RESEARCH PAPER

Differing effects of exogenous and endogenous hydrogen sulphide in carrageenan-induced knee joint synovitis in the rat

E Ekundi-Valentim¹, KT Santos¹, EA Camargo^{1,2}, A Denadai-Souza¹, SA Teixeira¹, CI Zanoni¹, AD Grant³, JL Wallace⁴, MN Muscará¹ and SK Costa¹

¹Department of Pharmacology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, SP, Brazil, ²Department of Physiology, Federal University of Sergipe, Aracaju-SE, Brazil, ³Wolfson Centre for Age-related Diseases, King's College, London, UK and ⁴Farncombe Institute, Department of Medicine, McMaster University, Hamilton, ON, Canada

Background and purpose: Recent findings suggest that the noxious gas H₂S is produced endogenously, and that physiological concentrations of H₂S are able to modulate pain and inflammation in rodents. This study was undertaken to evaluate the ability of endogenous and exogenous H₂S to modulate carrageenan-induced synovitis in the rat knee.

Experimental approach: Synovitis was induced in Wistar rats by intra-articular injection of carrageenan into the knee joint. Sixty minutes prior to carrageenan injection, the rats were pretreated with indomethacin, an inhibitor of H₂S formation (DL-propargylglycine) or an H₂S donor [Lawesson's reagent (LR)].

Key results: Injection of carrageenan evoked knee inflammation, pain as characterized by impaired gait, secondary tactile allodynia of the ipsilateral hindpaw, joint swelling, histological changes, inflammatory cell infiltration, increased synovial myeloperoxidase, protein nitrotyrosine residues, inducible NOS (iNOS) activity and NO production. Pretreatment with LR or indomethacin significantly attenuated the pain responses, and all the inflammatory and biochemical changes, except for the increased iNOS activity, NO production and 3-NT. Propargylglycine pretreatment potentiated synovial iNOS activity (and NO production), and enhanced macrophage infiltration, but had no effect on other inflammatory parameters.

Conclusions and implications: Whereas exogenous H₂S delivered to the knee joint can produce a significant anti-inflammatory and anti-nociceptive effect, locally produced H₂S exerts little immunomodulatory effect. These data further support the development and use of H₂S donors as potential alternatives (or complementary therapies) to the available anti-inflammatory compounds used for treatment of joint inflammation or relief of its symptoms.

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Abbreviations: CBS, cystathionine- β -synthase; CMC, carboxymethylcellulose; CSE, cystathionine- γ -lyase; i.art., intra-articular; ICAM-1, intercellular adhesion molecule 1; IL-1, interleukin-1; IL-6, interleukin-6; iNOS, inducible NOS; K_{ATP} channels, ATP-sensitive K⁺ channels; LPS, lipopolysaccharide; LR, Lawesson's reagent; MPO, myeloperoxidase; NO_x⁻, nitrite + nitrate; PBS, phosphate-buffered saline solution; PGly, DL-propargylglycine; RA, rheumatoid arthritis; TNF- α , tumour necrosis factor- α

Introduction

The toxic effects of gaseous hydrogen sulphide (H₂S) are well known and have been studied for more than a century. However, over the past three decades, evidence has emerged that H₂S is produced physiologically during cystathionine

metabolism (Stipanuk and Beck, 1982), by the action of the pyridoxal-5'-phosphate-dependent enzymes cystathionine- γ -lyase (CSE) and cystathionine- β -synthase (CBS). CSE and CBS have different tissue distributions; for example, CBS is approximately 30 times more abundantly expressed in the brain than CSE (Awata *et al.*, 1995), while CSE shows higher expression in liver and kidney (Stipanuk and Beck, 1982; Zhao *et al.*, 2001). H₂S is not simply a metabolic by-product, but actively participates in signalling pathways (Kimura, 2002; Kimura *et al.*, 2005; Szabó, 2007) and, along with nitric oxide (NO) and carbon monoxide (CO), H₂S is now recognized as a

Correspondence: SK Costa, Av Prof Lineu Prestes, 1524, São Paulo, 05508-900, SP, Brazil. E-mail: scosta@icb.usp.br

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member of the growing family of the endogenous gaseous mediators, involved in important physiological effects (Wang, 2002). Interestingly, and similarly to NO, H₂S has been implicated in the modulation of both pro- and anti-inflammatory events. Studies with inhibitors of H₂S biosynthesis, such as DL-propargylglycine (PGly) and H₂S donors, such as Na₂S or NaHS at acid pH, show that physiological H₂S concentrations (30–100 μ M) produce anti-inflammatory, anti-apoptotic and anti-nociceptive effects (Zanardo *et al.*, 2006; Li *et al.*, 2007; Cunha *et al.*, 2008; Sivarajah *et al.*, 2009). Other groups reported that elevated H₂S levels are detrimental, exerting pro-inflammatory (Bhatia *et al.*, 2005a,b; Collin *et al.*, 2005; Zhang *et al.*, 2007) and pro-nociceptive effects (Cunha *et al.*, 2008).

The precise sites and mechanisms of action of H₂S as an inflammatory mediator are not well established, although the existing data indicate diverse targets. It directly stimulates capsaicin-sensitive primary afferent neurons in the rat urinary bladder via an unknown molecular interaction (Patacchini *et al.*, 2004). Some anti-inflammatory and anti-nociceptive effects of H₂S seem to be mediated via activation of ATP-sensitive K⁺ channels (K_{ATP}), as these effects were effectively prevented by glibenclamide, a K_{ATP} channel blocker (Zanardo *et al.*, 2006; Cunha *et al.*, 2008). Other anti-inflammatory effects of H₂S occur via up-regulation of haem oxygenase-1 and CO production, leading to inhibition of the nuclear factor- κ B (NF κ B) pathway and down-regulation of inducible NO synthase (iNOS) expression and NO production by inflammatory stimuli (Oh *et al.*, 2006).

Rheumatoid arthritis (RA) is a leading cause of disability worldwide and is characterized by severe pain, oedema and destruction of the cartilage and bone (see Yelin, 2007). The great economic and social burden of this disease has stimulated many investigations of the pathological mechanisms involved in the aetiology of RA, and adequate treatment remains the main goal of arthritis research. Recently, sulphide compounds have been suggested to reduce leucocyte infiltration (Andruski *et al.*, 2008) and cyclooxygenase-2 expression (Lee *et al.*, 2009) in animal models of joint inflammation, but no effects on joint pain were reported (Andruski *et al.*, 2008).

In addition to the experimental evidence, clinical findings show that the therapeutic use of thermal baths with H₂S water has anti-oxidant *in vitro* effects on erythrocytes obtained from patients with RA (Grabski *et al.*, 2004; Wozakowska-Kaplon *et al.*, 2006). It also produces anti-inflammatory effects, as well as stimulating general and immune reactivity, in patients with osteoarthritis (Ibadova *et al.*, 2005).

Our present data demonstrated that whereas exogenous H₂S delivered to the knee joint can produce powerful anti-inflammatory and anti-nociceptive effects, locally produced endogenous H₂S seemed to exert little immunomodulatory effect, mainly regulating macrophage migration and NO production in the articular cavity.

Methods

Animals

All animal care and experimental procedures are in accordance with the ethical principles for animal research set down in the Animals (Scientific Procedures) Act, UK, 1986, and were

approved by the local ethics committee at the University of São Paulo (protocol no. 64, book no. 2/2007). A total of 189 adult male Wistar rats (180–200 g) from the local animal care facilities were used in this study. Rats were kept in polypropylene cages (five per cage) under standard controlled conditions (22°C; 12 h light/dark cycle) with free access to commercial rodent chow and tap water. The British Journal of Pharmacology guide to receptor/channels was used (Alexander *et al.*, 2009).

Carrageenan-induced acute knee joint inflammatory response (synovitis)

The rats were transiently anaesthetized with halothane (1.5% v/v in O₂; Takaoka, Mod KT-20, São Paulo, Brazil), and the skin around the knee joints was shaved. Using a 0.1 mL hypodermic syringe with a 30 G needle (BD, Franklin Lakes, NJ, USA), the left knee received an intra-articular (i.art.) injection of carrageenan (50 μ L of a 3% solution in a sterile 0.9% saline solution) or the same volume of sterile saline, which constituted a separate control group.

Experimental design

One hour prior to i.art. injection of carrageenan, anaesthetized animals received the i.art. injection of a 50 μ L volume containing either the CSE inhibitor, PGly; (53 μ mol per knee joint), H₂S donor, Lawesson's reagent (LR; 3.6 μ mol per joint) or the vehicle carboxymethylcellulose (CMC 0.2%). Another group of animals were pretreated with the non-selective cyclooxygenase inhibitor indomethacin (6 mg·kg⁻¹, i.p.) 60 min before carrageenan (positive treatment control).

Measurement of knee swelling

Measurements of the mediolateral diameter (at the level of the patellar ligament) of each rat knee joint were performed immediately in conscious animals before anaesthesia for carrageenan injection using a digital calliper (Starret, São Paulo, Brazil). A further reading was performed 4 h after carrageenan injection. Knee swelling was expressed as the change in knee diameter (in mm) before and following the induction of inflammation.

Assessment of pain behaviour and secondary tactile allodynia

Knee joint pain and ipsilateral secondary tactile allodynia were evaluated by the functional measurement of animal behaviour using a modified gait score based on walking pattern and by using a modified von Frey digital device comprised of a hand-held force transducer fitted with a 0.7 mm² polypropylene tip, respectively (Insight, Ribeirão Preto, SP, Brazil), before and 4 h after, the i.art. injection of carrageenan (Otsuki *et al.*, 1986; see Neugebauer *et al.*, 2007; Denadai-Souza *et al.*, 2009). In a quiet, dimmed room, each rat was placed on an open bench that enabled the animal to walk freely, before and after the carrageenan injection. The severity of disturbances of walking was graded as score 0 (normal; rat runs and walks normally), score 1 (mild disability; rat runs and walks with difficulty), score 2 (rat walks with difficulty due to intermittent loading of inflamed paw) or score 3 (rat

stands on only three paws). In a separate set of experiments, rats were conditioned to a perspex box (12 × 20 × 17 cm) with a wire grid base. The secondary tactile allodynia in the hindpaw of the injected knee (ipsilateral) was evaluated by recording the threshold of force intensity (g) that evoked hindpaw withdrawal with a hand-held force transducer. A tilted mirror below the grid provided a clear view of the rat hindpaw. The force threshold (in g) value was recorded before the i.art. injection of either carrageenan or vehicle (basal value), and 4 h later. Measurements of force thresholds were performed by averaging three measurements, and the values before and after carrageenan injection were averaged. Secondary tactile allodynia in the ipsilateral hindpaw was expressed by delta (Δ) withdrawal threshold (in g) by subtracting the basal measurement from measurements obtained after carrageenan. All experimental measurements were made by investigators unaware of the treatments.

Synovial lavage fluid and membrane collection

Under deep anaesthesia (induced by the intraperitoneal injection of 80 mg·kg⁻¹ ketamine and 20 mg·kg⁻¹ xylazine), a blood sample was obtained by cardiac puncture using a heparinized syringe. The rats were then killed by cervical dislocation, the skin overlying the knee was excised, the patellar ligament was dissected and a 30 G needle connected to a 0.3 mL syringe was inserted through the joint capsule. The knee joint cavity was washed twice by injecting and immediately aspirating 100 μ L of phosphate-buffered saline solution (PBS) containing 4 mM EDTA. The resulting synovial lavages were combined and immediately used for cell counting, or centrifuged (at 1500× g, 10 min) and stored at -20°C for further analysis (see below). The knee joints or synovial membranes were surgically removed. The former were paraffin embedded and routinely processed for histological analysis, whereas the latter were reserved for biochemical assays as required.

Leucocyte quantification in synovial lavage fluid

Aliquots of 50 μ L of synovial lavage fluid were diluted with 150 μ L of PBS. The number of total white cells was determined using a haemocytometer (Neubauer chamber); differential cell counting was performed after cytocentrifugation of the suspension (Citospin, Fanem, Sao Paulo, Brazil), staining with May-Grünwald-Giemsa and examination of the slides under a light microscope for identification of the cells (as neutrophils, lymphocytes or macrophages/monocytes) based on their characteristic nuclear morphology. The results were expressed as number of cells (×10⁵) per cavity.

Biochemical analysis

Myeloperoxidase (MPO) activity, a marker of neutrophil accumulation, was measured in the synovial lavage samples as previously described (Denadai-Souza *et al.*, 2009), based on the method originally described by Bradley *et al.* (1982). The concentrations of tumour necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β) and interleukin 6 (IL-6) in lavage fluid were measured by ELISA, using commercially available kits and according to instructions supplied by the manufacturer (R&D

Systems, Minneapolis, MN, USA). After deproteinization of the synovial lavages by ultrafiltration (10 kDa; Microcon centrifugal filter units), total nitrite/nitrate (NO_x⁻) concentrations were determined by the Griess reaction for nitrite, after the nitrate reductase-catalysed reduction of nitrate to nitrite, according to Grisham *et al.* (1996).

For measurement of total sulphide concentration, plasma and synovial lavages were diluted with 0.02 mM NaOH (1:10 and 1:5, respectively) and further analysed based on the formation of methylene blue, as described by Bian *et al.* (2006). The absorbance of the resulting chromophore was read at 670 nm (Spectra Max Plus, Molecular Devices, Sunnyvale, CA, USA), and sulphide concentrations were extrapolated from a calibration curve (prepared with aqueous Na₂S standard solutions within the concentration range 0.78–100 μ M).

Furthermore, synovial membranes were weighed and homogenized with cold Tris-HCl buffer (50 mM, pH 7.4) containing 1 mM phenylmethanesulphonylfluoride (PMSF) and 1 mM L-citrulline. The homogenates were centrifuged (at 1500× g, 10 min), and both Ca²⁺-dependent and -independent NOS activity present were determined in the supernatant samples based on the [³H]-L-arginine to [³H]-L-citrulline conversion (Teixeira *et al.*, 2002).

The synovial membrane contents of proteins with 3-nitrotyrosine (NT) residues were determined, via Western blot analysis, in order to estimate the occurrence of this oxidative modification, as previously described (Teixeira *et al.*, 2002). Immunoreactive bands on synovial membrane NT formation were detected by chemiluminescence (Immun-Star, Bio-Rad, Hercules, CA, USA), and their intensities were estimated by summation of band intensities of proteins within the 27–197 kDa MW range.

Collection of knee samples and histopathological analysis

The knee joints were removed from four to five animals per group, fixed in 4% formaldehyde buffered in PBS (pH 7.4), decalcified in 10% EDTA for 20 days, dehydrated and paraffin embedded. Two paraffin sagittal medial 5–6 μ m thick slices per joint were stained with haematoxylin and eosin. The assessment of the histological acute joint inflammatory scores was performed by an investigator unaware of the details of the study using semiquantitative grading scales of 0–3 (Roth *et al.*, 2005). The degree of infiltration of the synovial membrane by leucocytes and exudation of granulocytes in the joint space was evaluated in each case, as follows: 0 = no changes, 1 = mild changes, 2 = moderate changes and 3 = severe changes. Either the presence (score 1) or absence (score 0) of fibrin exudation in the joint space and periarticular inflammation was assessed, resulting in a maximum total score of 8 for acute inflammation.

Data analysis and statistical procedures

All results were expressed as mean $\pm$ SEM, for *n* animals. Differences among the groups were analysed by one-way ANOVA followed by Bonferroni's multiple comparison test. Medians obtained from the gait score test or histopathological grading score were analysed by non-parametric statistics applying the Kruskal-Wallis test followed by Dunn's test for

multiple comparisons (using the software GraphPad Prism Co., version 4.0, San Diego, CA, USA). Values of *P* lower than 0.05 were considered as significant.

Materials

CMC was obtained from Cromoline Química Fina Ltda (Diadema, São Paulo, Brazil). Carrageenan, DL-propargylglycine (2-amino-4-pentynoic acid), indomethacin (1-[4-chlorobenzoyl]-5-methoxy-2-methyl-3-indoleacetic acid), LR (2,4-bis[4-methoxyphenyl]-1,3,2,4-dithiadiphosphatane 2,4-disulphide), L-citrulline and PMSF were purchased from Sigma Chemical Co. (St Louis, MO, USA). EDTA was purchased from Sigma-Aldrich (Milwaukee, WI, USA). ELISA kits for TNF- α , IL-1 β and IL-6 determination were obtained from R&D Systems. Halothane was obtained from Cristália (Itapira, São Paulo, Brazil), and both ketamine and xylazine were purchased from König (Avellaneda, Argentina). L-[2,3,4,5-³H]-Arginine monohydrochloride and Na₂S·9H₂O were obtained from Amersham Biosciences do Brazil Ltda (São Paulo, Brazil) and Reagentes Químicos Dinâmica (São Paulo, Brazil) respectively. Microcon centrifugal filter units (kDa) and Immobilon Western Chemiluminescent AP substrate were obtained from Millipore Corporation (Bedford or Billerica, MA, USA). Anti-NT mouse monoclonal antibody 1A6 was obtained from Upstate (Lake Placid, NY, USA), and goat anti-mouse IgG (H + L) AP conjugated was purchased from Bio-Rad.

Results

Clinical evaluation

The i.art. injection of carrageenan resulted in significant secondary tactile allodynia in the ipsilateral hindpaw (*n* = 5) and impairment of the normal walking pattern as shown by the increased gait score obtained from the animals 4 h after the carrageenan injection (*n* = 7) in comparison to the saline solution injection (Figure 1A and B). Pretreatment of the animals with either the H₂S donor LR (3.6 μ mol per knee i.art., *n* = 8) or indomethacin (6 mg·kg⁻¹ i.p., *n* = 6) 1 h before carrageenan, ameliorated both the impaired mobility (decreased gait score by 54 $\pm$ 9% and 85 $\pm$ 11%, respectively)

and secondary tactile allodynia in the ipsilateral hindpaw (Figure 1A and B). On the other hand, pretreatment of the carrageenan-injected animals with the CSE inhibitor PGly (53 μ mol per knee i.art.) did not affect the gait score significantly (Figure 1A, *n* = 8) and failed to significantly affect carrageenan-induced secondary tactile allodynia in the ipsilateral hindpaw, compared with vehicle-treated rats (Figure 1B, *n* = 5).

The i.art. injection of saline solution into the rat joint did not alter the knee diameter, when measured after 4 h (Figure 1C). In contrast, the i.art. injection of carrageenan resulted in a significant increase of the knee joint diameter

Figure 1 Effect of treatment with LR and DL-propargylglycine (PGly) on carrageenan-induced knee joint pain, secondary tactile allodynia in the ipsilateral hindpaw and knee swelling. Carrageenan-injected animals were pretreated with LR (3.6 μ mol i.art., *n* = 8), PGly (53 μ mol i.art., *n* = 8), indomethacin (6 mg·kg⁻¹ i.p., *n* = 6) or vehicle (*n* = 7). A separate control group received only i.art. injection of saline injection (*n* = 4). (A) The walking behaviours scored on a scale from 0 (normal) to 3 (total joint immobility). In a separate set of animals, the secondary tactile allodynia in the ipsilateral hindpaw of vehicle- and pretreated groups was evaluated (B; *n* = 5 for all groups). (C) The joint swelling of the change in articular diameter in mm following carrageenan. Data from (B) and (C) are expressed as mean $\pm$ SEM. In (A), data are presented as a scatter plot with the median values for each experimental group. In (A), differences among groups were analysed by the non-parametric Kruskal–Wallis statistic test followed by Dunn's test for multiple comparisons. Data shown in (B) and (C) were analysed by one-way ANOVA followed by Bonferroni's test for multiple comparisons. **P* < 0.05–****P* < 0.001 versus vehicle-treated animals.

Figure 2 Effect of treatment with LR and DL-propargylglycine on carrageenan-induced leucocyte influx in the knee joint. Carrageenan-injected animals were pretreated with LR (3.6 $\mu\text{mol i.art.}$, $n = 5$), PGly (53 $\mu\text{mol i.art.}$, $n = 4$), indomethacin (6 $\text{mg}\cdot\text{kg}^{-1}$ i.p.; $n = 5$) or vehicle ($n = 5$). A separate control group received only i.art. injection of saline ($n = 4$). Total leucocyte (A), neutrophil (B), lymphocyte (C) and macrophage (D) counts are expressed as mean $\pm$ SEM of cells ($\times 10^5$ per cavity). * $P < 0.05$ –*** $P < 0.001$ versus vehicle-treated animals. (One-way ANOVA followed by Bonferroni's multiple comparison test).

(40 $\pm$ 1% relative to the value before carrageenan), which, similarly to the nociceptive assays, was significantly reduced by pretreating the animals with either LR or indomethacin, but not affected by PGly (Figure 1C).

Leucocyte recruitment into the articular cavity and MPO activity

As shown in Figure 2, synovial lavage fluid collected from rats with carrageenan-induced synovitis showed a significantly higher number of total leucocytes when compared with those obtained from the saline solution-injected group (Figure 2A), and this increase was due to neutrophils (Figure 2B) and lymphocytes mainly (Figure 2C), but not monocytes/macrophages (Figure 2D). Pretreatment of the animals with LR or indomethacin resulted in a significant reduction in both neutrophils and lymphocytes in the articular cavity, whereas pretreatment with PGly not only failed to affect these numbers but also caused a significant increase in the number of macrophages (Figure 2D).

Likewise, increased MPO activity was found in the synovial lavage fluid collected from rats with carrageenan-induced synovitis, compared with that obtained from the saline solution-injected group (Table 1, $n = 6$ each group), and this increase was significantly reduced by pretreatment of the animals with LR (Table 1, $n = 6$) or indomethacin (Table 1,

Table 1 Effect of treatment with LR and DL-propargylglycine (PGly) on MPO activity, IL-1 β and NO_x⁻ concentrations in synovial lavage fluid after the i.art. injection of carrageenan

Group	MPO activity (U·mL ⁻¹)	IL-1 β (pg·mL ⁻¹)	NO _x ⁻ (μM)
Saline	5 $\pm$ 1	7 $\pm$ 1	26.2 $\pm$ 0.8
Vehicle + carrageenan	281 $\pm$ 42***	1632 $\pm$ 385***	55.5 $\pm$ 4.0***
LR + carrageenan	74 $\pm$ 10***	716 $\pm$ 131*	58.0 $\pm$ 2.0
PGly + carrageenan	257 $\pm$ 37	1605 $\pm$ 325	81.5 $\pm$ 6.4**
Indomethacin + carrageenan	79 $\pm$ 13**	721 $\pm$ 53**	45.5 $\pm$ 4.0

* $P < 0.05$ –*** $P < 0.001$ versus saline, and ** $P < 0.05$ –*** $P < 0.001$ versus carrageenan. (One-way anova followed by Bonferroni's multiple comparison t -test).

Carrageenan-injected animals were pretreated with LR (3.6 $\mu\text{mol i.art.}$), PGly (53 $\mu\text{mol i.art.}$), indomethacin (6 $\text{mg}\cdot\text{kg}^{-1}$ i.p.) or vehicle. After 4 h, MPO activity, IL-1 β and NO_x⁻ concentrations present in the synovial lavage fluids were measured. Data are expressed as mean $\pm$ SEM for n animals.

$n = 4$), whereas pretreatment with PGly had no effect (Table 1, $n = 4$).

Histological scores

Knee specimens from control rats had a normal histological appearance with scattered cell infiltration in the synovium and typical preserved lining layer (Figure 3A), resulting in a

Figure 3 Photomicrographs from rat knee joints stained with haematoxylin and eosin; light microscopy with low (50×, column 1); medium (100×, column 2) and high magnification (640×, column 3). (A) 1, 2 and 3 show the articular joints of rats injected with saline, which exhibit normal histological features, with intact synovial membranes and no evidence of inflammation; note the black arrows indicate resident cells. (B) 1, 2 and 3 illustrate the articular joints of carrageenan-injected rat, and reveal a pronounced inflammatory response. The square area in (B) 2 reveals [at high magnification in (B) 3] several polymorphonuclear cells (black arrows) and plasma exudates. (C) 1, 2 and 3 show that polymorphonuclear cell migration was decreased and more localized in animals pretreated with LR before carrageenan injection. Both the lining and sublining layers of synovium present low cellularity, and there are no signs of extensive exudates [see (C) 2 and 3]. (D) 1, 2 and 3 reveal high degree of leucocyte migration to the joint cavity of synovitic rats pretreated with PGly. The square area in (D) 2 is shown at high magnification in (D) 3, illustrating in detail polymorphonuclear cells (black arrows) along with a significant plasma exudate. (E) 1, 2 and 3 show the articular joints of rats pretreated with indomethacin prior i.art. injection with carrageenan. In (E) 2, it can be seen that the synovium presents a low inflammatory level, and leucocytes are restricted to small focal areas, as shown in (E) 3 (black arrows). Cartilage and bone were in normal condition in all samples analysed. Abbreviations: AC, articular cartilage; JC, joint cavity; M, meniscus; SM, synovial membrane; T, tibia.

Figure 4 Histopathological scores. Carrageenan-injected animals were pretreated with LR (3.6 μ mol i.art., $n = 5$), DL-propargylglycine (PGly; 53 μ mol i.art., $n = 5$), indomethacin (6 mg·kg⁻¹ i.p., $n = 4$) or vehicle ($n = 5$). A separate set of animals received only i.art. injection of saline ($n = 4$). In the specimens from rats pretreated with LR or indomethacin, a significant reduction of the acute inflammation score was observed as compared to the control group. No significant differences were observed between PGly-treated group and control rats. Results are expressed as box and whisker plots (median, IQR, minimum and maximum values of histopathological score). Differences among the experimental groups were assessed using Kruskal–Wallis non-parametric analysis followed by Dunn's test for multiple comparisons. * $P < 0.05$ versus vehicle-treated animals.

score of 0 (Figure 4). At 4 h after i.art. injection of carrageenan, the synovial membranes in the rat knee cavity displayed an intense and widespread cell infiltration, mostly due to neutrophils and occasional macrophages. The lining layers were atypical due to the presence of a leucocyte infiltration, in addition to a marked presence of fibrin-containing exudate (oedema) in the synovium and joint cavity (Figure 3B), resulting in a significant acute inflammatory score (>6; Figure 4). In synovitic rats treated with LR (Figure 3C) or indomethacin (Figure 3E), both neutrophil infiltration and oedematous areas (fibrinous exudate) were reduced, producing a significantly lower histological score (<3) compared to vehicle-treated group (Figure 4), and no hyperplasia of the lining layer was detected. Specimens from rats pretreated with PGly showed morphological changes, including leucocyte infiltra-

tion, oedema and atypical lining layer (Figure 3D), with a histological score similar to that seen in vehicle-treated rats (Figure 4).

Total sulphide concentrations

Neither carrageenan alone nor following pretreatment with LR or indomethacin altered the concentrations of total sulphide (i.e. S²⁻ + HS⁻ + H₂S) measured in synovial lavage fluid samples. However, PGly pretreatment resulted in significant reduction of total sulphide concentration (approximately 75%) in comparison with the control group ($P < 0.05$; Figure 5A). None of the treatments had any effect on the circulating concentrations of total sulphide species ($n = 5$ –6 animals; Figure 5B).

Synovial NO_x⁻ concentration

The concentrations of NO_x⁻ (nitrite + nitrate) in the synovial lavage fluid samples obtained from carrageenan-injected rats were significantly higher ($P < 0.001$) than those found in the control animals (Table 1; $n = 4$). Pretreatment with either the H₂S donor or indomethacin did not affect the NO_x⁻ production in response to carrageenan, but PGly pretreatment increased the NO_x⁻ concentration measured in the synovial lavage fluid from synovitic rats (Table 1; $n = 4$ each group).

Synovial NOS activity

Ca²⁺-dependent NOS activity was significantly reduced by approximately 80% in the synovial membrane homogenates obtained from the carrageenan-injected rats, either untreated or pretreated with PGly or indomethacin (Figure 6A). However, this response was partially, although significantly, reversed by pretreatment with LR (Figure 6A).

In terms of synovial Ca²⁺-independent NOS activity, the i.art. injection of carrageenan led to a significant augmentation of this activity (approximately five times higher than in the control animals; $P < 0.05$; Figure 6B), which was unaffected by pretreatment of the animals with either the LR reagent or indomethacin. On the other hand, pretreatment with the CSE inhibitor PGly resulted in a twofold increase of this Ca²⁺-independent NOS activity when compared with the untreated carrageenan group ($P < 0.01$; Figure 6B).

Protein NT levels

The i.art. injection of carrageenan markedly increased the content of NT in synovial membranes, compared to that in

Figure 5 Effect of treatment with LR and DL-propargylglycine on total sulphide concentration in synovial lavage fluid and plasma. Carrageenan-injected animals were pretreated with LR (3.6 μmol i.art., $n = 4-5$), DL-propargylglycine (PGly; 53 μmol i.art., $n = 4$), indomethacin (6 $\text{mg}\cdot\text{kg}^{-1}$ i.p., $n = 4-5$) or vehicle ($n = 4-5$). A separate control group received only i.art. injection of saline ($n = 4-5$). The total sulphide concentrations in articular lavage fluid (A; $n = 4$) or plasma (B; $n = 5$) were expressed as mean $\pm$ SEM of concentrations in μM . * $P < 0.05$ versus saline and # $P < 0.05$ versus vehicle-treated animals. (One-way ANOVA followed by Bonferroni's multiple comparison test).

samples from the saline-injected group (198 ± 23 vs. $100 \pm 7.13\%$, respectively; $P < 0.01$, $n = 5$ for each group). None of the pretreatments (e.g. LR, PGly or indomethacin) significantly affected this response (LR: 166 ± 17 , PGly: 173 ± 16 and indomethacin: 123 ± 45 , as % of saline group; $n = 5$ for each group).

Cytokine production in the rat knee joint

The i.art. injection of carrageenan into the rat knee cavity resulted in significant increase of IL-1 β (Table 1; $n = 5$), in the synovial lavage fluid, which was significantly reduced by pretreatment of the animals with LR (Table 1; $n = 5$) or indomethacin (Table 1; $n = 9$), but remained unchanged in those animals treated with the CSE inhibitor, PGly (Table 1; $n = 9$). Under our experimental conditions, neither TNF- α nor IL-6 concentrations were detectable (the detection limits were 1.95 and 0.98 $\text{pg}\cdot\text{mL}^{-1}$ respectively).

Figure 6 Effect of treatment with LR and DL-propargylglycine (PGly) on NO synthase activity in synovial membranes. Carrageenan-injected animals were pretreated with LR (3.6 μmol i.art., $n = 5$), PGly (53 μmol i.art., $n = 5$), indomethacin (6 $\text{mg}\cdot\text{kg}^{-1}$ i.p., $n = 5$) or vehicle ($n = 5$). A separate control group received only i.art. injection of saline ($n = 5$). The calcium-dependent NOS (A) or calcium-independent NOS (B) activities were expressed as mean $\pm$ SEM of enzyme activity in $\text{pmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ of protein. * $P < 0.05$ –*** $P < 0.001$ versus saline, and ^{##} $P < 0.01$ versus vehicle-treated animals. (One-way ANOVA followed by Bonferroni's multiple comparison test).

Discussion and conclusions

A number of both *in vivo* and *in vitro* studies provide evidence that the gaseous transmitter H₂S plays an important role as a modulator of inflammatory processes in various tissues, by acting on multiple targets. In this work, we show that the i.art. administration of H₂S donor LR results in significant amelioration of the inflammatory, pain process and secondary tactile allodynia evoked by carrageenan injected into the rat knee joint. The anti-nociceptive properties of LR in our model of synovitis joint pain were functionally assessed by both the gait score and electronic von Frey tests. We also show that the inhibition of CSE, one of the enzymes responsible for the production of endogenous H₂S by PGly, does not affect the progression of the disease in terms of joint swelling, pain or leucocyte migration, except for the higher number of

macrophages and increased iNOS-derived NO production that this treatment causes.

To the best of our knowledge, there are no previous reports on the protective effects of H₂S on nociception or tactile allodynia secondary to acute joint inflammation in the rat. Our identification of a protective effect of H₂S against behavioural joint pain, secondary tactile allodynia in the ipsilateral hindpaw and knee inflammation are in contrast with those from Andruski *et al.* (2008) who, despite detecting an anti-inflammatory effect of H₂S, failed to show significant alterations in the mouse pain behaviour secondary to carrageenan/kaolin-induced arthritis. The reason for this discrepancy is unknown, but as they have used mice and we have used rats, this may indicate a species difference in the modulatory roles of H₂S during joint inflammation. In addition, while our knee joint inflammation model was evoked by a single i.art. injection of carrageenan, and both the pain behaviour and secondary allodynia were measured 4 h later, these authors produced experimental arthritis in mice by i.art. injection of a carrageenan and kaolin mixture, and pain was assessed 24 h later.

A similar divergence among anti-nociceptive and pro-nociceptive effects of H₂S donors has emerged from other experimental models. Using the colorectal distension model (CRD) that mimics some features of irritable bowel syndrome, Distrutti *et al.* (2006) showed that H₂S inhibits CRD-induced nociception in both healthy and post-colitic rats. This inhibition was suggested to occur via activation of K_{ATP} channels and NO production. According to Cunha *et al.* (2008), the systemic administration of NaHS reduced both the LPS- and PGE₂-induced mechanical hypersensitivity in mice, without affecting the thermal nociceptive threshold in the hot plate test, thus indicating a peripheral anti-nociceptive mechanism for H₂S. Pro-nociceptive effects of H₂S have been proposed to occur through sensitization/activation of T-type Ca²⁺ channels (Maeda *et al.*, 2009). Collectively, these studies indicate that H₂S plays a dual role in the pathogenesis of inflammatory pain, with the balance between pro- and anti-nociceptive effects determined by the amount and site of release, and influenced by leucocyte migration and species difference.

The carrageenan-induced recruitment of neutrophils and lymphocytes into the knee joint was also markedly reduced in animals pretreated with either LR or indomethacin. In addition to the cell counting and MPO activity measurements, the histological analysis shows the presence of exudate and leucocyte (mainly neutrophils) accumulation within the lining and the sublining layers of the inflamed knee synovium. These effects were significantly attenuated in rats pretreated with LR or indomethacin, but not with PGly, thus suggesting that H₂S modulates the process of leucocyte recruitment. Supporting this finding, Zanardo *et al.* (2006) demonstrated that leucocyte infiltration in the rodent air pouch model was inhibited by H₂S donors (via activation of K_{ATP} channels) and exacerbated by the inhibition of endogenous H₂S synthesis. Likewise, Andruski *et al.* (2008) showed that H₂S donors reduced leucocyte infiltration in the mouse knee possibly via local vasoconstriction caused by H₂S. This was a surprising result, as the vasodilatory effects of H₂S are well known and systemic H₂S can cause hypotension in anaesthetized rats (Zhao *et al.*, 2001). An alternative mechanism by which H₂S

could inhibit leucocyte infiltration seems to be through inhibition of the intercellular adhesion molecule (ICAM)-1 and/or promotion of neutrophil apoptosis (Mariggiò *et al.*, 1998). In a rodent model of non-steroidal anti-inflammatory drug gastropathy, NaHS attenuated the gastric mucosal lesions, as well as decreasing the inflammatory markers of this condition, such as TNF- α and ICAM-1 (Fiorucci *et al.*, 2005).

Furthermore, elevated NO_x⁻ concentrations were found in the synovial cavity of rats with synovitis, as well as increased Ca²⁺-independent iNOS and decreased Ca²⁺-dependent NOS activity in the synovial homogenates. These results are in agreement with previous reports that show increased nitrite concentrations in synovial fluid and serum samples from patients with RA or osteoarthritis (Farrell *et al.*, 1992). Increased iNOS expression was also detected in joint synovio-cytes, macrophages and chondrocytes from patients with RA (Yonekura *et al.*, 2003), and that treatment with iNOS inhibitors results in an effective reduction of arthritis severity (Connor *et al.*, 1995; Stefanovic-Racic *et al.*, 1995; Pelletier *et al.*, 1998). Interestingly, carrageenan-induced increased iNOS activity and NO_x⁻ concentration in the knee cavity were potentiated by PGly, whereas neither LR nor indomethacin had a significant effect on these parameters. This indicates the importance of negative inhibition of iNOS-derived NO production by endogenous H₂S on the prevention of macromolecular damage caused by prolonged exposure to high concentrations of NO or its reaction products. Our *in vivo* results are supported by studies showing that H₂S donors decrease recombinant iNOS activity (Kubo *et al.*, 2007), in addition to reducing iNOS protein expression and NO_x⁻ production by RAW264.7 macrophages *in vitro* (Oh *et al.*, 2006). The lack of effects of indomethacin on NO production (and iNOS activity) is in agreement with a previous study, in which the authors suggested that aspirin (but not indomethacin) inhibited increased iNOS expression and NO production in response to LPS-stimulated macrophages, and that was exclusively due to irreversible COX-2 acetylation (Amin *et al.*, 1995).

Subsequently, we found that i.art. administration of carrageenan results in increased nitration of protein tyrosine residues (3-NT) in the synovial membrane, and none of the treatments (e.g. LR, indomethacin or PGly) were capable of affecting this response, thus confirming that the observed beneficial effects of either LR or indomethacin occur independently, and in spite of the augmented iNOS activity, NO production and tyrosine nitration (probably via NO-derived peroxynitrous acid).

We showed that Ca²⁺-dependent NOS activity was significantly reduced (>80%) in the carrageenan-treated animals, either untreated or pretreated with PGly or indomethacin, and pretreatment with LR partially prevented this reduction. Interestingly, the preservation of constitutive endothelial NOS activity can control leucocyte-endothelium interactions (Kubes *et al.*, 1991; Wahl *et al.*, 2003), and thus account for the reduced number of infiltrating cells in the knee cavity of LR-treated rats. In line with these observations, the higher number of macrophages found in the joint cavity of PGly-treated rats may indicate increased macrophage-endothelium interaction secondary to the removal of endogenous H₂S, and as a consequence, augmented synovial iNOS due to the higher

number of activated macrophages. It is thus evident that the interplay between H₂S and NOS isoforms is extremely complex, considering that in addition to H₂S and NOS isoforms and activity discussed above, the cross-talk between iNOS and the other NOS isoforms must also be taken into account (Persichini *et al.*, 2006).

A number of studies show the relative importance of IL-1 β in the pathophysiology of RA, because of its greater capacity (compared to TNF- α) to increase matrix degradation by inducing the production of iNOS, receptor activator of NF- κ B ligand, matrix metalloproteinases and eicosanoids in synovial cells (Dayer, 2002). IL-1 β also stimulates production of mediators involved in the destruction of the extracellular matrix, the cartilage and bone resorption. Over the time period studied following carrageenan-induced synovitis, concentrations of IL-1 β , but not TNF- α or IL-6, were significantly raised in the synovial lavage, and pretreatment with indomethacin or LR (but not with PGly) partially prevented this increase. However, we cannot establish a direct relationship between IL-1 β and iNOS activity as, on one hand, pretreatment with LR resulted in lower IL-1 β levels and unaltered iNOS activity (or synovial NO_x⁻ concentrations), and on the other hand, carrageenan-injected rats showed higher synovial iNOS activity (and increased synovial NO_x⁻ concentrations) with unaltered IL-1 β serum levels after PGly treatment. IL-1 β gene transcription is regulated by several pathways, including the activation of NF- κ B (Koenders *et al.*, 2006) and mitogen-activated protein kinase p38 (MAPK; Baldassare *et al.*, 1999). In a rat model of myocardial ischaemia/reperfusion, Sivarajah *et al.* (2009) reported that the cardioprotective effects of H₂S donors are mediated, at least in part, by inhibition of both p38 MAPK phosphorylation and translocation from the cytosol to the nucleus of the p65 subunit of NF- κ B. This mechanism may also directly reduce carrageenan-induced IL-1 β production in the rat knee joint, in addition to the diminished IL-1 β production due to decreased leucocyte migration to the knee joint.

Following carrageenan-induced synovitis, pretreatment with PGly did not result in systemic effects, but effectively inhibited local H₂S production, as shown by the reduced concentrations of total sulphide measured in the knee cavity. Treatment with LR did not result in a corresponding increase of articular sulphide, despite its pharmacological effects. There is evidence that H₂S is rapidly cleared by rapid reaction with oxygen- and nitrogen-derived anions, such as superoxide (Chang *et al.*, 2008), peroxynitrite (Whiteman *et al.*, 2004) or hypochlorite (Whiteman *et al.*, 2005). Considering that these species are usually present in high amounts during inflammatory processes, in addition to their high reactivity towards proteins, lipids and nucleotides, it is possible that the beneficial effects of the H₂S donor were partly due to chemical scavenging of deleterious reactive species. In fact, H₂S prevents peroxynitrite anion production (and the resulting tyrosine nitration) via the formation of an intermediate nitrosothiol (Whiteman *et al.*, 2006). Thus, the occurrence of these reactions explains the consumption of LR-derived H₂S, and then the lack of increase of total sulphide concentrations in the synovial lavages. Acute measurement of sulphide species in the rat knee following the i.art. injection of LR identified a rapid increase followed by an exponential decay with a half-

life of approximately 22 min (data not shown). This seems to be also the case after the administration of pure H₂S (Dorman *et al.*, 2002). Indeed, Whitfield *et al.* (2008) show that exogenous sulphide was rapidly removed from blood and plasma *in vitro* and from intact trout *in vivo*. We measured total sulphide concentrations in the knee cavity 5 h following LR pretreatment, and found that the concentration of sulphide in the knee cavity was at the basal level, reinforcing the finding of rapid H₂S metabolism. Taken together, we can speculate that the biochemical reactions underlying the protective effect of LR on carrageenan-induced synovitis must occur during the first 2 h following injection of this compound (~5 half-lives to achieve the basal sulphide concentrations), of which the last 60 min was after carrageenan injection. This argument is strengthened by the notion that LR is similar to other H₂S donors (e.g. NaSH or Na₂S), which are important modulators of inflammatory events in other tissues (Zanardo *et al.*, 2006; Wallace, 2007; Medeiros *et al.*, 2009).

Overall, even though the effects of the H₂S donor, LR, are very short lived, we showed a long-lasting effect on carrageenan-induced pain behavioural responses, secondary allodynia and knee inflammation, thus reinforcing the importance of H₂S as an anti-inflammatory and analgesic mediator. This result also shows the differences between endogenously produced and exogenously administered H₂S in terms of their actions on the various components of the knee joint inflammatory pathophysiology. While exogenous H₂S supply in the rat knee can inhibit pain, swelling, IL-1 β synthesis and neutrophil/lymphocyte influx in response to i.art. injection of carrageenan, endogenous H₂S seems to regulate macrophage migration and NO production.

Furthermore, it is possible that the early supply of H₂S in conditions such as RA might reduce the severity and speed of progression of the disease, and can be an attractive possible mechanism to explain the benefit of LR treatment on carrageenan-induced synovitis. Interestingly, the novel hydrogen sulphide donors slow-releasing molecules (Li *et al.*, 2009; Whiteman *et al.*, 2009) in addition to the anti-inflammatory drugs that are modified to release H₂S (Wallace, 2007) have important implications for anti-inflammatory drug development, and might be a useful insight to the therapy of acute and chronic joint inflammation. However, their effects in different arthritis models must be studied further before any firm conclusions can be drawn.

It is clear that H₂S can interact with inflammatory cells and mediators, and the study of these interactions is further complicated by their reciprocal cross-talk. Nevertheless, we conclude that our results support the development and use of H₂S donors as potential alternatives (or complementary) to the available anti-inflammatory compounds used for treatment of arthritis or relief of its symptoms.

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Conflicts of interest

None.

References

- Alexander SPH, Mathie A, Peters JA (2009). Guide to Receptors and Channels (GRAC), 4th edn. *Br J Pharmacol* **158** (Suppl. 1): S1–S254.
- Amin AR, Vyas P, Attur M, Leszczynska-Piziak J, Patel IR, Weissmann G *et al.* (1995). The mode of action of aspirin-like drugs: effect on inducible nitric oxide synthase. *Proc Natl Acad Sci USA* **92**: 7926–7930.
- Andruski B, McCafferty DM, Ignacy T, Millen B, McDougall JJ (2008). Leukocyte trafficking and pain behavioral responses to a hydrogen sulfide donor in acute monoarthritis. *Am J Physiol Regul Integr Comp Physiol* **295** (3): R814–R820.
- Awata S, Nakayama K, Suzuki I, Sugahara K, Kodama H (1995). Changes in cystathionine gamma-lyase in various regions of rat brain during development. *Biochem Mol Biol Int* **35**: 1331–1338.
- Baldassare JJ, Bi Y, Bellone CJ (1999). The role of p38 mitogen-activated protein kinase in IL-1 beta transcription. *J Immunol* **162** (9): 5367–5373.
- Bhatia M, Sidhapuriwala J, Moolchhala SM, Moore PK (2005a). Hydrogen sulphide is a mediator of carrageenan-induced hindpaw oedema in the rat. *Br J Pharmacol* **145** (2): 141–144.
- Bhatia M, Wong FL, Fu D, Lau HY, Moolchhala SM, Moore PK (2005b). Role of hydrogen sulphide in acute pancreatitis and associated lung injury. *FASEB J* **19** (6): 623–625.
- Bian JS, Yong QC, Pan TT, Feng ZN, Ali MY, Zhou S *et al.* (2006). Role of hydrogen sulphide in the cardioprotection caused by ischemic preconditioning in the rat heart and cardiac myocytes. *J Pharmacol Exp Ther* **316** (2): 670–678.
- Bradley PP, Priebe DA, Christensen RD, Rothstein G (1982). Measurement of cutaneous inflammation: estimation of neutrophil content with an enzyme marker. *J Invest Dermatol* **78** (3): 206–209.
- Chang L, Geng B, Yu F, Zhao J, Jiang H, Du J *et al.* (2008). Hydrogen sulfide inhibits myocardial injury induced by homocysteine in rats. *Amino Acids* **34** (4): 573–585.
- Collin M, Anuar FB, Murch O, Bhatia M, Moore PK, Thiemermann C (2005). Inhibition of endogenous hydrogen sulfide formation reduces the organ injury caused by endotoxemia. *Br J Pharmacol* **146** (4): 498–505.
- Connor JR, Manning PT, Settle SL, Moore WM, Jerome GM, Webber RK *et al.* (1995). Suppression of adjuvant-induced arthritis by selective inhibition of inducible nitric oxide synthase. *Eur J Pharmacol* **273** (1–2): 15–24.
- Cunha TM, Dal-Secco D, Verri WA Jr, Guerrero AT, Souza GR, Vieira SM *et al.* (2008). Dual role of hydrogen sulfide in mechanical inflammatory hypernociception. *Eur J Pharmacol* **590** (1–3): 127–135.
- Dayer JM (2002). Evidence for the biological modulation of IL-1 activity: the role of IL-1Ra. *Clin Exp Rheumatol* **20** (5 Suppl. 27): S14–S20.
- Denadai-Souza A, Camargo LD, Ribela MT, Keeble JE, Costa SK, Muscará MN (2009). Participation of peripheral tachykinin NK(1) receptors in the carrageenan-induced inflammation of the rat temporomandibular joint. *Eur J Pain* **13** (8): 812–819.
- Distrutti E, Sediari L, Mencarelli A, Renga B, Orlandi S, Antonelli E *et al.* (2006). Evidence that hydrogen sulphide exerts antinociceptive effects in the gastrointestinal tract by activating KATP channels. *J Pharmacol Exp Ther* **316** (1): 325–335.
- Dorman DC, Moulin FJ, McManus BE, Mahle KC, James RA, Struve MF (2002). Cytochrome oxidase inhibition induced by acute hydrogen sulfide inhalation: correlation with tissue sulfide concentrations in the rat brain, liver, lung, and nasal epithelium. *Toxicol Sci* **65**: 18–25.
- Farrell AJ, Blake DR, Palmer RM, Moncada S (1992). Increased concentrations of nitrite in synovial fluid and serum samples suggest increased nitric oxide synthesis in rheumatic diseases. *Ann Rheum Dis* **51** (11): 1219–1222.
- Fiorucci S, Antonelli E, Distrutti E, Rizzo G, Mencarelli A, Orlandi S *et al.* (2005). Inhibition of hydrogen sulfide generation contributes to gastric injury caused by anti-inflammatory nonsteroidal drugs. *Gastroenterology* **129** (4): 1210–1224.
- Grabski M, Wozakowska-Kaplon B, Kedziora J (2004). Hydrogen sulphide water balneum effect on erythrocyte superoxide dismutase activity in patients with rheumatoid arthritis – *in vitro* study. *Przegl Lek* **61** (12): 1405–1409.
- Grisham MB, Johnson GG, Lancaster JR Jr (1996). Quantitation of nitrate and nitrite in extracellular fluids. *Methods Enzymol* **268**: 237–246.
- Ibadova GD, Mamishev SN, Zavara MI, Sarian LA (2005). Combined use of hydrogen sulphide baths and galvanopeloid therapy with magnetolaser radiation in osteoarthritis patients at a health resort. *Vopr Kurortol Fizioter Lech Fiz Kult* **4**: 23–25.
- Kimura H (2002). Hydrogen sulphide as a neuromodulator. *Mol Neurobiol* **26** (1): 13–19.
- Kimura H, Nagai Y, Umemura K, Kimura Y (2005). Physiological roles of hydrogen sulphide: synaptic modulation, neuroprotection, and smooth muscle relaxation. *Antioxid Redox Signal* **7** (5–6): 795–803.
- Koenders MI, Joosten LAB, Van den Berg WB (2006). Potential new targets in arthritis therapy: interleukin (IL)-17 and its relation to tumour necrosis factor and IL-1 in experimental arthritis. *Ann Rheum Dis* **65** (Suppl. 3): 29–33.
- Kubes P, Suzuki M, Granger DN (1991). Nitric oxide: an endogenous modulator of leukocyte adhesion. *Proc Natl Acad Sci USA* **88** (11): 4651–4655.
- Kubo S, Kurokawa Y, Doe I, Masuko T, Sekiguchi F, Kawabata A (2007). Hydrogen sulphide inhibits activity of three isoforms of recombinant nitric oxide synthase. *Toxicology* **241** (1–2): 92–97.
- Lee HS, Lee CH, Tsai HC, Salter DM (2009). Inhibition of cyclooxygenase 2 expression by diallyl sulphide on joint inflammation induced by urate crystal and IL-1β. *Osteoarthritis Cartilage* **17** (1): 91–99.
- Li L, Rossoni G, Sparatore A, Lee LC, Del Soldato P, Moore PK (2007). Anti-inflammatory and gastrointestinal effects of a novel diclofenac derivative. *Free Radic Biol Med* **42** (5): 706–719.
- Li L, Salto-Tellez M, Tan CH, Whiteman M, Moore PK (2009). GYY4137, a novel hydrogen sulfide-releasing molecule, protects against endotoxic shock in the rat. *Free Radic Biol Med* **47** (1): 103–113.
- Maeda Y, Aoki Y, Sekiguchi F, Matsunami M, Takahashi T, Nishikawa H *et al.* (2009). Hyperalgesia induced by spinal and peripheral hydrogen sulphide: evidence for involvement of Cav3.2 T-type calcium channels. *Pain* **142**: 127–132.
- Mariggiò MA, Minunno V, Riccardi S, Santacroce R, De Rinaldis P, Fumarulo R (1998). Sulphide enhancement of PMN apoptosis. *Immunopharmacol Immunotoxicol* **20**: 399–408.
- Medeiros JV, Bezerra VH, Gomes AS, Barbosa AL, Lima-Júnior RC, Soares PM *et al.* (2009). Hydrogen sulfide prevents ethanol-induced gastric damage in mice: role of ATP-sensitive potassium channels and capsaicin-sensitive primary afferent neurons. *J Pharmacol Exp Ther* **330**: 764–770.
- Neugebauer V, Han JS, Adwanikar H, Fu Y, Ji G (2007). Techniques for assessing knee joint pain in arthritis. *Mol Pain* **28**: 3–8.
- Oh GS, Pae HO, Lee BS, Kim BN, Kim JM, Kim HR *et al.* (2006). Hydrogen sulphide inhibits nitric oxide production and nuclear factor-kappaB via heme oxygenase-1 expression in RAW264.7 macrophages stimulated with lipopolysaccharide. *Free Radic Biol Med* **41**: 106–119.

- Otsuki T, Nakahama H, Niizuma H, Suzuki J (1986). Evaluation of the analgesic effects of capsaicin using a new rat model for tonic pain. *Brain Res* **365** (2): 235–240.
- Patacchini R, Santicioli P, Giuliani S, Maggi CA (2004). Hydrogen sulfide (H₂S) stimulates capsaicin-sensitive primary afferent neurons in the rat urinary bladder. *Br J Pharmacol* **142** (1): 31–34.
- Pelletier JP, Mineau F, Fernandes JC, Duval N, Martel-Pelletier J (1998). Diacerhein and rhein reduce the interleukin 1beta stimulated inducible nitric oxide synthase level and activity while stimulating cyclooxygenase-2 synthesis in human osteoarthritic chondrocytes. *J Rheumatol* **25** (12): 2417–2424.
- Persichini T, Cantoni O, Suzuki H, Colasanti M (2006). Cross-talk between constitutive and inducible NO synthase: an update. *Anti-oxid Redox Signal* **8** (5–6): 949–954.
- Roth A, Mollenhauer J, Wagner A, Fuhrmann R, Straub A, Venbrocks RA *et al.* (2005). Intra-articular injections of high-molecular-weight hyaluronic acid have biphasic effects on joint inflammation and destruction in rat antigen-induced arthritis. *Arthritis Res Ther* **7**: R677–R686.
- Sivarajah A, Collino M, Yasin M, Benetti E, Gallicchio M, Mazzon E *et al.* (2009). Antiapoptotic and anti-inflammatory effects of hydrogen sulphide in a rat model of regional myocardial I/R. *Shock* **31** (3): 267–274.
- Stefanovic-Racic M, Stadler J, Evans CH (1995). Nitric oxide and arthritis. *Arthritis Rheum* **38** (10): 1529–1531.
- Stipanuk MH, Beck PW (1982). Characterization of the enzymic capacity for cysteine desulphydration in liver and kidney of the rat. *Biochem J* **206** (2): 267–277.
- Szabó C (2007). Hydrogen sulphide and its therapeutic potential. *Nat Rev Drug Discov* **6** (11): 917–935.
- Teixeira SA, Castro GM, Papes F, Martins ML, Rogério F, Langone F *et al.* (2002). Expression and activity of nitric oxide synthase isoforms in rat brain during the development of experimental allergic encephalomyelitis. *Brain Res Mol Brain Res* **99** (1): 17–25.
- Wahl SM, McCartney-Francis N, Chan J, Dionne R, Ta L, Orenstein JM (2003). Nitric oxide in experimental joint inflammation. Benefit or detriment? *Cells Tissues Organs* **174** (1–2): 26–33.
- Wallace JL (2007). Hydrogen sulfide-releasing anti-inflammatory drugs. *Trends Pharmacol Sci* **28** (10): 501–505.
- Wang R (2002). Two's company, three's a crowd: can H₂S be the third endogenous gaseous transmitter? *FASEB J* **16** (13): 1792–1798.
- Whiteman M, Armstrong JS, Chu SH, Jia-Ling S, Wong BS, Cheung NS *et al.* (2004). The novel neuromodulator hydrogen sulphide: an endogenous peroxynitrite 'scavenger'? *J Neurochem* **90** (3): 765–768.
- Whiteman M, Cheung NS, Zhu YZ, Chu SH, Siau JL, Wong BS *et al.* (2005). Hydrogen sulphide: a novel inhibitor of hypochlorous acid-mediated oxidative damage in the brain? *Biochem Biophys Res Commun* **326** (4): 794–798.
- Whiteman M, Li L, Kostetski I, Chu SH, Siau JL, Bhatia M *et al.* (2006). Evidence for the formation of a novel nitrosothiol from the gaseous mediators nitric oxide and hydrogen sulphide. *Biochem Biophys Res Commun* **343** (1): 303–310.
- Whiteman M, Li L, Rose P, Tan CH, Parkinson D, Moore P (2009). The effect of hydrogen sulfide donors on lipopolysaccharide-induced formation of inflammatory mediators in macrophages. *Antioxid Redox Signal* [Epub ahead of print] PMID: 19769459.
- Whitfield NL, Kreimier EL, Verdial FC, Skovgaard N, Olson KR (2008). Reappraisal of H₂S/sulfide concentration in vertebrate blood and its potential significance in ischemic preconditioning and vascular signaling. *Am J Physiol Regul Integr Comp Physiol* **294**: R1930–R1937.
- Wozakowska-Kaplon B, Grabski M, Kedziora J (2006). Hydrogen sulphide water balneum effect on erythrocyte catalase activity in patients with rheumatoid arthritis – *in vitro* study. *Wiad Lek* **59** (1–2): 72–77.
- Yelin E (2007). Work disability in rheumatic diseases. *Curr Opin Rheumatol* **19** (2): 91–96.
- Yonekura Y, Koshiishi I, Yamada K, Mori A, Uchida S, Nakamura T *et al.* (2003). Association between the expression of inducible nitric oxide synthase by chondrocytes and its nitric oxide-generating activity in adjuvant arthritis in rats. *Nitric Oxide* **8** (3): 164–169.
- Zanardo RC, Brancalone V, Distrutti E, Fiorucci S, Cirino G, Wallace JL (2006). Hydrogen sulphide is an endogenous modulator of leukocyte-mediated inflammation. *FASEB J* **20** (12): 2118–2120.
- Zhang H, Zhi L, Mochhala S, Moore PK, Bhatia M (2007). Hydrogen sulfide acts as an inflammatory mediator in cecal ligation and puncture-induced sepsis in mice by upregulating the production of cytokines and chemokines via NF-kappaB. *Am J Physiol Lung Cell Mol Physiol* **292** (4): L960–L971.
- Zhao W, Zhang J, Lu Y, Wang R (2001). The vasorelaxant effect of H₂S as a novel endogenous gaseous K(ATP) channel opener. *EMBO J* **20** (21): 6008–6016.