, 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′-CATGGATGAAGTGTATGG AGGC-3′, and the reverse primer sequence was 5′-CGGCAG CAGAGGTAACAATCG-3′. The PCR product size for CSE 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 36 cycles of PCR reaction consisting of 94°C (30 s) for denaturation, 58.5°C (30 s) for primer-specific annealing, and 72°C (30 s) for extension. The reaction without RNA template (none template control) was also performed as a negative control. PCR products were analysed by 1.5% w v^{-1} agarose ethidium bromide gel electrophoresis and imaged under ultraviolet light by the MultiGenius Bioimaging system (Syngene, Cambridge, U.K.). The band intensity was semiquantified by densitometry using Gel analysis software (Syngene, Cambridge, U.K.).

Measurement of MPO activity

Neutrophil sequestration in liver and kidney was quantified by measuring tissue MPO activity. Tissue samples were thawed, homogenized 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% 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 × *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-Aldrich Corp., St Louis, MO, U.S.A.), 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 using a 96-well microplate reader (Tecan Systems Inc., San Jose, CA, U.S.A.). This absorbance was then corrected for the DNA content of the tissue sample and results are expressed as enzyme activity (fold increase over control).

Preparation of nuclear extracts and determination of NF-κB activation

Nuclear extracts of livers (100 mg) were prepared by using the Compartmental Protein Extraction Kit as described by the manufacturer (CHEMICON International Inc., Temecula, CA, U.S.A.). Protein concentrations in nuclear extracts were determined using the Bradford assay (Bio-Rad Ltd, Hercules, CA, U.S.A.). To monitor NF-κB activation in liver tissues, we used a TransAM NF-κB p65 Transcription Factor Assay Kit (Active Motif, Tokyo, Japan). The kit consists of a 96-well plate into which oligonucleotide containing the NF-κB consensus site (5′-GGGACTTTCC-3′) is bound. The active form of NF-κB in the nuclear extract specifically binds to this consensus site and is recognised by a primary antibody. A horseradish peroxidase-conjugated secondary antibody provides the basis for the colorimetric quantification. The absorbance of the resulting solution was measured 5 min thereafter (450 nm with a reference wavelength of 655 nm), using a 96-well microplate reader (Tecan Systems Inc., San Jose, CA, U.S.A.). The wild-type consensus oligonucleotide is provided as a competitor for NF-κB binding in order to monitor the specificity of the assay. The oligonucleotide will prevent NF-κB binding to the probe immobilized on the plate. Conversely, the mutated consensus oligonucleotide should have no effect on NF-κB binding. To allow for optimum competition, the oligonucleotide was added to the well prior to the addition of nuclear extract. Results were expressed as percentages of the positive control (TNF-α-stimulated HeLa whole cell extract) provided by the manufacturer.

Statistical analysis

Data show mean ± s.e.m. Statistical analysis of data was by one-way ANOVA followed by *post hoc* Tukey's test. A *P*-value

of <0.05 was taken to indicate a statistically significant difference.

Results

Effect of flurbiprofen and nitroflurbiprofen on plasma NO_x, TNF-α and IL-1β in LPS rats

Low concentrations of TNF-α, IL-1β, and nitrite/nitrate (NO_x) (Figure 1a–c) were detected in plasma of rats at 6 h after injection with saline. Conversely, LPS-induced septic shock was associated with significantly increased plasma concentrations of TNF-α, IL-1β and NO_x (Figure 1a–c). Treatment of LPS rats with flurbiprofen did not affect the resulting rise in plasma TNF-α, IL-1β and NO_x concentrations (Figure 1a–c). In contrast, nitroflurbiprofen administration resulted in a significant ($P<0.05$) dose-dependent inhibition of LPS-mediated increase in plasma TNF-α and IL-1β (c.f. LPS rats) (Figure 1a and b). Treatment with nitroflurbiprofen also resulted in a significant ($P<0.05$) inhibition of plasma NO_x concentrations at 6 h after LPS-induced septic shock (Figure 1c).

Effect of flurbiprofen and nitroflurbiprofen on liver iNOS expression in LPS rats

Blots were reacted with antibodies against iNOS, a protein whose expression is known to be increased by LPS. Rats injected with LPS and killed 6 h thereafter showed significant induction in the expression of iNOS in the liver (Figure 2a and b). However iNOS expression was not detected in tissues from rats injected with saline in the liver (Figure 2a and b). Treatment of LPS rats with flurbiprofen did not affect the resulting increase in the expression of iNOS in the liver (Figure 2a and b). In contrast, nitroflurbiprofen administration resulted in a significant ($P<0.05$) inhibition of LPS-mediated increase in iNOS expression (Figure 2a and b).

Effect of flurbiprofen and nitroflurbiprofen on liver and kidney H_2S biosynthesis in LPS rats

Incubation of liver and kidney homogenates (30 min at 37°C) with cysteine (10 mM) and pyridoxal 5′-phosphate (2 mM) resulted in the formation of significant amounts of H_2S as determined spectrophotometrically (Figure 3). Interestingly, the formation of H_2S by liver and kidney homogenates from rats injected with LPS was significantly greater ($P<0.05$) than in homogenates prepared from rats injected with saline (Figure 3). Treatment of LPS rats with flurbiprofen did not affect the resulting rise in formation of significant amounts of H_2S both in the liver and kidney (Figure 3). In contrast, nitroflurbiprofen administration resulted in a significant ($P<0.05$) dose-dependent inhibition of LPS-mediated increase in H_2S formation both in the liver and kidney (c.f. LPS rats) (Figure 3).

Effect of flurbiprofen and nitroflurbiprofen on liver and kidney CSE mRNA in LPS rats

Rat liver and kidney homogenates from sham animals contained detectable amounts of mRNA for CSE (Figure 4). Rats injected with LPS and killed 6 h thereafter showed significant ($P<0.05$) increase in CSE mRNA expression in liver and kidney (c.f. sham rats) (Figure 4). Treatment with flurbiprofen did not affect the CSE mRNA expression for both liver and kidney in LPS-injected animals (Figure 4). Conversely, nitroflurbiprofen administration resulted in a significant ($P<0.05$) dose-dependent inhibition of LPS-mediated increase in CSE mRNA expression for both liver and kidney (c.f. LPS rats) (Figure 4).

Effect of flurbiprofen and nitroflurbiprofen on liver and kidney injury in LPS rats

Evidence of liver and kidney injury in rats injected with LPS and killed 6 h thereafter was indicated by a significant ($P<0.05$) increase in liver and kidney MPO as a measure of neutrophil infiltration (c.f. sham rats) (Figure 5). Treatment with flurbiprofen did not inhibit MPO activity either in the liver or kidney (Figure 5). However, nitroflurbiprofen administration in LPS injected rats resulted in a significant ($P<0.05$) dose-dependent inhibition of MPO activity in the liver but not in the kidney (Figure 5).

Effect of flurbiprofen and nitroflurbiprofen on liver NF-κB activation in LPS rats

Rats injected with LPS and killed 6 h thereafter showed significant ($P<0.05$) increase in NF-κB activation in the liver (c.f. sham rats) (Figure 6). Treatment with flurbiprofen did not affect the NF-κB activation in the liver of LPS-injected animals (Figure 6). On the other hand, nitroflurbiprofen administration resulted in a significant ($P<0.05$) dose-dependent inhibition of LPS-mediated increase in NF-κB activation in the liver (c.f. LPS rats) (Figure 6).

Discussion

We have previously reported that LPS administration in the mouse results in increased plasma H_2S concentration coupled with upregulated liver and kidney CSE mRNA expression (Li *et al*., 2005) and have further shown that DL-propargylglycine (an inhibitor of CSE enzyme activity) abrogates endotoxic shock in the rat as evidenced by a reduction in plasma markers of liver (aspartate aminotransferase), pancreas (lipase) and skeletal muscle (creatinine kinase) damage (Collin *et al*., 2005). Furthermore, H_2S administration (by injection of NaHS – a water-soluble H_2S donor drug) resulted in augmented liver and kidney MPO activity as well as microscopic changes in the lung, liver and kidney typical of an inflammatory insult (Li *et al*., 2005). Interestingly, similar airways inflammation has been noted in man following accidental environmental exposure to H_2S (for review, see Knight & Presnell, 2005). Based on these studies we have proposed that H_2S acts as a proinflammatory mediator in endotoxic shock. A similar proinflammatory effect of H_2S has been reported in other inflammatory conditions including caerulein-induced pancreatitis (Bhatia *et al*., 2005b) and carrageenan-induced hindpaw oedema (Bhatia *et al*., 2005a).

The aetiology of LPS-evoked endotoxic shock is complex and undoubtedly involves the overproduction of a number of different mediators other than H_2S. For example, it has long

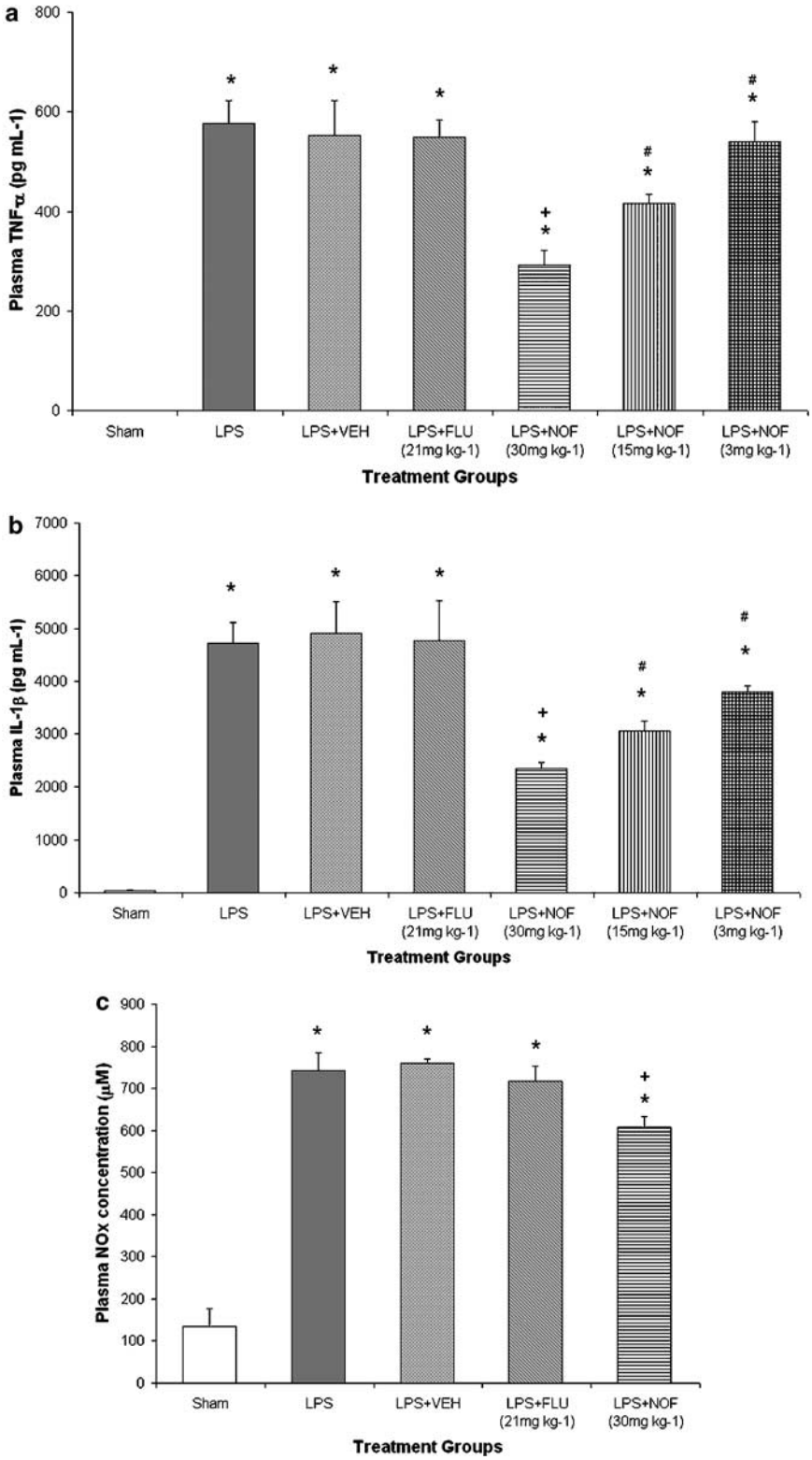

Figure 1 Plasma concentration of (a) TNF-α, (b) IL-1β, (c) NO_x of rats killed 6 h after injection of either saline (sham), *E. coli* LPS (LPS) or LPS pretreatment with either equivalent volume of vehicle (2 ml kg^{-1}, i.p., VEH), flurbiprofen (21 mg kg^{-1}, i.p., FLU) or nitroflurbiprofen (3–30 mg kg^{-1}, i.p., NOF). Results show plasma concentration and are mean $\pm$ s.e.m., $n=5$, $^{*}P<0.05$ c.f. Sham, $^{+}P<0.05$ c.f. LPS, $^{\#}P<0.05$ c.f. LPS + NOF (30 mg kg^{-1}).

been recognised that the excessive formation of NO, as a consequence of upregulation of iNOS, plays an important role in the cardiovascular depression and organ damage associated with endotoxic and septic shock (Stein *et al.*, 1996). Furthermore, several recent reports have suggested that H_2S and NO interact together in such a way as to modify the

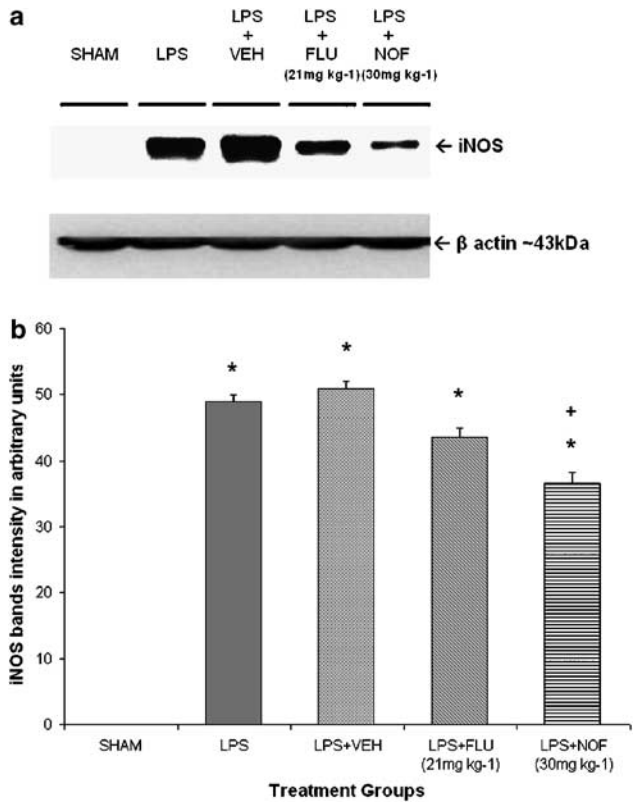

Figure 2 (a) Representative blot from one liver showing the presence of iNOS (~130 kDa). (b) Quantitation of liver (shown in (a)) iNOS. Homogenates were prepared from liver removed from rats killed 6 h after injection of either saline (sham), *E. coli* LPS (LPS) or LPS pretreatment with either equivalent volume of vehicle (2 ml kg^{-1}, i.p., VEH), flurbiprofen (21 mg kg^{-1}, i.p., FLU) or nitroflurbiprofen (30 mg kg^{-1}, i.p., NOF). Results indicate the relative intensities in arbitrary units, and are mean ± s.e.m., $n = 5$, $^{*}P < 0.05$ c.f. sham, $^{+}P < 0.05$ c.f. LPS.

biological activity of each mediator (see Introduction for references). However, whether such an interaction takes place in pathological states such as endotoxic shock, or indeed in other examples of inflammatory disease, is not known. As such, it seemed of interest to determine whether exogenously provided NO affected the upregulated biosynthesis of H_2S which we have reported in LPS-injected animals. Nitroflurbiprofen was used as the NO donor in the present series of experiments. This particular NO donor was chosen since LPS-induced endotoxic shock in the rat becomes evident over a relatively long time period (up to 6 h) and as such administration of classical NO donors such as sodium nitroprusside which produce a rapid, but transient (min), release of NO is inappropriate. It is now well established in the literature that the administration of nitroflurbiprofen (or indeed other NO-NSAID) to animals (Aldini *et al*., 2002) or man (Zacharowski *et al*., 2004) results in esteratic cleavage of the parent molecule and leads to the slow release (maximum plasma concentrations at 2–4 h) of both component molecules, that is, NO and flurbiprofen. An added advantage of the use of nitroflurbiprofen in these experiments is that it does not alter blood pressure in the rat (Keeble *et al*., 2001) most probably due to the slow time course of NO release. Such a vasodepressor effect would likely confound the interpretation of the present results.

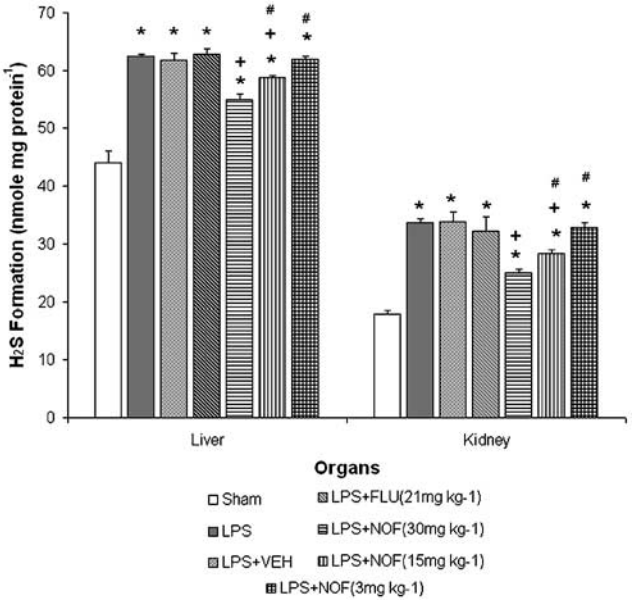

Figure 3 Formation of H_2S from cysteine (10 mM) in the presence of pyridoxal 5′-phosphate (1 mM) following incubation (37°C, 30 min). Homogenates were prepared from liver or kidney removed from rats killed 6 h after injection of either saline (sham), *E. coli* LPS (LPS) or LPS pretreatment with either equivalent volume of vehicle (2 ml kg^{-1}, i.p., VEH), flurbiprofen (21 mg kg^{-1}, i.p., FLU) or nitroflurbiprofen (3–30 mg kg^{-1}, i.p., NOF). Results show H_2S formation as nmol formed mg $protein^{-1}$, and are mean ± s.e.m., $n = 5$, $^{*}P < 0.05$ c.f. sham, $^{+}P < 0.05$ c.f. LPS, $^{\#}P < 0.05$ c.f. LPS + NOF (30 mg kg^{-1}).

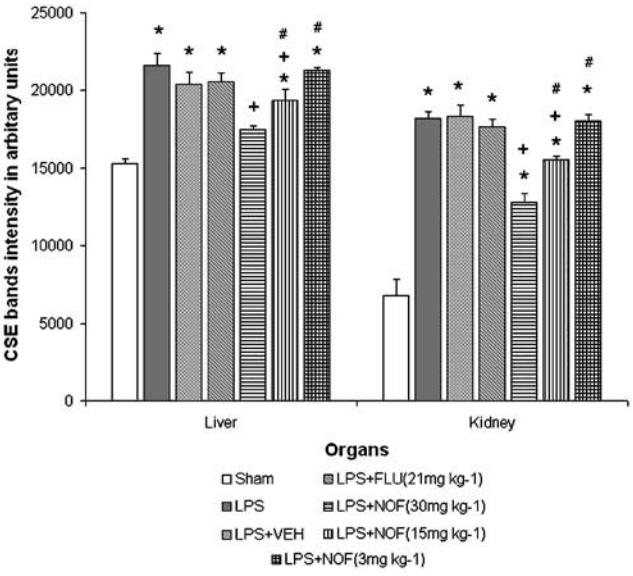

Figure 4 Quantitation of liver and kidney CSE. Homogenates were prepared from liver or kidney removed from rats killed 6 h after injection of either saline (sham), *E. coli* LPS (LPS) or LPS pretreatment with either equivalent volume of vehicle (2 ml kg^{-1}, i.p., VEH), flurbiprofen (21 mg kg^{-1}, i.p., FLU) or nitroflurbiprofen (3–30 mg kg^{-1}, i.p., NOF). Data indicate the relative intensities in arbitrary units, and are mean ± s.e.m., $n = 5$, $^{*}P < 0.05$ c.f. sham, $^{+}P < 0.05$ c.f. LPS, $^{\#}P < 0.05$ c.f. LPS + NOF (30 mg kg^{-1}).

We demonstrate here that nitroflurbiprofen, produces a dose-related inhibition of LPS-mediated endotoxic shock in the rat as evidenced by reduced plasma TNF-α and IL-1β

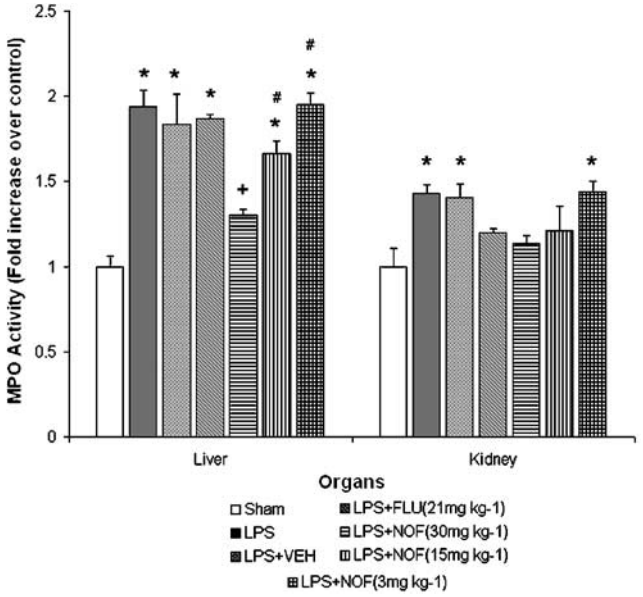

Figure 5 Effect of LPS injection on MPO activity. Homogenates were prepared from liver or kidney removed from rats killed 6 h after injection of either saline (sham), *E. coli* LPS (LPS) or LPS pretreatment with either equivalent volume of vehicle (2 ml kg^{-1}, i.p., VEH), flurbiprofen (21 mg kg^{-1}, i.p., FLU) or nitroflurbiprofen (3–30 mg kg^{-1}, i.p., NOF). Results indicate the relative MPO activity fold increase over control in arbitrary units, and are mean ± s.e.m., $n=5$, $^{*}P<0.05$ c.f. sham, $^{+}P<0.05$ c.f. LPS, $^{\#}P<0.05$ c.f. LPS + NOF (30 mg kg^{-1}).

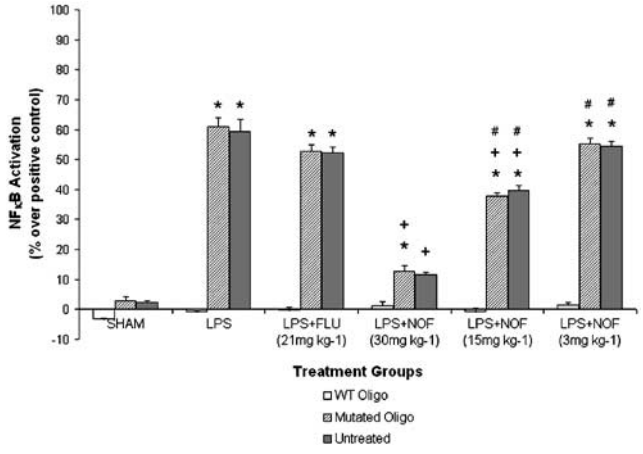

Figure 6 Effect of flurbiprofen (21 mg kg^{-1}, i.p., FLU) and nitroflurbiprofen (3–30 mg kg^{-1}, i.p., NOF) on NF-κB activation in the liver of rats at 6 h after LPS injection. Results indicate the relative percentage of NF-κB activation over the positive control provided by the manufacturer, and are mean ± s.e.m., $n=5$, $^{*}P<0.05$ c.f. sham, $^{+}P<0.05$ c.f. LPS, $^{\#}P<0.05$ c.f. LPS + NOF (30 mg kg^{-1}).

concentration and liver and kidney MPO activity (indicative of neutrophil infiltration in these organs) coupled with a significant fall in plasma NO_x concentration and liver iNOS protein expression. The decrease in plasma NO_x concentration was not as great as the decline in plasma TNF-α and IL-1β levels which presumably reflects the ability of other cytokines to uprgeulate iNOS expression. Flurbiprofen pretreatment was without effect on all parameters studied indicating that NO, released following cleavage of nitroflurbiprofen, was the molecule responsible for the changes observed. These data suggest that at least part of the mechanism of action of nitroflurbiprofen may be attributed to an NO-mediated downregulation of iNOS with consequent diminished biosynthesis of proinflammatory and vasodilator NO. In support of this possibility, we have previously shown that nitroflurbiprofen inhibits LPS-induced generation of TNF-α and IL-1β in whole human blood (Marshall & Moore, 2004) while others have observed a similar inhibitory effect of this molecule in cultured macrophages and neutrophils exposed to LPS (Mariotto *et al*., 1995a, b).

Of even greater significance, we also provide evidence that nitroflurbiprofen administration affects the metabolism of endogenous H_2S. Animals administered nitroflurbiprofen exhibited reduced liver and kidney H_2S synthesising activity (measured *ex vivo*) associated with decreased liver and kidney CSE mRNA expression. Again, flurbiprofen was devoid of such activity indicating that NO, released from nitroflurbiprofen *in vivo*, was responsible for the changes in H_2S metabolism observed. These data suggest that exogenous NO (provided by nitroflurbiprofen) downregulates not only the biosynthesis of NO but also that of H_2S. Whether endogenous NO exerts a similar effect on H_2S metabolism in either physiological or pathophysiological states warrants further study.

The molecular mechanism by which NO (released from nitroflurbiprofen) downregulates iNOS protein and CSE mRNA expression has also been investigated. We observed that pretreatment of LPS-injected animals with nitroflurbiprofen (but not flurbiprofen) caused a dose-related inhibition of liver NF-κB activation. There are several reports in the literature that NO modulates NF-κB activity either by interfering with the phosphorylation of IκB (Peng *et al*., 1995) and/or by inhibition of NF-κB-DNA-binding affinity by nitrosation of the p50 subunit (Matthews *et al*., 1996). Interestingly, nitroaspirin (a structural analogue of nitroflurbiprofen) has previously been reported to reduce transduction *via* the NF-κB pathway in cultured macrophages (Cui *et al*., 2001) as has nitroflurbiprofen in cultured L929 cells (Fratelli *et al*., 2003). The present data may additionally be taken to suggest that NO also inhibits the expression of CSE mRNA (and consequent ability to synthesise H_2S) at least in the liver and kidney of LPS-injected rats. While we, and other researchers, have reported changes in CSE mRNA expression and/or H_2S synthesising activity in a number of animal models of disease including hypertension (Zhong *et al*., 2003), diabetes mellitus (Yusuf *et al*., 2005) and pancreatitis (Bhatia *et al*., 2005b), the molecular basis underlying this effect has not been examined. The present data suggesting that both iNOS and CSE mRNA expression may be regulated by exogenous NO is therefore of potential significance in a range of different disease states.

Notwithstanding the interaction between NO and H_2S identified in this work the present experiments also offer an opportunity to probe further the complex anti-inflammatory effect of nitroflurbiprofen (for review, see Scatena, 2004). On a mol for mol basis, nitroflurbiprofen has been reported to exhibit greater anti-inflammatory activity in animal models of Alzheimer's disease (injection of preaggregated Aβ 1–42 into the nucleus basalis in the rat) (Prosperi *et al*, 2004) and

experimental autoimmune encephalomyelitis (Furlan *et al*., 2004). Nitroflurbiprofen also reduced brain inflammation resulting form chronic infusion of LPS into the fourth ventricle in the rat (Wenk *et al*., 2002). Since the brain contains high levels of H_2S (approx. 100–150 μM) and is an excellent source of cystathionine β synthetase (which, like CSE, acts on cysteine to form H_2S) it is tempting to suggest that the augmented anti-inflammatory effect of nitroflurbiprofen (c.f. flurbiprofen) noted in the brain may also be due, at least in part, to inhibition of H_2S biosynthesis.

In conclusion, we show here that NO (provided by nitroflurbiprofen) abrogates the upregulation of both NO and H_2S biosynthesis following LPS injection in the rat. The mechanism involved may be by inhibition of transduction *via* the NF-κB pathway. The data suggests, (i) that NO and H_2S interact together to promote inflammatory changes in endotoxic shock and (ii) lend further support to our proposal that inhibition of H_2S formation or action may represent a novel approach to the treatment of this and perhaps other inflammatory disorders.

We thank the Agency for Science, Technology and Research (A*STAR) for the award of a Graduate Scholarship to FA and the Office of Life Science (OLS) of the National University of Singapore for financial support (Grant no. R-184-000-074-712). We also thank NicOx SA, Sophia-Antipolis, France for the generous gift of nitroflurbiprofen.

References

ALDINI, G., CARINI, M., ORIOLI, M., FACINO, R.M. & WENK, G.L. (2002). Metabolic profile of NO-flurbiprofen (HCT1026) in rat brain and plasma: a LC-MS study. *Life Sci.,* **71,** 1487–1500.

BHATIA, M., SIDHAPURIWALA, J., MOOCHHALA, S.M. & MOORE, P.K. (2005a). Hydrogen sulphide is a mediator of carrageenan-induced hindpaw oedema in the rat. *Br. J. Pharmacol.,* **145,** 141–144.

BHATIA, M., WONG, F.L., FU, D., LAU, H.Y., MOOCHHALA, S.M. & MOORE, P.K. (2005b). Role of hydrogen sulfide in acute pancreatitis and associated lung injury. *FASEB J.,* **19,** 623–625. E-pub 2005 Jan 25.

COLLIN, M., ANUAR, F.B., MURCH, O., BHATIA, M., MOORE, P.K. & THIEMERMANN, C. (2005). Inhibition of endogenous hydrogen sulfide formation reduces the organ injury caused by endotoxemia. *Br. J. Pharmacol.,* **146,** 498–505.

CUI, Z., DEL SOLDATO, P., MOORE, P.K. & BAYDOUN, A.R. (2001). Inhibition of NF-κB activation by nitro-paracetamol and nitro-aspirin in J774 macrophages. *Inflamm. Res.,* **50,** S152.

EVANS, C.H. (1995). Nitric oxide: what role does it play in inflammation and tissue destruction? *Agents Actions Suppl.,* **47,** 107–116.

FRATELLI, M., MINTO, M., CRESPI, A., ERBA, E., VANDENABEELE, P., DEL SOLDATO, P. & GHEZZI, P. (2003). Inhibition of nuclear factor-kappaB by a nitro-derivative of flurbiprofen: a possible mechanism for antiinflammatory and antiproliferative effect. *Antioxid. Redox Signal.,* **5,** 229–235.

FURLAN, R., KURNE, A., BERGAMI, A., BRAMBILLA, E., MAUCCI, R., GASPARINI, L., BUTTI, E., COMI, G., ONGINI, E. & MARTINO, G. (2004). A nitric oxide releasing derivative of flurbiprofen inhibits experimental autoimmune encephalomyelitis. *J. Neuroimmunol.,* **150,** 10–19.

GRABSKI, M., WOZAKOWSKA-KAPLON, B. & KEDZIORA, J. (2004). Hydrogen sulfide water balneum effect on erythrocyte superoxide dismutase activity in patients with rheumatoid arthritis – *in vitro* study. *Przegl. Lek.,* **61,** 1405–1409.

KEEBLE, J., AL-SWAYEH, O.A. & MOORE, P.K. (2001). Vasorelaxant effect of nitric oxide releasing steroidal and nonsteroidal anti-inflammatory drugs. *Br. J. Pharmacol.,* **133,** 1023–1028.

KNIGHT, L.D. & PRESNELL, S.E. (2005). Death by sewer gas: case report of a double fatality and review of the literature. *Am. J. Forensic Med. Pathol.,* **26,** 181–185.

LI, L., BHATIA, M., ZHU, Y.Z., ZHU, Y.C., RAMNATH, R.D., WANG, Z.J., ANUAR, F.B., WHITEMAN, M., SALTO-TELLEZ, M. & MOORE, P.K. (2005). Hydrogen sulfide is a novel mediator of lipopolysaccharide-induced inflammation in the mouse. *FASEB J.,* **19,** 1196–1198. E-pub 2005 Apr 29.

LOPEZ, A., PRIOR, M., LILLIE, L.E., GULAYETS, C. & ATWAL, O.S. (1988). Histologic and ultrastructural alterations in lungs of rats exposed to sub-lethal concentrations of hydrogen sulfide. *Vet. Pathol.,* **25,** 376–384.

MARIOTTO, S., CUZZOLIN, L., ADAMI, A., DEL SOLDATO, P., SUZUKI, H. & BENONI, G. (1995a). Effect of a new non-steroidal anti-inflammatory drug, nitroflurbiprofen, on the expression of inducible nitric oxide synthase in rat neutrophils. *Br. J. Pharmacol.,* **115,** 225–226.

MARIOTTO, S., CUZZOLIN, L., ADAMI, A., DEL SOLDATO, P., SUZUKI, H. & BENONI, G. (1995b). Inhibition by sodium nitroprusside of the expression of inducible nitric oxide synthase in rat neutrophils. *Br. J. Pharmacol.,* **114,** 1105–1106.

MARSHALL, M. & MOORE, P.K. (2004). Effect of nitric oxide releasing paracetamol and flurbiprofen on cytokine production in human blood. *Eur. J. Pharmacol.,* **483,** 317–322.

MATTHEWS, J.R., BOTTING, C.H., PANICO, M., MORRIS, H.R. & HAY, R.T. (1996). Inhibition of NF-kappaB DNA binding by nitric oxide. *Nucleic Acids Res.,* **24,** 2236–2242.

MILBY, T.H. & BASELT, R.C. (1999). Hydrogen sulfide poisoning: clarification of some controversial issues. *Am. J. Ind. Med.,* **35,** 192–195.

MOK, Y.Y., ATAN, M.S., YOKE PING, C., ZHONG JING, W., BHATIA, M., MOOCHHALA, S.M. & MOORE, P.K. (2004). Role of hydrogen sulphide in haemorrhagic shock in the rat: protective effect of inhibitors of hydrogen sulphide biosynthesis. *Br. J. Pharmacol.,* **143,** 881–889. E-pub 2004 Oct 25.

MOORE, P.K., BHATIA, M. & MOOCHHALA, S. (2003). Hydrogen sulphide: from the smell of the past to the mediator of the future? *Trends Pharm. Sci.,* **24,** 609–611.

PENG, H.B., LIBBY, P. & LIAO, J.K. (1995). Induction and stabilization of I kappa B alpha by nitric oxide mediates inhibition of NF-kappa B. *J. Biol. Chem.,* **270,** 14214–14219.

PROSPERI, C., SCALI, C., BARBA, M., BELLUCCI, A., GIOVANNINI, M.G., PEPEU, G. & CASAMENTI, F. (2004). Comparison between flurbiprofen and its nitric oxide-releasing derivatives HCT-1026 and NCX-2216 on Abeta(1–42)-induced brain inflammation and neuronal damage in the rat. *Int. J. Immunopathol. Pharmacol.,* **17,** 317–330.

RICHARDSON, C.J., MAGEE, E.A. & CUMMINGS, J.H. (2000). A new method for the determination of sulphide in gastrointestinal contents and whole blood by microdistillation and ion chromatography. *Clin. Chim. Acta.,* **293,** 115–125.

ROEDIGER, W.E. & BABIDGE, W.J. (2000). Nitric oxide effect on colonocyte metabolism: co-action of sulfides and peroxide. *Mol. Cell Biochem.,* **206,** 159–167.

SCATENA, R. (2004). Nitroflurbiprofen (NicO*x*). *Curr. Opin. Invest. Drugs,* **5,** 551–556.

SEARCY, D.G. & LEE, S.H. (1998). Sulfur reduction by human erythrocytes. *J. Exp. Zool.,* **282,** 310–322.

SORENSEN, J., TIEDJE, J.M. & FIRESTONE, R.B. (1980). Inhibition by sulfide of nitric and nitrous oxide reduction by denitrifying Pseudomonas fluorescens. *Appl. Environ. Microbiol.,* **39,** 105–108.

STEIN, B., FRANK, P., SCHMITZ, W., SCHOLZ, H. & THOENES, M. (1996). Endotoxin and cytokines induce direct cardiodepressive effects in mammalian cardiomyocytes via induction of nitric oxide synthase. *J. Mol. Cell Cardiol.,* **28,** 1631–1639.

TREVISANI, M., PATACCHINI, R., NICOLETTI, P., GATTI, R., GAZZIERI, D., LISSI, N., ZAGLI, G., CREMINON, C., GEPPETTI, P. & HARRISON, S. (2005). Hydrogen sulfide causes vanilloid receptor 1-mediated neurogenic inflammation in the airways. *Br. J. Pharmacol.,* **145,** 1123–1131.

WENK, G.L., ROSI, S., MCGANN, K. & HAUSS-WEGRZYNIAK, B. (2002). A nitric oxide-donating flurbiprofen derivative reduces neuroinflammation without interacting with galantamine in the rat. *Eur. J. Pharmacol.,* **453,** 319–324.

WHITEMAN, M., ARMSTRONG, J.S., CHEUNG, N.S., SIAU, J.L., ROSE, P., SCHANTZ, J.T., JONES, D.P. & HALLIWELL, B. (2004). Peroxynitrite mediates calcium-dependent mitochondrial dysfunction and cell death via activation of calpains. *FASEB J.,* **18,** 395–397.

WHITEMAN, M., ROSE, P., SIAU, J.L., CHEUNG, N.S., TAN, G.S., HALLIWELL, B. & ARMSTRONG, J.S. (2005). Hypochlorous acid-mediated mitochondrial dysfunction and apoptosis in human hepatoma HepG2 and human fetal liver cells: role of mitochondrial permeability transition. *Free Radic. Biol. Med.,* **38,** 1571–1584.

YUSUF, M., KWONG HUAT, B.T., HSU, A., WHITEMAN, M., BHATIA, M. & MOORE, P.K. (2005). Streptozotocin-induced diabetes in the rat is associated with enhanced tissue hydrogen sulfide biosynthesis. *Biochem. Biophys. Res. Commun.,* **333,** 1146–1152.

ZACHAROWSKI, P., ZACHAROWSKI, K., DONNELLAN, C., JOHNSTON, A., VOJNOVIC, I., FORTE, P., DEL SOLDATO, P., BENJAMIN, N. & O'BYRNE, S. (2004). The effects and metabolic fate of nitroflurbiprofen in healthy volunteers. *Clin. Pharmacol. Ther.,* **76,** 350–358.

ZHAO, W., NDISANG, J.F. & WANG, R. (2003). Modulation of endogenous production of H_2S in rat tissues. *Can. J. Physiol. Pharmacol.,* **81,** 848–853.

ZHAO, W., ZHANG, J., LU, Y. & WANG, R. (2001). The vasorelaxant effect of H_2S as a novel endogenous gaseous K(ATP) channel opener. *EMBO J.,* **20,** 6008–6016.

ZHONG, G., CHEN, F., CHENG, Y., TANG, C. & DU, J. (2003). The role of hydrogen sulfide generation in the pathogenesis of hypertension in rats induced by inhibition of nitric oxide synthase. *J. Hypertens.,* **21,** 1879–1885.

(Received December 1, 2005
Revised January 16, 2006
Accepted January 18, 2006
Published online 20 February 2006)