Hydrogen sulfide is an endogenous modulator of leukocyte-mediated inflammation

Renata C. O. Zanardo,* Vincenzo Brancaleone,† Eleonora Distrutti,‡ Stefano Fiorucci,‡ Giuseppe Cirino,† and John L. Wallace*,[1]

*Inflammation Research Network, University of Calgary, Calgary, Alberta, Canada; †Department of Experimental Pharmacology, University of Naples, Naples, Italy; and ‡Division of Gastroenterology and Hepatology, University of Perugia, Perugia, Italy

ABSTRACT **Hydrogen sulfide (H_2S) is increasingly recognized as an important signaling molecule in the cardiovascular and nervous systems. Recently, H_2S donors were reported to induce neutrophil apoptosis and to suppress expression of some leukocyte and endothelial adhesion molecules. Using rats, we examined the possibility that H_2S is an endogenous regulator of key inflammatory events at the leukocyte-endothelial interface. Via intravital microscopy, we observed that H_2S donors (NaHS and Na_2S) inhibited aspirin-induced leukocyte adherence in mesenteric venules (ED_{50} of 5.0 μmol/kg for Na_2S), likely via activation of ATP-sensitive K^+ (K_{ATP}) channels. Inhibition of endogenous H_2S synthesis elicited leukocyte adherence. Leukocyte infiltration in an air pouch model was also suppressed by H_2S donors (NaHS, Lawesson's reagent, and N-acetylcysteine; ED_{50} of 42.7, 1.3, and 29.9 μmol/kg, respectively) and exacerbated by inhibition of endogenous H_2S synthesis. Carrageenan-induced paw edema was suppressed by H_2S donors (NaHS and Na_2S; ED_{50}s of 35 and 28 μmol/kg, respectively) to the same extent as by diclofenac and enhanced by an inhibitor of H_2S synthesis. Suppression of edema formation by H_2S donors was mimicked by a K_{ATP} channel agonist and reversed by an antagonist of this channel. These results suggest that endogenous H_2S is an important mediator of acute inflammation, acting at the leukocyte-endothelium interface. These findings have important implications for anti-inflammatory drug development.—Zanardo, R. C. O., Brancaleone, V., Distrutti, E., Fiorucci, S., Cirino, G., Wallace, J. L. Hydrogen sulfide is an endogenous modulator of leukocyte-mediated inflammation. *FASEB J.* 20, E1411–E1418 (2006)**

Key Words: CNS · H_2S donor · leukocyte adhesion

GASES SUCH AS NO and carbon monoxide play important roles in various tissues in both health and disease. Recently a third gaseous mediator, hydrogen sulfide (H_2S), has become recognized as an important endogenous vasodilator and neuromodulator (1, 2). H_2S is synthesized from L-cysteine primarily via two enzymes: cystathionine-γ-lyase (CSE) and cystathionine-β-synthetase (CBS). In some tissues, CSE and CBS are both required for H_2S synthesis, whereas in others only one of these enzymes is necessary (1). CSE appears to be the predominant enzymatic source of H_2S in the vasculature and heart (1), but in the central nervous system (CNS) CBS predominates (1, 3). The ability of H_2S to relax vascular smooth muscle most likely occurs through activation of ATP-sensitive K^+ (K_{ATP}) channels (1).

Several recent reports provide evidence suggesting a role for H_2S in inflammation. H_2S can scavenge peroxynitrite (4) and can interfere with the ability of neutrophils, through hypochlorous acid, to kill microbes and other cells (5). H_2S can also induce neutrophil apoptosis, thereby contributing to resolution of inflammatory reactions (6). We recently demonstrated that H_2S can exert analgesic effects in a visceral pain model (7). Nonsteroidal anti-inflammatory drugs (NSAIDs) suppress endogenous H_2S synthesis by reducing expression of CSE (8). This may contribute to the production of damage in the stomach induced by NSAIDs, since administration of exogenous H_2S reduced the ability of these agents to cause gastric injury. Particularly relevant to a potential role in inflammation, the H_2S donor suppressed NSAID-induced granulocyte infiltration, expression of endothelial and leukocyte adhesion molecules, and expression of tumor necrosis factor α (8). Leukocyte adherence to the vascular endothelium induced by aspirin was also suppressed by an H_2S donor.

Recent data also suggest that H_2S may contribute to inflammatory processes. For example, significant increases in H_2S production and up-regulation of CSE expression were observed in studies of rodent models of acute pancreatitis (9) and endotoxemia (10), whereas irreversible inhibition of CSE activity with DL-propargylglycine reduced the severity of pancreatitis and endotoxic shock. This inhibitor was also found to suppress edema formation and granulocyte infiltration in a rat model of hindpaw inflammation (9).

Given these apparently conflicting observations, we

[1] Correspondence: Department of Pharmacology and Therapeutics, University of Calgary, 3330 Hospital Dr. NW, Calgary, Alberta, T2N 4N1, Canada. E-mail: wallacej@ucalgary.ca

doi: 10.1096/fj.06-6270fje

performed a detailed study of the effects of a number of different H_2S donors in several *in vivo* models of inflammation, using multiple distinct phlogistic agents. We also examined the effects on several inflammatory parameters of inhibition of endogenous H_2S synthesis and of blockade or activation of K_{ATP} channels (the putative target of the vascular actions of H_2S). In particular, we examined the role of H_2S in modulating leukocyte adhesion to the vascular endothelium, leukocyte infiltration, and edema formation. Our studies implicate H_2S as an important endogenous inhibitor of these key elements of acute inflammatory reactions.

MATERIALS AND METHODS

Animals

Male Wistar rats weighing 175–200 g were obtained from Charles River Breeding Farms (Montreal, Canada, and Monza, Italy). For 18 h prior to an experiment, the rats were deprived of food, but not water. All experimental procedures described below were approved by the institutional animal care committees and were performed in accordance with the guidelines of the National Council on Animal Care.

Intravital microscopy

Examination of leukocyte-endothelial interactions *in vivo* was performed as described in detail (11). Postcapillary mesenteric venules with a length of at least 150 μm and diameters ranging from 25 to 40 μm were selected for the study. A video camera mounted on the microscope (Panasonic digital 5000) projected the image onto a monitor, and the images were recorded for playback analysis using a videocassette recorder. Images of the mesenteric microcirculation were recorded over 5 min periods starting immediately before (baseline) and after aspirin administration or initiation of fMLP superfusion, and at 15 min intervals thereafter for 60 min. Aspirin was administered intragastrically at a dose of 50 mg/kg, whereas fMLP (10 μM) was dissolved in the buffer that superfused the mesenteric venules. In controls, vehicle (1% CMC) was given intragastrically instead of aspirin and vessels were superfused with buffer not containing fMLP. Leukocyte adhesion was blindly quantified as the number of leukocytes that adhered to the vessel wall for at least 30 s per 100 μm venule length. Rolling leukocytes were defined as white blood cells moving at a velocity less than that of the erythrocytes in the same stream. The rolling leukocyte velocity was determined by the time required for a leukocyte to traverse a given distance along the length of a venule.

To assess the effects of H_2S on aspirin- and fMLP-induced leukocyte adherence, rats were pretreated intragastrically with Na_2S (1–100 μmol/kg), NaHS (100 μmol/kg), or Lawesson's reagent (0.1 to 3 μmol/kg) 30 min before aspirin or fMLP administration. Control rats received vehicle at the same time. In another group of experiments, glibenclamide was given 1 h prior to Na_2S or vehicle. In other experiments, rats were given a reversible inhibitor of CSE (β-cyano-alanine, 50 mg/kg i.p.) 1 h prior to aspirin. This dose of β-cyano-alanine (BCA) has been shown to significantly inhibit CSE activity in the rat (12).

Carrageenan air pouch model

An air pouch was induced as described previously (13, 14). Briefly, 20 μl of air was injected subcutaneously on the back of the rat on the first day. Two days later, another 10 μl of air was injected at the same site. On the fifth day after the first injection, another 10 μl of air was injected into the pouch. Twenty-four hours later, carrageenan (2 ml of a 1% w/v solution in sterile saline) or the vehicle was injected into the air pouch. All of the injections were performed after the rats had been anesthetized with 5% (v/v) halothane. Six hours after the carrageenan injection, rats were anesthetized with sodium pentobarbital (60 mg/kg; i.p.) and 1 μl of heparinized saline was injected into the pouch. The pouch was then carefully opened by a small incision. The exudate was collected, the volume determined gravimetrically, and an aliquot was used to quantify leukocyte concentration using a Sysmex KX-21N hematology analyzer. Another aliquot was applied to a glass slide and stained with Wright's stain to determine the relative numbers of different leukocyte subtypes.

The effects of H_2S on leukocyte infiltration into the pouch were assessed by treating rats (i.p.) 30 min before carrageenan injection with vehicle (0.9% saline) or one of the following H_2S donors: NaHS (1–100 μmol/kg), Lawesson's reagent (0.1–3 μmol/kg), or N-acetylcysteine (0.5–50 μmol/kg). In other experiments rats were treated with BCA (50 mg/kg) 30 min before administration of N-acetylcysteine (50 μmol/kg). These experiments permitted us to evaluate whether or not N-acetylcysteine might affect leukocyte infiltration of the air pouch independent of metabolism via CSE. Additional experiments were performed in which rats received glibenclamide (10 mg/kg i.p.) or vehicle (dimethyl sulfoxide, 0.1 ml, i.p.) 30 min before an H_2S donor to determine whether the effects of the donors were mediated via K_{ATP} channels.

Several drugs were tested in the air pouch model as positive controls, as we had found them to significantly reduce carrageenan-induced leukocyte infiltration. These included an NSAID (diclofenac, 10 mg/kg i.p.), a NOS inhibitor (L-NAME; NG-nitro-L-arginine methyl ester; 25 mg/kg i.p.), and dexamethasone (1 mg/kg i.p.). Diclofenac and L-NAME were administered 30 min prior to carrageenan, and dexamethasone was administered 2 h before carrageenan. As dexamethasone produced what was deemed to be a "maximal" reduction of leukocyte infiltration in this model, we calculated ED_{50} values for each of the H_2S donors relative to the response induced by dexamethasone.

Paw edema

Carrageenan (100 μl of a 1% w/v solution, prepared in sterile saline) was injected into a hind footpad of rats under halothane anesthesia. Paw volume was measured prior to any treatment, immediately before carrageenan administration, and at intervals of 1 h for 5 h thereafter using a Ugo Basile Model 7140 plethysmometer (Comerio, Italy). The person performing these measurements was unaware of the treatments the rats had received. Groups of at least 5 rats each were treated intraperitoneally 30 min before carrageenan administration with an H_2S donor (NaHS at 25–150 μmol/kg or Na_2S at 100 μmol/kg), an NSAID as positive control (diclofenac, 10 mg/kg), or a K_{ATP} channel agonist (pinacidil, 10 mg/kg). Other rats received BCA (50 mg/kg i.p.) 30 min before carrageenan administration. Additional experiments were performed in which groups of 5 rats each received glibenclamide (10 mg/kg) or vehicle (dimethyl sulfoxide) i.p. 30 min before administration of one of the H_2S donors.

Expression of CSE and CBS mRNA

Samples of rat portal vein and mesenteric venules were used to measure CSE and CBS mRNA expression by RT-polymer-

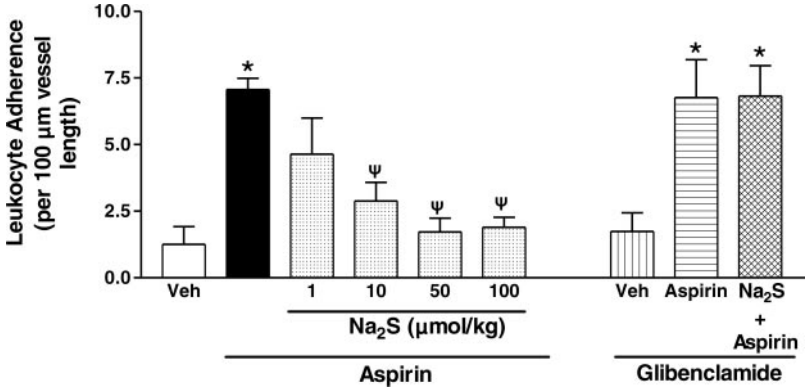

Figure 1. Hydrogen sulfide inhibits aspirin-induced leukocyte adherence in mesenteric venules through activation of K_{ATP} channels. Na_2S dose-dependently suppressed leukocyte adherence induced by intragastric aspirin (50 mg/kg). The inhibition of aspirin-induced adherence by Na_2S (100 μmol/kg) was abolished by pretreatment with glibenclamide (10 mg/kg), a K_{ATP} channel antagonist. $^*P<0.05$ vs. the corresponding vehicle-treated group. $^{\Psi}P< 0.05$ vs. the corresponding group receiving aspirin alone. Each group consisted of at least 5 rats. The results are plotted as mean ± SE.

ase chain reaction (RT-PCR), as described previously (8). Expression of β-actin was determined as a control.

MATERIALS

Unless otherwise stated, all drugs were suspended in 1% carboxymethylcellulose. Aspirin, diclofenac sodium, N-formyl-Met-Leu-Phe, glibenclamide, PAG, BCA, λ-carrageenan, NaHS, Na_2S, pinacidil, N-acetylcysteine, and Lawesson's reagent (2,4-bis(4-methoxyphenyl)-1,3,2,4-dithiadiphosphetane 2,4-disulfide) were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

RESULTS

H_2S donors decrease ASA-induced leukocyte adhesion through the activation of K_{ATP} channels

Oral administration of aspirin (50 mg/kg) induced a significant time-dependent increase in leukocyte adherence compared with rats that received vehicle (**Fig. 1**). Pretreatment of rats with Na_2S dose-dependently decreased aspirin-induced leukocyte adherence to the mesenteric microcirculation (ED_{50} of 5.0 μmol/kg). The reduction of leukocyte adherence by Na_2S likely occurred through activation of K_{ATP} channels, since pretreatment with an antagonist of those channels, glibenclamide, reversed the effects of the H_2S donor. Glibenclamide given to rats prior to vehicle or ASA did not alter basal leukocyte adherence or that induced by aspirin (data not shown). NaHS (100 μmol/kg) also inhibited aspirin-induced leukocyte adherence. As for Na_2S, the inhibition of leukocyte adherence by NaHS could be inhibited by glibenclamide (data not shown).

H_2S donors inhibit fMLP-induced leukocyte adherence

Pretreatment with Na_2S or NaHS (each at 100 μmol/kg) abolished fMLP-induced leukocyte adherence to the mesenteric microcirculation (**Fig. 2**). Lawesson's reagent also inhibited leukocyte adhesion when administered at a dose of 1 μmol/kg. At a dose of 0.3 umol/kg, Lawesson's reagent did not affect fMLP-induced leukocyte adherence (data not shown).

Inhibition of CSE activity promotes leukocyte adhesion

Mesenteric venules in the rat express both CSE and CBS (mRNA), whereas the portal vein exhibits a greater expression of CBS than of CSE (**Fig. 3**, upper panel). Administration of BCA at a dose shown to suppress CSE activity in the rat (13) resulted in a marked increase in leukocyte adherence that continued to increase throughout the 60 min experiment (Fig. 3, middle panel). BCA also elicited a sharp decline in leukocyte velocity (Fig. 3, lower panel).

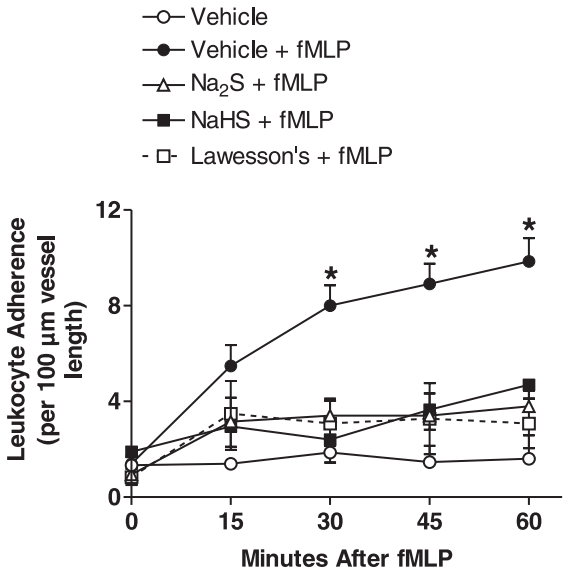

Figure 2. Hydrogen sulfide inhibits fMLP-induced leukocyte adherence in mesenteric venules. Superfusion of the vessels with N-formylated-Met-Leu-Phe (fMLP; 10 μM) induced a time-dependent increase in leukocyte adherence. Hydrogen sulfide donors, given 30 min before fMLP, suppressed the increase in leukocyte adherence to levels not significantly different from control levels of adherence ($^*P<0.05$ vs. the group receiving vehicle in place of fMLP). Na_2S and NaHS were given at 100 μmol/kg, and Lawesson's reagent was given at 1 μmol/kg. Each group consisted of at least 5 rats. The results are plotted as mean ± SE.

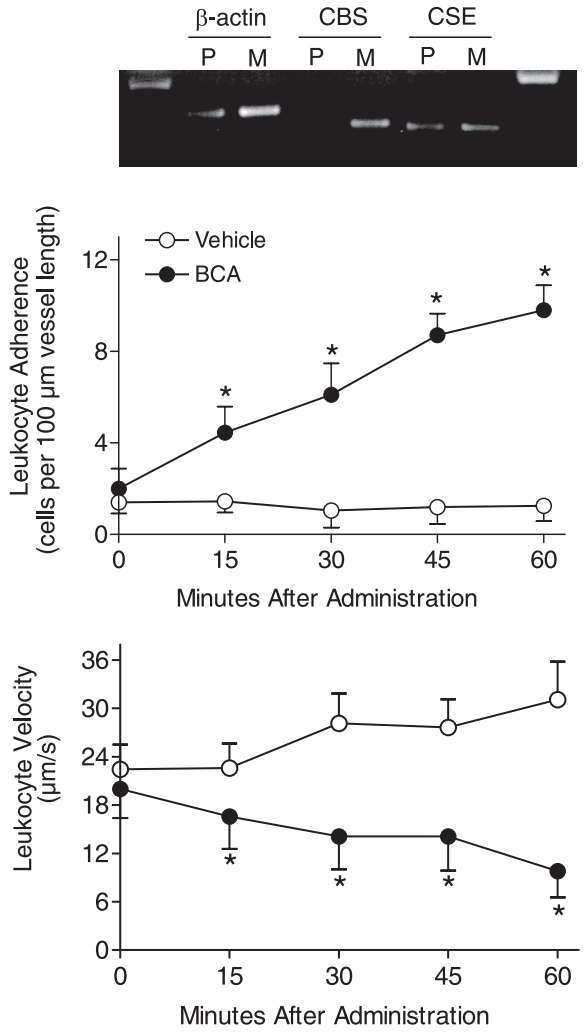

Figure 3. Inhibition of cystathionine-γ-lyase (CSE) reduces leukocyte rolling velocity and increases leukocyte adherence in rat mesenteric venules. *Upper panel*) rat mesentery expresses both CSE and cystathionine-β-synthetase (CBS) mRNA. Expression of these enzymes in the portal vein is also shown. This gel is representative of gels for 3 healthy rats. Within 15 min of administration of β-cyanoalanine (50 mg/kg i.p.), an inhibitor of CSE, leukocyte rolling velocity (*lower panel*) had declined significantly (*$P<0.05$) compared with vehicle-treated controls; significant leukocyte adherence was evident (*middle panel*). Rolling velocity remained at a reduced state throughout the 60 min experiment, whereas leukocyte adherence increased steadily with time. Each group consisted of at least 5 rats. The results are plotted as mean ± SE.

H_2S donors inhibit leukocyte infiltration through the activation of K_{ATP} channels

Administration of carrageenan into a rat air pouch results in infiltration of substantial numbers of neutrophils (**Fig. 4**). Most of the leukocytes were neutrophils (87.0±1.1%) and lymphocytes (12.3±0.8%). Pretreatment with H_2S donors (NaHS, N-acetylcysteine, Lawesson's reagent) dose-dependently reduced the numbers of leukocytes infiltrating into the air pouch in response to carrageenan (ED_{50} values of 42.7, 29.9, and 1.3 μmol/kg, respectively, defining the response to dexamethasone as maximal). With the highest dose of each H_2S donor tested, the reduction in leukocyte infiltration was comparable to that achieved by an NSAID (diclofenac), a NOS inhibitor (L-NAME), and a corticosteroid (dexamethasone).

The ability of N-acetylcysteine to reduce carrageenan-induced leukocyte infiltration was dependent on CSE activity. As shown in **Fig. 5** (top panel), prior

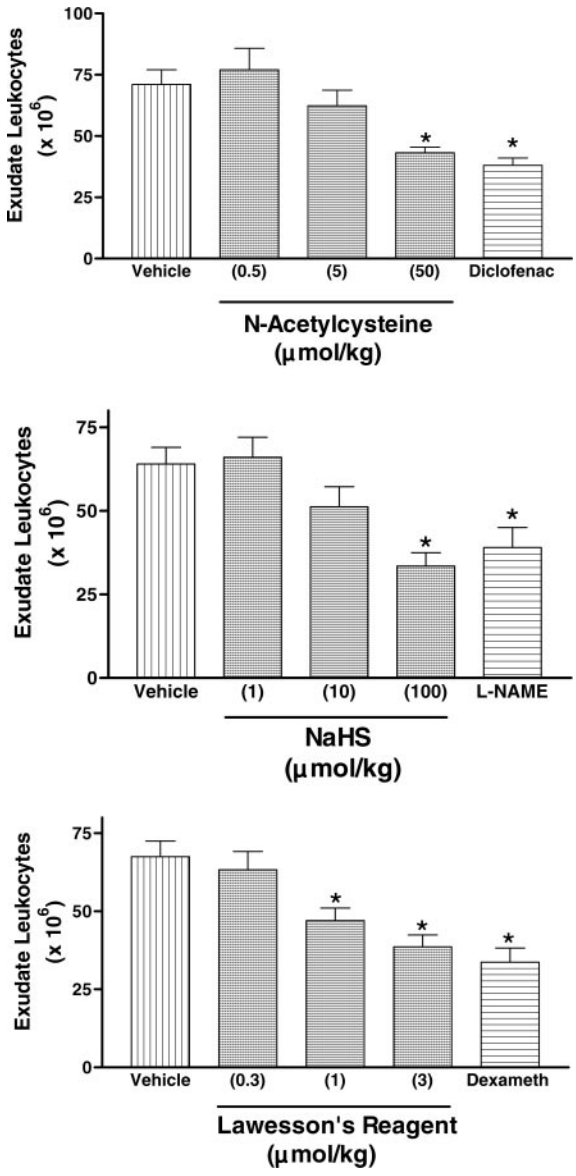

Figure 4. Hydrogen sulfide reduces leukocyte infiltration induced in an air pouch by carrageenan. Rats were treated i.p. with one of the H_2S donors 30 min before injection of carrageenan into the air pouch, and the exudates were collected 6 h later for quantification of leukocyte numbers. N-acetylcysteine (*top panel*), NaHS (*middle panel*), and Lawesson's reagent (*bottom panel*) each caused a dose-dependent reduction of leukocyte infiltration (*$P<0.05$ vs. controls). As positive controls, diclofenac (10 mg/kg), L-NAME (25 mg/kg), and dexamethasone (1 mg/kg) were also tested in the same manner for effects on carrageenan-induced leukocyte infiltration. Each bar represents the mean ± SE, with at least 5 rats per group.

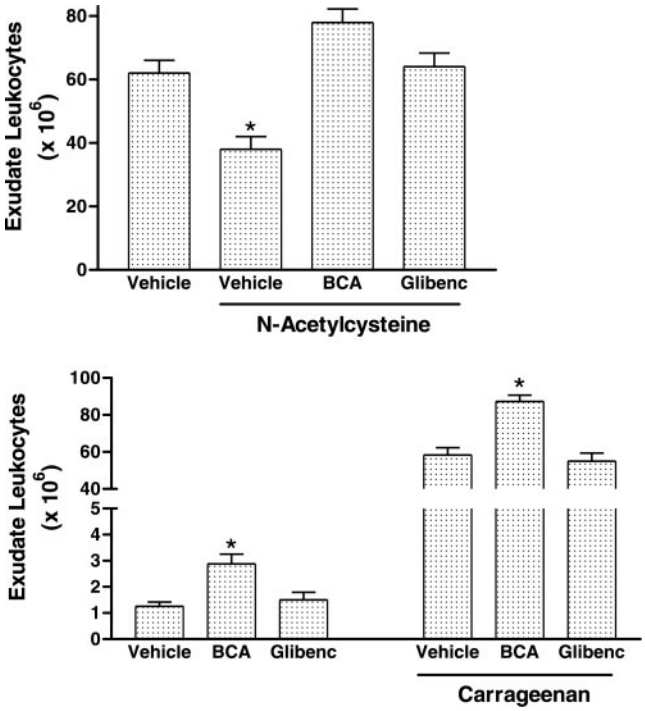

Figure 5. Reduction of leukocyte infiltration by N-acetylcysteine is mediated via cystathionine-γ-lyase-dependent hydrogen sulfide synthesis and K_{ATP} channels. Top panel: N-acetylcysteine (50 μmol/kg i.p.) given 30 min before carrageenan resulted in a significant reduction in leukocyte infiltration (*$P<0.05$ vs. the group treated only with vehicle). This effect was reversed by pretreatment with BCA (β-cyanoalanine; 50 mg/kg i.p.), an inhibitor of cystathionine-γ-lyase. The inhibition of leukocyte infiltration by N-acetylcysteine was blocked by prior treatment with glibenclamide (10 mg/kg, 30 min before), a K_{ATP} channel antagonist. Bottom panel: treatment with BCA augmented basal infiltration of leukocytes in the air pouch model (i.e., in the absence of administration of carrageenan). Treatment with BCA significantly augmented carrageenan-induced leukocyte adherence. The effect of BCA was inhibited by prior treatment with glibenclamide. *$P < 0.05$ vs. the corresponding vehicle-treated group. Each bar represents the mean ± SE, with at least 5 rats per group.

treatment with BCA, an inhibitor of CSE, reversed the effects of N-acetylcysteine. Moreover, the reduction of leukocyte infiltration by N-acetylcysteine could be reversed by glibenclamide, a K_{ATP} channel antagonist.

Inhibition of H_2S synthesis promotes leukocyte infiltration

Administration of BCA resulted in a significant increase in "basal" leukocyte numbers in the air pouch (i.e., without carrageenan administration) (Fig. 5, lower panel). Pretreatment with BCA also significantly enhanced leukocyte infiltration into the air pouch in response to carrageenan. Thus, endogenous H_2S synthesis, via CSE, acts to down-regulate leukocyte infiltration.

H_2S modulates edema formation via effects on K_{ATP} channels

Injection of carrageenan into the hind footpads of rats resulted in a rapid and marked increase in paw volume as a consequence of edema formation (**Fig. 6**, upper

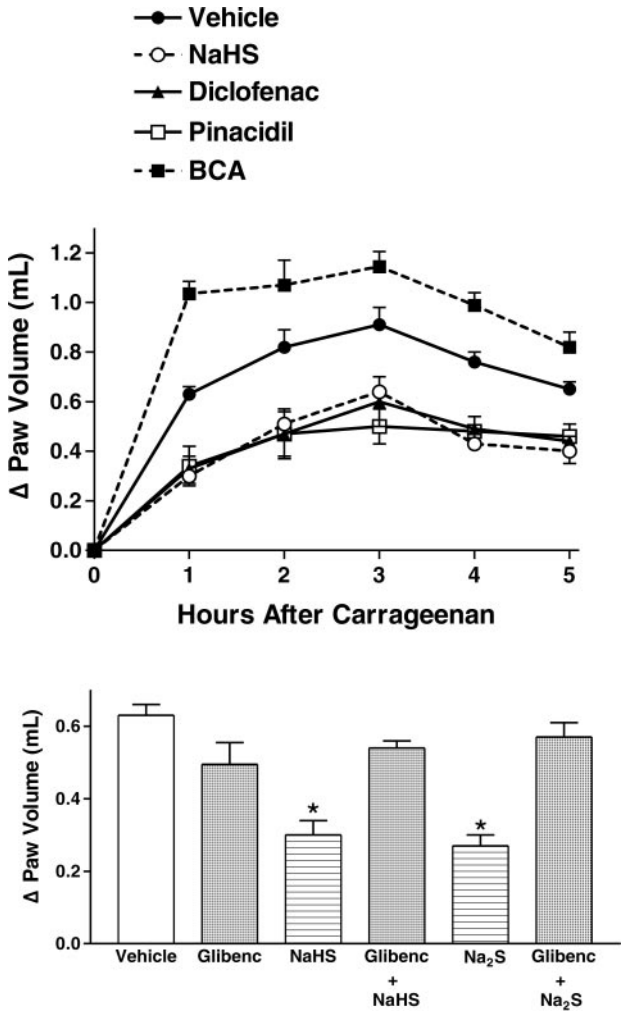

Figure 6. Hydrogen sulfide suppresses edema formation in the rat paw in a K_{ATP} channel-dependent manner. Top panel: injection of carrageenan into the rat hindpaw resulted in significant edema formation over the ensuing 5 h, as indicated by the increase in paw volume. Pretreatment with either of two H_2S donors (NaHS; 150 μmol/kg i.p.) significantly reduced paw edema at each time point ($P<0.05$), as did pretreatment with a conventional nonsteroidal anti-inflammatory drug (diclofenac, 10 mg/kg i.p.) and as did a K_{ATP} channel agonist (pinacidil; 10 mg/kg i.p.). In contrast, administration of an inhibitor of cystathionine-γ-lyase (β-cyanoalanine; BCA; 10 mg/kg i.p.) significantly increased the edema formation induced by carrageenan at all time points ($P<0.05$). Bottom panel: The increase in paw edema occurring during the first hour after carrageenan administration is shown. The reduction of paw edema by either of two hydrogen sulfide donors (NaHS and Na_2S, each at 150 μmol/kg) was abolished by pretreatment with a K_{ATP} channel antagonist, glibenclamide (10 mg/kg i.p. 30 min before carrageenan), whereas glibenclamide alone did not alter carrageenan-induced edema formation. Data are shown as mean ± SE, with at least 5 rats per group.

panel). The increase in paw volume could be significantly reduced by pretreatment with diclofenac (an NSAID). Pretreatment with NaHS or Na_2S similarly decreased carrageenan-induced paw edema (ED_{50}s of 35 and 28 μmol/kg, respectively), as did pinacidil, a K_{ATP} channel agonist. In contrast, suppression of endogenous H_2S synthesis, through administration of BCA, resulted in a significantly greater paw swelling response to carrageenan. The reduction paw edema by either of the H_2S donors (NaHS or Na_2S) could be reversed by pretreatment with glibenclamide (**Fig. 7**, lower panel).

DISCUSSION

Studies over the past 5 years have provided convincing evidence that H_2S is an important modulator of vascular tone and acts as a neuromodulator (1, 2). The results of the present study suggest that H_2S also plays important roles in the context of inflammation. H_2S is generated at sites of inflammation and can influence the ability of neutrophils to cause tissue injury (4); it was recently shown to reduce visceral pain perception (7). In the present study, we have demonstrated that several H_2S donors can suppress leukocyte adherence to the vascular endothelium and can reduce leukocyte infiltration and edema formation. These effects of H_2S were seen irrespective of the inflammatory stimulus used (carrageenan, aspirin, fMLP). Suppression of endogenous H_2S synthesis, through blockade of CSE, resulted in enhanced leukocyte adhesion, leukocyte infiltration, and edema formation. These actions appeared to be mediated via K_{ATP} channels, as they were reversed by pretreatment with glibenclamide and mimicked by pinacidil. Our findings therefore suggest an important role for endogenous H_2S as a modulator of some of the key components of acute inflammatory responses, particularly those occurring at the leukocyte-endothelial interface (Fig. 7).

As for other gaseous mediators (carbon monoxide, NO), H_2S was recognized for its toxicity long before its importance in physiological processes was described. H_2S is synthesized, primarily from L-cysteine, through actions of the enzymes CSE and CBS. In rats, blood and plasma levels of H_2S are in the 10–100 μM range (15). In the present study, we used three different H_2S donors at doses that would approximate concentrations of H_2S that fall within this physiological range. Differences in the potency of Lawesson's reagent vs. Na_2S and NaHS in suppressing leukocyte adherence/infiltration are consistent with observed differences in their ability to elicit H_2S-mediated vascular smooth muscle relaxation (unpublished observation). Moreover, the observation that suppression of endogenous H_2S synthesis with β-cyanoalanine led to increased leukocyte adherence and infiltration is consistent with a role for this mediator as a tonic inhibitor of leukocyte adherence/extravasation. Our observation that leukocyte rolling velocity decreased sharply after administration of the CSE inhibitor is consistent with previous observations that P-selectin expression can be regulated by H_2S (8). As leukocyte expression/affinity of LFA-1 has also been shown to be suppressed by H_2S (8), it is possible that the actions of H_2S with respect to leukocyte-endothelial adherence are exerted on both cell types (Fig. 7).

We recently reported that NSAIDs suppress H_2S synthesis by reducing expression of CSE (8). The accompanying reduction of H_2S synthesis may contribute to the increase in leukocyte adherence that is seen after NSAID administration (16, 17), which has been shown to contribute significantly to the gastric injury induced by this class of drugs (18–20). Indeed, coadministration of an H_2S donor with an NSAID resulted in inhibition of NSAID-induced leukocyte adherence and reduction of the severity of gastric damage (8).

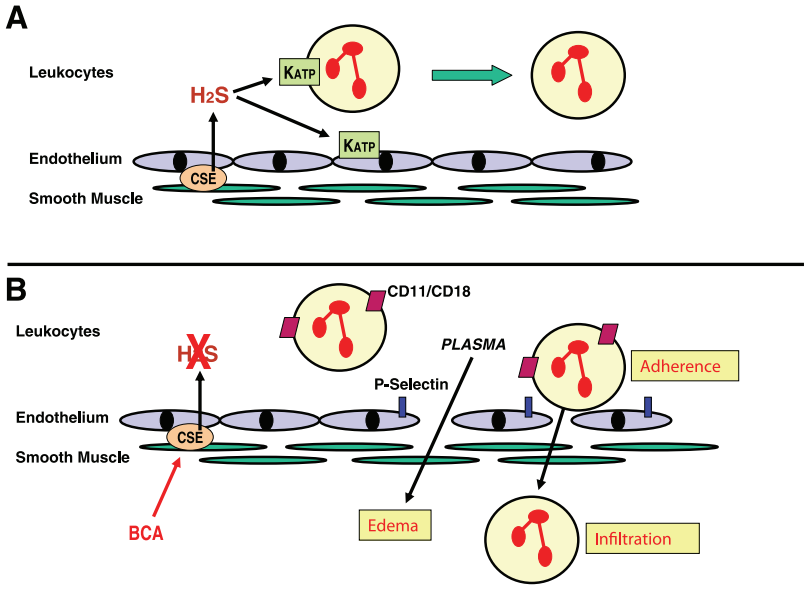

Figure 7. Hydrogen sulfide modulates inflammatory processes at the leukocyte-endothelial interface. *A)* Under normal conditions, H_2S is synthesized in blood vessels primarily via cystathionine-γ-lyase (CSE), which is expressed in endothelial cells and smooth muscle cells. H_2S tonically down-regulates leukocyte adherence via activation of ATP-activated potassium channels (K_{ATP}) on leukocytes and the endothelium. *B)* When endogenous H_2S synthesis is inhibited, such as with β-cyanoalanine (bicinchoninic acid), leukocyte rolling and adherence to the vascular endothelium increase, likely due in part to elevated expression of adhesion molecules on leukocytes (CD11/CD18) and endothelial cells (P-selectin). Marked increases in endothelial permeability, resulting in edema formation, also occur when H_2S synthesis is suppressed.

Pertinent to the present study, administration of an H_2S donor prevented many of the other "proinflammatory" effects of NSAIDs, including the elevation of ICAM-1 and LFA-1 expression and the increase in mucosal TNFα expression (8).

Of the four H_2S donors used in this study, only N-acetylcysteine requires metabolism in order for H_2S to be released. N-acetylcysteine is a precursor of L-cysteine (21), which is the substrate for H_2S generation via CSE and/or CBS. The observation that the anti-inflammatory actions of N-acetylcysteine were reversed by an inhibitor of CSE (β-cyanoalanine) is consistent with the effects being mediated by H_2S.

NO is another gaseous mediator that exerts many effects in common with H_2S in the cardiovascular and nervous systems. Moreover, there is evidence of cross-talk between H_2S and NO on many levels. For example, H_2S promotes the release of NO from vascular endothelium (22), whereas an NO donor was shown to increase the conversion of L-cysteine to H_2S, at least in part by increasing the expression of CSE, one of the key enzymes for H_2S synthesis (23) Hemoglobin (Hb) has been referred to as a common "sink" for H_2S, NO, and carbon monoxide. Thus, saturation of Hb binding to one of these gaseous mediators could lead to enhanced plasma levels and to biological effects from the others (1, 24). The extent to which NO may contribute to some of the observed actions of H_2S in the present study has not yet been examined.

As was the case for studies of NO for many years, evaluation of the contributions of H_2S to various processes is hampered by a paucity of precise pharmacological and genetic tools. Irreversible inhibitors of CSE and CBS have been reported to interfere with other enzymes (25, 26). As with any pharmacological agent, we cannot exclude the possibility that the reversible inhibitor of CSE, β-cyanoalanine, could exert nonspecific effects. For these reasons we chose to study four different H_2S donors in order to increase the veracity of our conclusions. Genetic deletion of CSE and CBS are lethal, ruling out the use of these "knockouts." CBS heterozygotes are viable, expressing half as much CBS as wild-type (WT). When fed a diet high in homocysteine, $CBS^{\pm}$ mice have been shown to exhibit increased leukocyte adherence, increased P-selectin expression, and increased vascular permeability (in the brain) (27), all consistent with a role for H_2S in mediating acute inflammation. However, use of these mice for direct studies of inflammation is of questionable value, as they have drastically altered vascular responsiveness to cholinergics and bradykinin (28).

Although our findings point to a role for H_2S as an endogenous modulator of inflammation, there are reports suggesting that this mediator may contribute to inflammatory processes. In addition to reports that irreversible inhibition of CSE can attenuate the severity of experimental pancreatitis (9) and endotoxemia (10), administration of DL-propargylglycine has been shown to dose-dependently reduce carrageenan-induced paw edema (29). The different outcomes of the latter study and the present one may be related to differences in selectivity of the inhibitors used or to the fact that one involved an irreversible inhibitor of CSE and the other a reversible inhibitor. With a very high level of suppression of H_2S synthesis, a significant decrease in blood flow would be anticipated, which would result in reduced edema formation. A similar scenario has been described with respect to another vasodilator, NO. Although NO exerts many anti-inflammatory effects (30), suppression of NO synthesis has been shown to reduce paw edema via reduced blood flow to the tissue (31).

A consistent finding in the various models used in the present study was that the anti-inflammatory effects of H_2S appeared to be mediated via activation of K_{ATP} channels. It is also the case that the analgesic effects of H_2S donors are mediated through these channels (7). It is possible, therefore, that K_{ATP} channels represent a novel target for anti-inflammatory and analgesic agents.

In summary, the results of the present study have demonstrated a role for endogenous H_2S as a modulator of key inflammatory events occurring at the interface of leukocytes and the vascular endothelium. H_2S functions as a tonic regulator of leukocyte adherence to the endothelium and of endothelial permeability. The anti-inflammatory effects of H_2S appear to be mediated via activation of K_{ATP} channels. These results, and recent reports that H_2S donors can reduce pain perception and down-regulate adhesion molecule and proinflammatory cytokine expression, therefore identify H_2S, the key enzymes responsible for H_2S synthesis, and K_{ATP} channels as potential targets for novel anti-inflammatory therapies. FJ

This work was supported by a grant from the Canadian Institutes of Health Research (CIHR). Dr. Wallace is an Alberta Heritage Foundation for Medical Research Senior Scientist and holds a Canada Research Chair in Inflammation Research. Dr. Zanardo is supported by a Fellowship from the Canadian Association of Gastroenterology, AstraZeneca Canada and the CIHR. The authors are grateful to Michael Dicay and Webb McKnight for their assistance in performing these studies.

REFERENCES

1. Wang, R. (2002) Two's company, three's a crowd: can H_2S be the third endogenous gaseous mediator? *FASEB J.* **16,** 1792–1798
2. Abe, K., and Kimura, H. (1996) The possible role of hydrogen sulfide as an endogenous neuromodulator. *J. Neurosci.* **16,** 1066–1071
3. Boehning, D., and Snyder, S. H. (2003) Novel neural modulators. *Annu. Rev. Neurosci.* **26,** 105–131
4. Whiteman, M., Armstrong, J. S., Chu, S. H., Siau, J. L., Wong, B. S., Cheung, N. S., Halliwell, B., and Moore, P. K. (2004) The novel neuromodulator hydrogen sulfide: an endogenous peroxynitrite 'scavenger'? *J. Neurochem.* **90,** 765–768
5. Whiteman, M., Cheung, N. S., Zhu, Y. Z., Chu, S. H., Siau, J. L., Wong, B. S., Armstrong, J. S., and Moore, P. K. (2005) Hydrogen sulphide: a novel inhibitor of hypochlorous acid-mediated oxidative damage in the brain? *Biochem. Biophys. Res. Commun.* **326,** 794–798

6. Mariggio, M.A., Minunno, V., Riccardi, S., Santacroce, R., De Rinaldis, P., and Fumarulo, R. (1998) Sulfide enhancement of PMN apoptosis. *Immunopharmacol. Immunotoxicol.* **20,** 399–408
7. Distrutti, E., Sediari, L., Mencarelli, A., Renga, B., Orlandi, S., Antonelli, E., Roviezzo, F., Morelli, A., Wallace, J. L., Cirino, G., and Fiorucci, S. (2005) Evidence that hydrogen sulfide exerts antinociceptive effects in the gastrointestinal tract by activating K_{ATP} channels. *J. Pharmacol. Exp. Ther.* **316,** 325–335
8. Fiorucci, S., Antonelli E., Distrutti, E., Rizzo, G., Mencarelli, A., Orlandi, S., Zanardo, R., Renga, B., Di Sante, M., and Morelli, A., *et al.* (2005) Inhibition of hydrogen sulfide generation contributes to gastric injury caused by anti-inflammatory nonsteroidal drugs. *Gastroenterology* **129,** 1210–1224
9. Bhatia, M., Wong, F. L., Fu, D., Lau, H. Y., Moochaala, S. M., and Moore, P. K. (2005) Role of hydrogen sulfide in acute pancreatitis and associated lung injury. *FASEB J.* **19,** 623–625
10. Li., L., Bhatia, M., Zhu, Y. Z., Zhu, Y. C., Ramnath, R. D., Wang, Z. J., Anuar, F. B. M., Whitman, M., Salto-Telles, M., and Moore, P. K. (2005) Hydrogen sulfide is a novel mediator of lipopolysaccharide-induced inflammation in the mouse. *FASEB J.* **19,** 1196–1198
11. Wallace, J. L., McKnight, W., Miyasaka, M., Tamatani, T., Paulson, J., Anderson, D. C., Granger, D. N., and Kubes, P. (1993) Role of endothelial adhesion molecules in NSAID-induced gastric mucosal injury. *Am. J. Physiol.* **265,** G993–G998
12. Mok, Y. Y., Atan, M. S., Yoke, P. C., Zhong J. W., Bhatia, M., Moochhala, S., and 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
13. Sedgwick, A. D., Moore, A. R., Al-Duaij, A. Y., Edwards, J. C., and Willoughby, D. A. (1985) The immune response to pertussis in the 6-day air pouch: a model of chronic synovitis. *Br. J. Exp. Pathol.* **66,** 455–464
14. Wallace, J. L., Chapman, K., and McKnight, W. (1999) Limited anti-inflammatory efficacy of cyclo-oxygenase-2 inhibition in carrageenan-airpouch inflammation. *Br. J. Pharmacol.* **126,** 1200–1204
15. Richardson, C. J., Magee, E. A., and 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
16. Asako, H., Kubes, P., Wallace, J. L., Wolf, R. E., and Granger, D. N. (1992) Modulation of leukocyte adhesion in rat mesenteric venules by aspirin and salicylate. *Gastroenterology* **103,** 146–152
17. Wallace, J. L., McKnight, W., Miyasaka, M., Tamatani, T., Paulson, J., Anderson, D.C., Granger, D. N., and Kubes, P. (1993) Role of endothelial adhesion molecules in NSAID-induced gastric mucosal injury. *Am. J. Physiol.* **265,** G993–G998
18. Wallace, J. L., Keenan, C. M., and Granger, D. N. (1990) Gastric ulceration induced by nonsteroidal anti-inflammatory drugs is a neutrophil-dependent process. *Am. J. Physiol.* **259,** G462–G467
19. Wallace, J. L., Arfors, K. E., and McKnight, G. W. (1991) A monoclonal antibody against the CD18 leukocyte adhesion molecule prevents indomethacin-induced gastric damage in the rabbit. *Gastroenterology* **100,** 878–883
20. Wallace, J. L. (1997) Nonsteroidal anti-inflammatory drugs and gastroenteropathy: the second hundred years. *Gastroenterology* **112,** 1000–1016
21. Fujii, K., Sakuragawa, T., Kashiba, M., Sugiura, Y., Kondo, M., Maruyama, K., Goda, N., Nimura, Y., and Suematsu, M. (2005) Hydrogen sulphide as an endogenous modulator of biliary bicarbonate excretion in the rat liver. *Antioxid. Redox Signal.* **7,** 788–794
22. Hosoki, R., Matsuki, N., and Kimura, H. (1997) The possible role of hydrogen sulfide as an endogenous smooth muscle relaxant in synergy with nitric oxide. *Biochem. Biophys. Res. Commun.* **237,** 527–531
23. Zhao, W., and Wang, R. (2002) H_2S-induced vasorelaxation and underlying cellular and molecular mechanisms. *Am. J. Physiol.* **283,** H474–H480
24. Searcy, D. G., and Lee, S. H. (1998) Sulfur reduction by human erythrocytes. *J. Exp. Zool.* **282,** 310–322
25. Moore, P. K., Bhatia, M., and Moochhala, S. (2003) Hydrogen sulfide: from the smell of the past to the mediator of the future? *Trends Pharmacol. Sci.* **12,** 609–611
26. Smith, S. B., Briggs, S., Triebwasser, K. C., and Freedland, R. A. (1977) Re-evaluation of amino-oxyacetate as an inhibitor. *Biochem. J.* **162,** 453–455
27. Kamath, A. F., Chauhan, A. K., Kisucka, J., Dole, V. S., Loscalzo, J., Handy, D. E., and Wagner, D. D. (2006) Elevated levels of homocysteine compromise blood-brain barrier integrity in mice. *Blood* **107,** 591–593
28. Weiss, N., Heydrick, S., Zhang, Y. Y., Bierl, C., Cap, A., and Loscalzo, J. (2002) Cellular redox state and endothelial dysfunction in mildly hyperhomocysteinemic cystathione beta-synthase-deficient mice. *Arterioscler. Thromb. Vasc. Biol.* **22,** 34–41
29. Bhatia, M., Sidhapuriwala, J., Moochhala, S. M., and Moore, P. K. (2005) Hydrogen sulfide is a mediator of carrageenan-induced hindpaw oedema in the rat. *Br. J. Pharmacol.* **145,** 141–144
30. Wallace, J. L., and Del Soldato, P. (2003) The therapeutic potential of NO-NSAIDs. *Fundam. Clin. Pharmacol.* **17,** 11–20
31. Antunes, E., Mariano, M., Cirino, G., Levi, S., and de Nucci, G. (1990) Pharmacological characterization of polycation-induced rat hind-paw oedema. *Br. J. Pharmacol.* **101,** 986–990

Received for publication April 7, 2006.
Accepted for publication May 8, 2006.

Hydrogen sulfide is an endogenous modulator of leukocyte mediated inflammation

Renata C. O. Zanardo,* Vincenzo Brancaleone,† Eleonora Distrutti,‡ Stefano Fiorucci,‡ Giuseppe Cirino,† and John L. Wallace*,[1]

*Inflammation Research Network, University of Calgary, Calgary, Alberta, T2N 4N1, Canada; †Department of Experimental Pharmacology, University of Naples, Naples, Italy; and ‡Division of Gastroenterology and Hepatology, University of Perugia, Perugia, Italy

To read the full text of this article, go to http://www.fasebj.org/cgi/doi/10.1096/fj.06-6270fje

SPECIFIC AIMS

Hydrogen sulfide (H_2S) is increasingly recognized as a physiologically important signaling molecule, possibly contributing to innate immunity. The aims of this study were to *1*) determine whether H_2S modulates leukocyte adherence to the endothelium; *2*) determine whether H_2S inhibits leukocyte infiltration; and *3*) examine the contribution of H_2S to edema formation.

PRINCIPAL FINDINGS

1. H_2S modulates leukocyte adherence to the vascular endothelium

The effects of H_2S donors on leukocyte adherence to the vascular endothelium were examined in the rat using intravital microscopy. Leukocyte adherence within mesenteric venules was observed in response to intragastric administration of aspirin (50 mg/kg) or superfusion with f-Met-Leu-Phe (fMLP; 10 μM). Each agent induced a time-dependent increase in leukocyte adherence during the 60 min observation period (**Fig. 1**). Both H_2S donors (Na_2S and NaHS, given orally) inhibited aspirin-induced leukocyte adherence (ED_{50} of 5.0 μmol/kg for Na_2S). H_2S donors (Na_2S and NaHS at 100 μmol/kg; Lawesson's reagent at 3 μmol/kg) also suppressed fMLP-induced leukocyte adherence. Inhibition of leukocyte adherence by the H_2S donors was reversed by pretreatment with glibenclamide, an ATP-activated potassium channel (K_{ATP}) antagonist.

We next investigated the possibility that endogenous H_2S could modulate leukocyte adherence in the absence of any inflammatory stimulus. H_2S is synthesized primarily via two enzymes: cystathionine-γ-lyase (CSE) and cystathionine-β-synthetase (CSB). Both enzymes are expressed in the rat mesentery. Oral administration of a reversible inhibitor of CSE, β-cyanoalanine, resulted in a time-dependent increase in leukocyte adherence to the vascular endothelium, reaching > 10-fold above basal levels after 60 min. This inhibitor also caused a marked decrease in leukocyte rolling velocity (to ~50% of basal levels).

2. H_2S reduces leukocyte infiltration via K_{ATP} channel activation

Administration of carrageenan into a rat air pouch resulted in infiltration of substantial numbers ($\sim 6\times10^7$) of leukocytes over the next 6 h, most of which (87.0±1.1%) were neutrophils. Pretreatment with H_2S donors (NaHS, N-acetylcysteine, Lawesson's reagent; i.p.) dose-dependently reduced the numbers of leukocytes infiltrating into the air pouch (ED_{50} of 42.7, 29.9, and 1.3 μmol/kg, respectively, defining the response to dexamethasone as maximal). With the highest dose of each H_2S donor tested, the reduction in leukocyte infiltration was comparable to that achieved by an NSAID (diclofenac), a NOS inhibitor (L-NAME), or a corticosteroid (dexamethasone).

N-Acetylcysteine is a precursor for L-cysteine, the substrate from which H_2S is synthesized via CSE and CBS. The ability of N-acetylcysteine to reduce carrageenan-induced leukocyte infiltration was dependent on CSE activity. Prior treatment with an inhibitor of CSE (β-cyanoalanine) reversed the inhibitory effects of N-acetylcysteine. Moreover, β-cyanoalanine increased basal leukocyte infiltration and that induced by carrageenan, suggesting that endogenous H_2S synthesis down-regulates leukocyte infiltration. The anti-inflammatory effects of N-acetylcysteine were also reversed by glibenclamide, a K_{ATP} channel antagonist.

3. H_2S modulates edema formation via K_{ATP} channel activation

Injection of carrageenan into the hind footpads of rats resulted in a rapid and marked increase in paw volume

[1] Correspondence: Department of Pharmacology and Therapeutics, University of Calgary, 3330 Hospital Dr. NW, Calgary, Alberta, T2N 4N1, Canada. E-mail: wallacej@ucalgary.ca

doi: 10.1096/fj.06-6270fje

0892-6638/06/0020-2118 © FASEB

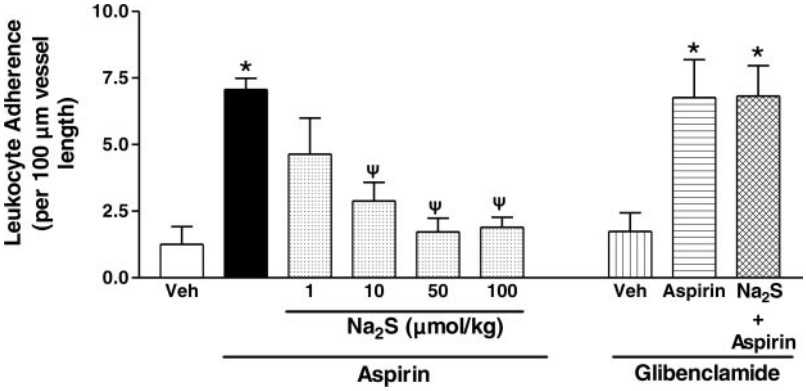

Figure 1. Hydrogen sulfide inhibits aspirin-induced leukocyte adherence in mesenteric venules via activation of K_{ATP} channels. Na_2S dose-dependently suppressed leukocyte adherence induced by intragastric aspirin (50 mg/kg). Inhibition of aspirin-induced adherence by Na_2S (100 μmol/kg) was abolished by pretreatment with glibenclamide (10 mg/kg), a K_{ATP} channel antagonist. $^*P < 0.05$ vs. corresponding vehicle-treated group. $^{\Psi}P < 0.05$ vs. corresponding group receiving aspirin alone. Each group consisted of at least 5 rats. Results are plotted as mean ±SE.

as a consequence of edema formation (**Fig. 2**, upper panel). The increase in paw volume could be significantly reduced by pretreatment with diclofenac, an NSAID. Pretreatment with NaHS or Na_2S similarly decreased carrageenan-induced paw edema (ED_{50} of 28 and 35 μmol/kg, respectively), as did pinacidil, a K_{ATP} channel agonist. In contrast, suppression of endogenous H_2S synthesis, through administration of β-cyanoalanine, resulted in a significantly greater paw swelling response to carrageenan. The reduction of carrageenan-induced paw edema by H_2S donors (NaHS or Na_2S) could be reversed by pretreatment with glibenclamide, a K_{ATP} channel antagonist (Fig. 2, lower panel).

CONCLUSIONS AND SIGNIFICANCE

H_2S is an important modulator of vascular tone and acts as a neuromodulator. The present study demonstrates that H_2S also plays important roles in inflammation (**Fig. 3**). Several H_2S donors were shown to suppress leukocyte adherence to the vascular endothelium and to reduce leukocyte infiltration and edema formation. The effects of H_2S were seen irrespective of the inflammatory stimulus used (carrageenan, aspirin, fMLP). Suppression of endogenous H_2S synthesis through blockade of CSE resulted in enhanced leukocyte adhesion, leukocyte infiltration, and edema formation. These actions appeared to be mediated via K_{ATP} channels, as they were reversed by glibenclamide and mimicked by pinacidil. Our observations therefore suggest an important role for endogenous H_2S as a modulator of several key components of acute inflammatory responses, particularly those occurring at the leukocyte-endothelial interface (Fig. 3).

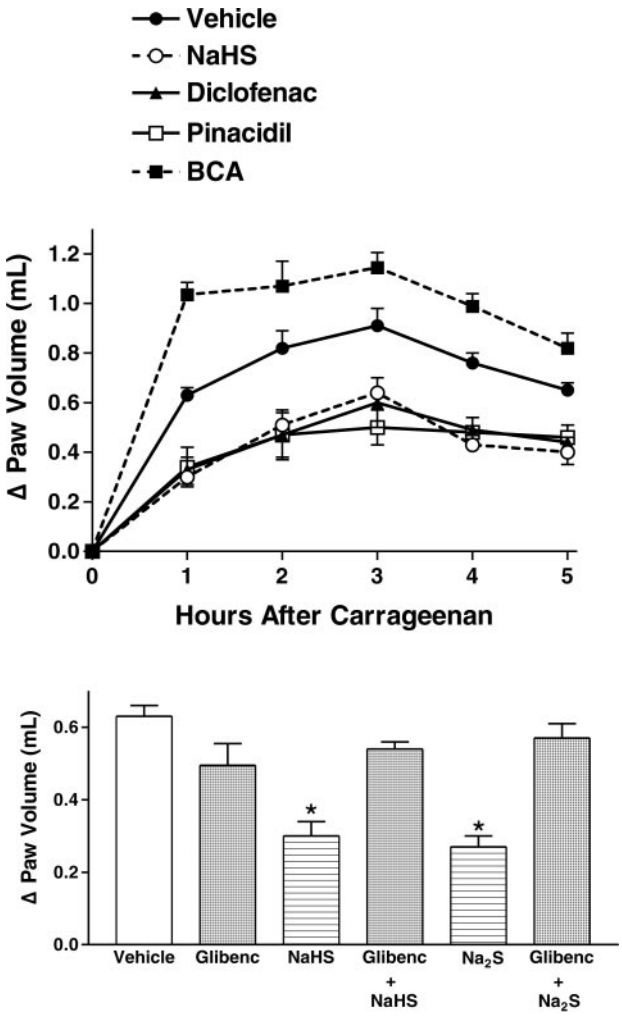

Figure 2. Hydrogen sulfide suppresses edema formation in the rat paw in a K_{ATP} channel-dependent manner. Top panel: injection of carrageenan into the rat hindpaw resulted in significant edema formation over the ensuing 5 h. Pretreatment with an H_2S donor (NaHS; 100 μmol/kg i.p.) significantly reduced paw edema at each time point ($P<0.05$), as did pretreatment with a conventional nonsteroidal anti-inflammatory drug (diclofenac, 10 mg/kg i.p.) and a K_{ATP} channel agonist (pinacidil; 10 mg/kg i.p.). In contrast, administration of an inhibitor of cystathionine γ-lyase (β-cyanoalanine; 10 mg/kg i.p.) significantly increased the edema formation induced by carrageenan at all time points ($P < 0.05$). Bottom: the increase in paw edema occurring during the first hour after carrageenan administration is shown. The reduction of paw edema by either of two hydrogen sulfide donors (NaHS and Na_2S, each at 100 μmol/kg) was abolished by pretreatment with glibenclamide, a K_{ATP} channel antagonist (10 mg/kg i.p. 30 min before carrageenan); glibenclamide alone did not alter carrageenan-induced edema formation. Data are shown as mean ± SE, with at least 5 rats per group.

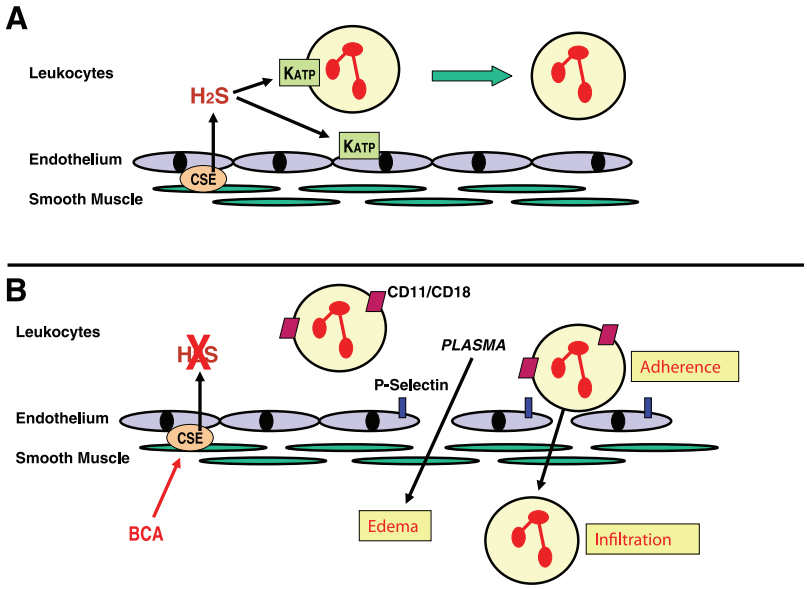

Figure 3. Hydrogen sulfide modulates inflammatory processes at the leukocyte-endothelial interface. *A)* H_2S is synthesized in blood vessels via cystathionine-γ-lyase (CSE), which is expressed in endothelial cells and smooth muscle cells. H_2S tonically down-regulates leukocyte adherence via activation of ATP-activated potassium channels (K_{ATP}) on leukocytes and endothelium. *B)* When endogenous H_2S synthesis is inhibited, such as with β-cyanoalanine, leukocyte rolling and adherence to the vascular endothelium increase, likely due partly to elevated expression of adhesion molecules on leukocytes (CD11/CD18) and endothelial cells (P-selectin). Marked increases in endothelial permeability that result in edema formation occur when H_2S synthesis is suppressed.

As for other gaseous mediators (carbon monoxide, NO), H_2S was recognized for its toxicity long before its importance in physiological processes was described. H_2S is synthesized, primarily from L-cysteine, through the actions of the enzymes CSE and CBS. In rats, blood and plasma levels of H_2S are in the 10–100 μM range. In the present study, we used three different H_2S donors at doses that would approximate concentrations of H_2S within the physiological range. The observation that suppression of endogenous H_2S synthesis with β-cyanoalanine led to increased leukocyte adherence and infiltration is consistent with a role for this mediator as a tonic inhibitor of leukocyte adherence/extravasation.

Of the four H_2S donors used in this study, only N-acetylcysteine requires metabolism for H_2S to be released. N-acetylcysteine is a precursor of L-cysteine, which is the substrate for H_2S generation via CSE and/or CBS. The observation that the anti-inflammatory actions of N-acetylcysteine were reversed by an inhibitor of CSE (β-cyanoalanine) is consistent with the effects being mediated by H_2S.

NO is another gaseous mediator that exerts many effects in common with H_2S in the cardiovascular and nervous systems. Moreover, there is evidence of cross-talk between H_2S and NO on many levels. For example, H_2S promotes the release of NO from vascular endothelium, whereas an NO donor was shown to increase the conversion of L-cysteine to H_2S at least in part by increasing the expression of CSE, a key enzyme for H_2S synthesis. Hemoglobin (Hb) has been referred to as a common "sink" for H_2S, NO, and carbon monoxide. Thus, saturation of Hb binding to one of these gaseous mediators could lead to enhanced plasma levels and to biological effects from the others.

While our findings point to a role for H_2S as an endogenous modulator of inflammation, there are reports suggesting that this mediator may contribute to inflammatory processes. In addition to reports that irreversible inhibition of CSE can attenuate the severity of experimental pancreatitis and endotoxemia, administration of DL-propargylglycine has been shown to dose-dependently reduce carrageenan-induced paw edema. It is possible that with a very high level of suppression of H_2S synthesis, a significant decrease in blood flow would be anticipated that would result in reduced edema formation.

In summary, the results of this study have demonstrated a role for endogenous H_2S as a modulator of key inflammatory events occurring at the interface of leukocytes and the vascular endothelium. The data suggest that H_2S is a tonic regulator of leukocyte adherence to the endothelium and of endothelial permeability. The anti-inflammatory effects of H_2S appear to be mediated through K_{ATP} channels. These results therefore identify H_2S, the key enzyme responsible for H_2S synthesis, and K_{ATP} channels as potential targets for novel anti-inflammatory therapies. FJ