

## Hydrogen sulfide is an endogenous modulator of leukocyte-mediated inflammation

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**ABSTRACT** Hydrogen sulfide (H<sub>2</sub>S) is increasingly recognized as an important signaling molecule in the cardiovascular and nervous systems. Recently, H<sub>2</sub>S 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<sub>2</sub>S is an endogenous regulator of key inflammatory events at the leukocyte-endothelial interface. Via intravital microscopy, we observed that H<sub>2</sub>S donors (NaHS and Na<sub>2</sub>S) inhibited aspirin-induced leukocyte adherence in mesenteric venules (ED<sub>50</sub> of 5.0 μmol/kg for Na<sub>2</sub>S), likely via activation of ATP-sensitive K<sup>+</sup> (K<sub>ATP</sub>) channels. Inhibition of endogenous H<sub>2</sub>S synthesis elicited leukocyte adherence. Leukocyte infiltration in an air pouch model was also suppressed by H<sub>2</sub>S donors (NaHS, Lawesson's reagent, and N-acetylcysteine; ED<sub>50</sub> of 42.7, 1.3, and 29.9 μmol/kg, respectively) and exacerbated by inhibition of endogenous H<sub>2</sub>S synthesis. Carrageenan-induced paw edema was suppressed by H<sub>2</sub>S donors (NaHS and Na<sub>2</sub>S; ED<sub>50</sub>s of 35 and 28 μmol/kg, respectively) to the same extent as by diclofenac and enhanced by an inhibitor of H<sub>2</sub>S synthesis. Suppression of edema formation by H<sub>2</sub>S donors was mimicked by a K<sub>ATP</sub> channel agonist and reversed by an antagonist of this channel. These results suggest that endogenous H<sub>2</sub>S 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<sub>2</sub>S 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<sub>2</sub>S), has become recognized as an important endogenous vasodilator and neuromodulator (1, 2). H<sub>2</sub>S 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<sub>2</sub>S synthesis, whereas in others only one of these enzymes is necessary (1). CSE appears to be the predominant enzymatic source of H<sub>2</sub>S in the vasculature and heart (1), but in the central nervous system (CNS) CBS predominates (1, 3). The ability of H<sub>2</sub>S to relax vascular smooth muscle most likely occurs through activation of ATP-sensitive K<sup>+</sup> (K<sub>ATP</sub>) channels (1).

Several recent reports provide evidence suggesting a role for H<sub>2</sub>S in inflammation. H<sub>2</sub>S can scavenge peroxynitrite (4) and can interfere with the ability of neutrophils, through hypochlorous acid, to kill microbes and other cells (5). H<sub>2</sub>S can also induce neutrophil apoptosis, thereby contributing to resolution of inflammatory reactions (6). We recently demonstrated that H<sub>2</sub>S can exert analgesic effects in a visceral pain model (7). Nonsteroidal anti-inflammatory drugs (NSAIDs) suppress endogenous H<sub>2</sub>S 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<sub>2</sub>S reduced the ability of these agents to cause gastric injury. Particularly relevant to a potential role in inflammation, the H<sub>2</sub>S 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<sub>2</sub>S donor.

Recent data also suggest that H<sub>2</sub>S may contribute to inflammatory processes. For example, significant increases in H<sub>2</sub>S 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

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performed a detailed study of the effects of a number of different H<sub>2</sub>S donors in several *in vivo* models of inflammation, using multiple distinct proinflammatory agents. We also examined the effects on several inflammatory parameters of inhibition of endogenous H<sub>2</sub>S synthesis and of blockade or activation of K<sub>ATP</sub> channels (the putative target of the vascular actions of H<sub>2</sub>S). In particular, we examined the role of H<sub>2</sub>S in modulating leukocyte adhesion to the vascular endothelium, leukocyte infiltration, and edema formation. Our studies implicate H<sub>2</sub>S 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<sub>2</sub>S on aspirin- and fMLP-induced leukocyte adherence, rats were pretreated intragastrically with Na<sub>2</sub>S (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<sub>2</sub>S 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<sub>2</sub>S 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<sub>2</sub>S 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<sub>2</sub>S donor to determine whether the effects of the donors were mediated via K<sub>ATP</sub> channels.

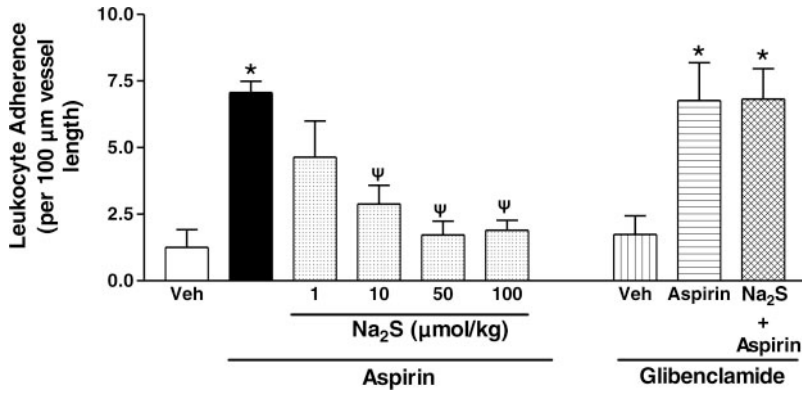
Several drugs were tested in the air pouch model as positive controls, as we had found them to significantly reduce carrageenan-induced leukocyte infiltration. These included an NSAID (diclofenac, 10 mg/kg i.p.), a NOS inhibitor (L-NAME; NG-nitro-L-arginine methyl ester; 25 mg/kg i.p.), and dexamethasone (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<sub>50</sub> values for each of the H<sub>2</sub>S 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<sub>2</sub>S donor (NaHS at 25–150 μmol/kg or Na<sub>2</sub>S at 100 μmol/kg), an NSAID as positive control (diclofenac, 10 mg/kg), or a K<sub>ATP</sub> 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<sub>2</sub>S 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  $\mu$ mol/kg) was abolished by pretreatment with glibenclamide (10 mg/kg), a  $K_{ATP}$  channel antagonist. \* $P < 0.05$  vs. the corresponding vehicle-treated group. † $P < 0.05$  vs. the corresponding group receiving aspirin alone. Each group consisted of at least 5 rats. The results are plotted as mean  $\pm$  SE.

ase chain reaction (RT-PCR), as described previously (8). Expression of  $\beta$ -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,  $\lambda$ -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  $\mu$ mol/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  $\mu$ mol/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).

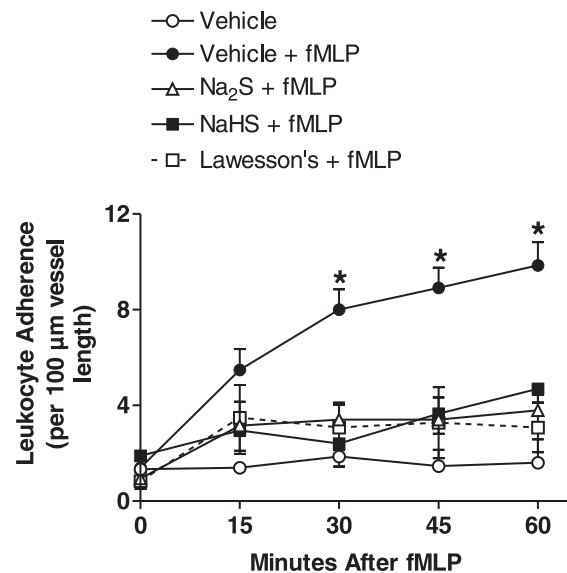
### $H_2S$ donors inhibit fMLP-induced leukocyte adherence

Pretreatment with  $Na_2S$  or  $NaHS$  (each at 100  $\mu$ mol/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  $\mu$ mol/kg. At a dose of 0.3

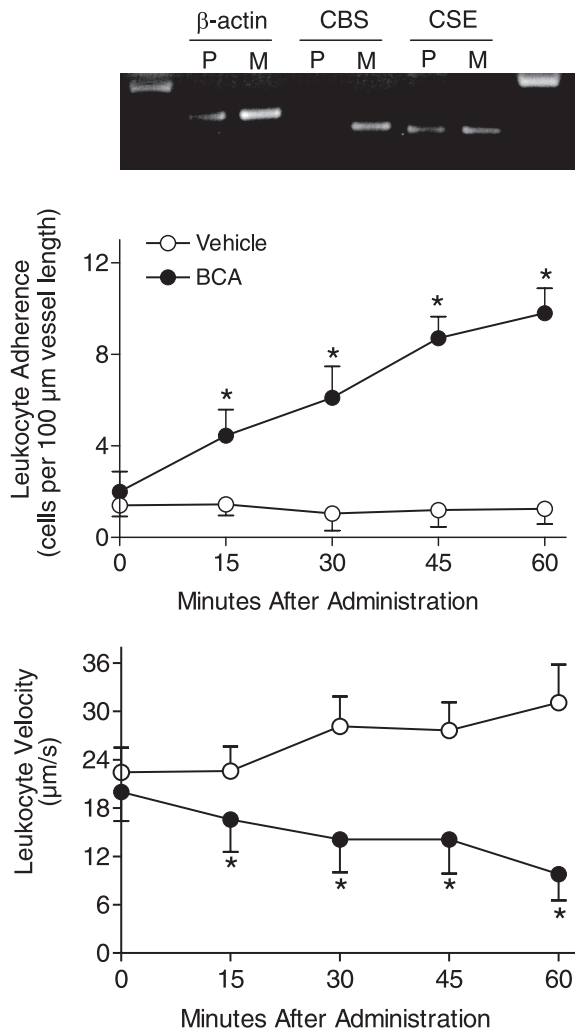
$\mu$ mol/kg, Lawesson's reagent did not affect fMLP-induced leukocyte adherence (data not shown).

### 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  $\mu$ M) 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  $\mu$ mol/kg, and Lawesson's reagent was given at 1  $\mu$ mol/kg. Each group consisted of at least 5 rats. The results are plotted as mean  $\pm$  SE.



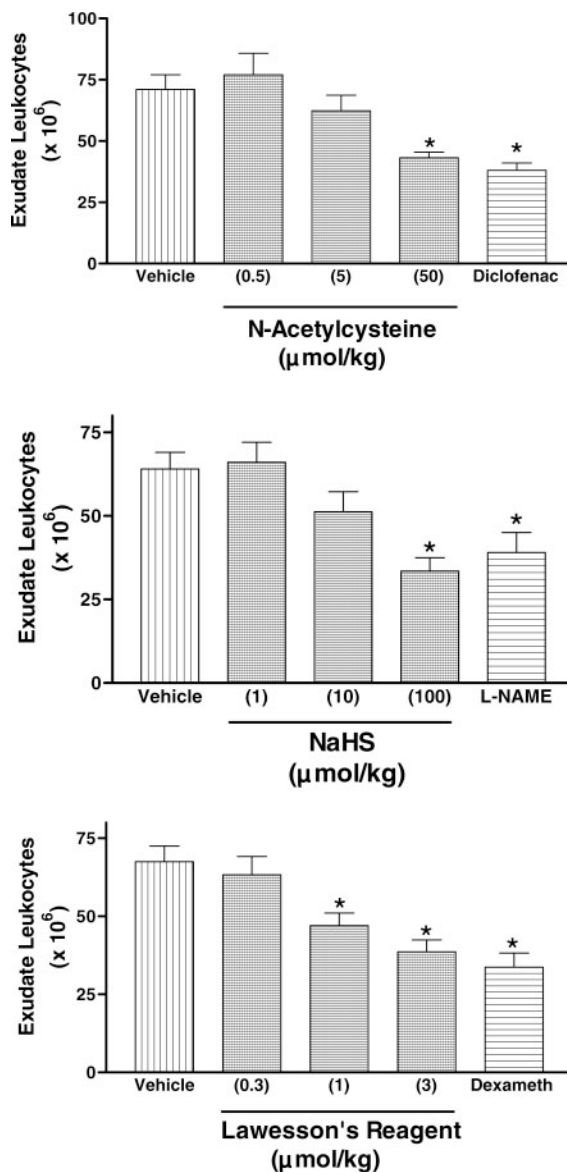
**Figure 3.** Inhibition of cystathionine- $\gamma$ -lyase (CSE) reduces leukocyte rolling velocity and increases leukocyte adherence in rat mesenteric venules. *Upper panel*) rat mesenteric venues express both CSE and cystathionine- $\beta$ -synthetase (CBS) mRNA. Expression of these enzymes in the portal vein is also shown. This gel is representative of gels for 3 healthy rats. Within 15 min of administration of  $\beta$ -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  $\pm$  SE.

### H<sub>2</sub>S donors inhibit leukocyte infiltration through the activation of K<sub>ATP</sub> channels

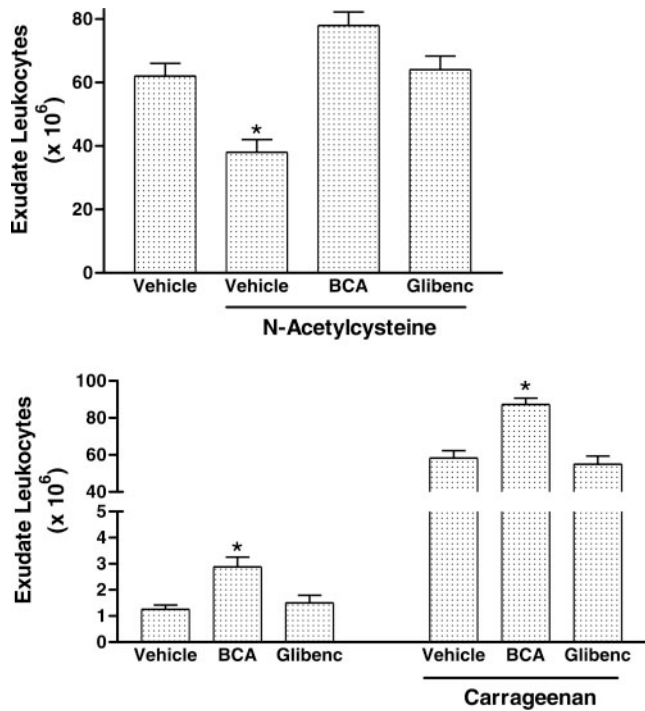
Administration of carrageenan into a rat air pouch results in infiltration of substantial numbers of neutrophils (Fig. 4). Most of the leukocytes were neutrophils (87.0  $\pm$  1.1%) and lymphocytes (12.3  $\pm$  0.8%). Pretreatment with H<sub>2</sub>S donors (NaHS, N-acetylcysteine, Lawesson's reagent) dose-dependently reduced the numbers of leukocytes infiltrating into the air pouch in response to carrageenan (ED<sub>50</sub> values of 42.7, 29.9, and 1.3

$\mu$ mol/kg, respectively, defining the response to dexamethasone as maximal). With the highest dose of each H<sub>2</sub>S 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<sub>2</sub>S 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  $\pm$  SE, with at least 5 rats per group.



**Figure 5.** Reduction of leukocyte infiltration by N-acetylcysteine is mediated via cystathionine- $\gamma$ -lyase-dependent hydrogen sulfide synthesis and  $K_{ATP}$  channels. Top panel: N-acetylcysteine (50  $\mu$ mol/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 ( $\beta$ -cyanoalanine; 50 mg/kg i.p.), an inhibitor of cystathionine- $\gamma$ -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  $\pm$  SE, with at least 5 rats per group.

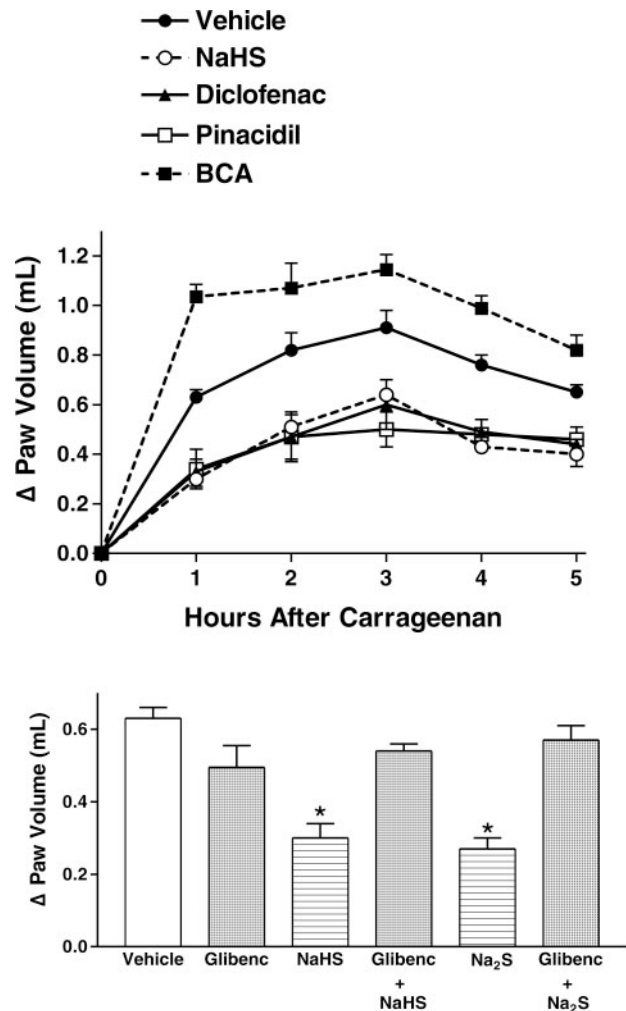
treatment with BCA, an inhibitor of CSE, reversed the effects of N-acetylcysteine. Moreover, the reduction of leukocyte infiltration by N-acetylcysteine could be reversed by glibenclamide, a  $K_{ATP}$  channel antagonist.

### 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  $\mu$ mol/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- $\gamma$ -lyase ( $\beta$ -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  $\mu$ mol/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  $\pm$  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<sub>2</sub>S similarly decreased carrageenan-induced paw edema (ED<sub>50</sub>s of 35 and 28 μmol/kg, respectively), as did pinacidil, a K<sub>ATP</sub> channel agonist. In contrast, suppression of endogenous H<sub>2</sub>S synthesis, through administration of BCA, resulted in a significantly greater paw swelling response to carrageenan. The reduction paw edema by either of the H<sub>2</sub>S donors (NaHS or Na<sub>2</sub>S) could be reversed by pretreatment with glibenclamide (Fig. 7, lower panel).

## DISCUSSION

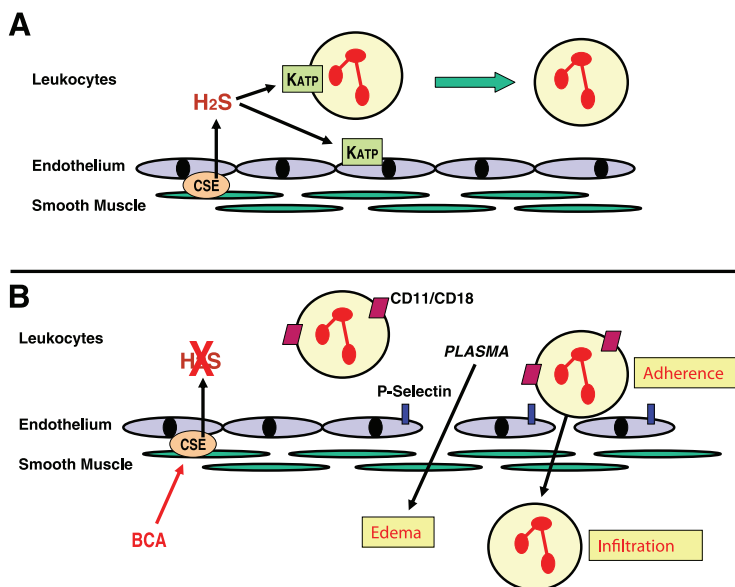
Studies over the past 5 years have provided convincing evidence that H<sub>2</sub>S is an important modulator of vascular tone and acts as a neuromodulator (1, 2). The results of the present study suggest that H<sub>2</sub>S also plays important roles in the context of inflammation. H<sub>2</sub>S is 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<sub>2</sub>S donors can suppress leukocyte adherence to the vascular endothelium and can reduce leukocyte infiltration and edema formation. These effects of H<sub>2</sub>S were seen irrespective of the inflammatory stimulus used (carrageenan, aspirin, fMLP). Suppression of endogenous H<sub>2</sub>S synthesis, through blockade of CSE, resulted in enhanced leukocyte adhesion, leukocyte infiltration, and edema formation. These actions appeared to be mediated via K<sub>ATP</sub> channels, as they were reversed by pretreatment with glibenclamide and mimicked by pinacidil. Our findings therefore suggest an important role for endogenous H<sub>2</sub>S 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<sub>2</sub>S was recognized for its toxicity long before its importance in physiological processes was described. H<sub>2</sub>S is synthesized, primarily from L-cysteine, through actions of the enzymes CSE and CBS. In rats, blood and plasma levels of H<sub>2</sub>S are in the 10–100 μM range (15). In the present study, we used three different H<sub>2</sub>S donors at doses that would approximate concentrations of H<sub>2</sub>S that fall within this physiological range. Differences in the potency of Lawesson's reagent vs. Na<sub>2</sub>S and NaHS in suppressing leukocyte adherence/infiltration are consistent with observed differences in their ability to elicit H<sub>2</sub>S-mediated vascular smooth muscle relaxation (unpublished observation). Moreover, the observation that suppression of endogenous H<sub>2</sub>S 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<sub>2</sub>S (8). As leukocyte expression/affinity of LFA-1 has also been shown to be suppressed by H<sub>2</sub>S (8), it is possible that the actions of H<sub>2</sub>S with respect to leukocyte-endothelial adherence are exerted on both cell types (Fig. 7).

We recently reported that NSAIDs suppress H<sub>2</sub>S synthesis by reducing expression of CSE (8). The accompanying reduction of H<sub>2</sub>S 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<sub>2</sub>S 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<sub>2</sub>S is synthesized in blood vessels primarily via cystathionine-γ-lyase (CSE), which is expressed in endothelial cells and smooth muscle cells. H<sub>2</sub>S tonically down-regulates leukocyte adherence via activation of ATP-activated potassium channels (K<sub>ATP</sub>) on leukocytes and the endothelium. *B*) When endogenous H<sub>2</sub>S 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<sub>2</sub>S synthesis is suppressed.



Pertinent to the present study, administration of an H<sub>2</sub>S 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 TNF $\alpha$  expression (8).

Of the four H<sub>2</sub>S donors used in this study, only N-acetylcysteine requires metabolism in order for H<sub>2</sub>S to be released. N-acetylcysteine is a precursor of L-cysteine (21), which is the substrate for H<sub>2</sub>S generation via CSE and/or CBS. The observation that the anti-inflammatory actions of N-acetylcysteine were reversed by an inhibitor of CSE ( $\beta$ -cyanoalanine) is consistent with the effects being mediated by H<sub>2</sub>S.

NO is another gaseous mediator that exerts many effects in common with H<sub>2</sub>S in the cardiovascular and nervous systems. Moreover, there is evidence of cross-talk between H<sub>2</sub>S and NO on many levels. For example, H<sub>2</sub>S promotes the release of NO from vascular endothelium (22), whereas an NO donor was shown to increase the conversion of L-cysteine to H<sub>2</sub>S, at least in part by increasing the expression of CSE, one of the key enzymes for H<sub>2</sub>S synthesis (23). Hemoglobin (Hb) has been referred to as a common “sink” for H<sub>2</sub>S, 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<sub>2</sub>S 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<sub>2</sub>S 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,  $\beta$ -cyanoalanine, could exert nonspecific effects. For these reasons we chose to study four different H<sub>2</sub>S 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<sup>±</sup> 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<sub>2</sub>S 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<sub>2</sub>S 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<sub>2</sub>S 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<sub>2</sub>S appeared to be mediated via activation of K<sub>ATP</sub> channels. It is also the case that the analgesic effects of H<sub>2</sub>S donors are mediated through these channels (7). It is possible, therefore, that K<sub>ATP</sub> 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<sub>2</sub>S as a modulator of key inflammatory events occurring at the interface of leukocytes and the vascular endothelium. H<sub>2</sub>S functions as a tonic regulator of leukocyte adherence to the endothelium and of endothelial permeability. The anti-inflammatory effects of H<sub>2</sub>S appear to be mediated via activation of K<sub>ATP</sub> channels. These results, and recent reports that H<sub>2</sub>S donors can reduce pain perception and down-regulate adhesion molecule and proinflammatory cytokine expression, therefore identify H<sub>2</sub>S, the key enzymes responsible for H<sub>2</sub>S synthesis, and K<sub>ATP</sub> channels as potential targets for novel anti-inflammatory therapies. **FJ**

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# Hydrogen sulfide is an endogenous modulator of leukocyte mediated inflammation

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## SPECIFIC AIMS

Hydrogen sulfide (H<sub>2</sub>S) 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<sub>2</sub>S modulates leukocyte adherence to the endothelium; 2) determine whether H<sub>2</sub>S inhibits leukocyte infiltration; and 3) examine the contribution of H<sub>2</sub>S to edema formation.

## PRINCIPAL FINDINGS

### 1. H<sub>2</sub>S modulates leukocyte adherence to the vascular endothelium

The effects of H<sub>2</sub>S 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<sub>2</sub>S donors (Na<sub>2</sub>S and NaHS, given orally) inhibited aspirin-induced leukocyte adherence (ED<sub>50</sub> of 5.0 μmol/kg for Na<sub>2</sub>S). H<sub>2</sub>S donors (Na<sub>2</sub>S 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<sub>2</sub>S donors was reversed by pretreatment with glibenclamide, an ATP-activated potassium channel (K<sub>ATP</sub>) antagonist.

We next investigated the possibility that endogenous H<sub>2</sub>S could modulate leukocyte adherence in the absence of any inflammatory stimulus. H<sub>2</sub>S 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<sub>2</sub>S reduces leukocyte infiltration via K<sub>ATP</sub> channel activation

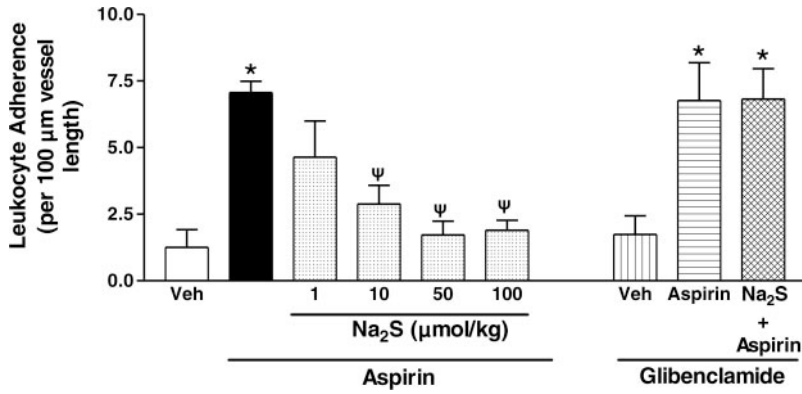
Administration of carrageenan into a rat air pouch resulted in infiltration of substantial numbers (~6×10<sup>7</sup>) of leukocytes over the next 6 h, most of which (87.0±1.1%) were neutrophils. Pretreatment with H<sub>2</sub>S donors (NaHS, N-acetylcysteine, Lawesson's reagent; i.p.) dose-dependently reduced the numbers of leukocytes infiltrating into the air pouch (ED<sub>50</sub> 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<sub>2</sub>S 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<sub>2</sub>S 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<sub>2</sub>S synthesis down-regulates leukocyte infiltration. The anti-inflammatory effects of N-acetylcysteine were also reversed by glibenclamide, a K<sub>ATP</sub> channel antagonist.

### 3. H<sub>2</sub>S modulates edema formation via K<sub>ATP</sub> channel activation

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

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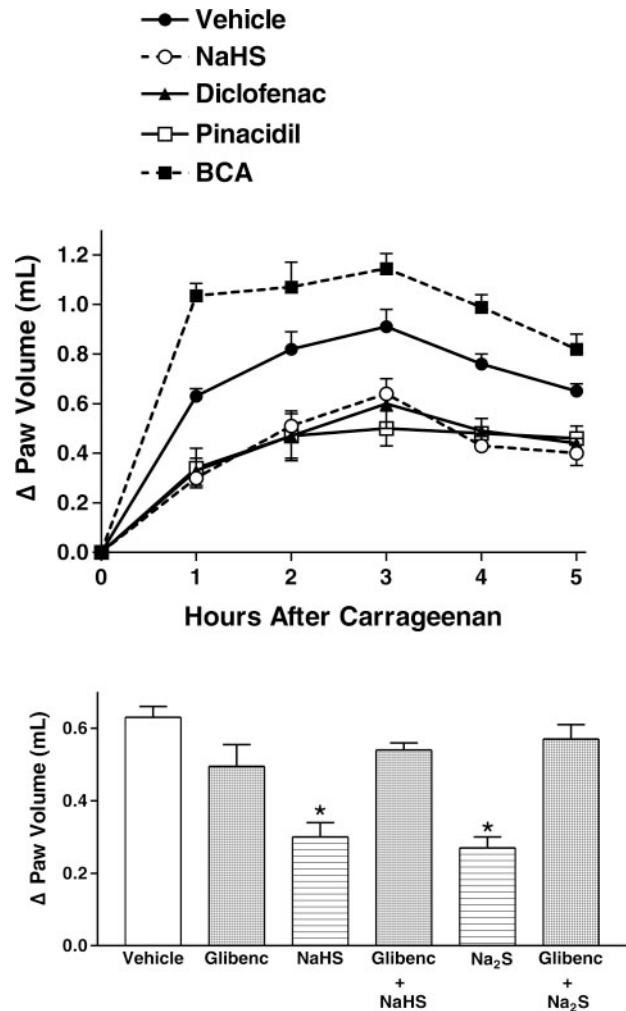
**Figure 1.** Hydrogen sulfide inhibits aspirin-induced leukocyte adherence in mesenteric venules via activation of  $K_{ATP}$  channels.  $Na_2S$  dose-dependently suppressed leukocyte adherence induced by intragastric aspirin (50 mg/kg). Inhibition of aspirin-induced adherence by  $Na_2S$  (100  $\mu$ mol/kg) was abolished by pretreatment with glibenclamide (10 mg/kg), a  $K_{ATP}$  channel antagonist. \* $P < 0.05$  vs. corresponding vehicle-treated group. † $P < 0.05$  vs. corresponding group receiving aspirin alone. Each group consisted of at least 5 rats. Results are plotted as mean  $\pm$  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  $\mu$ mol/kg, respectively), as did pinacidil, a  $K_{ATP}$  channel agonist. In contrast, suppression of endogenous  $H_2S$  synthesis, through administration of  $\beta$ -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

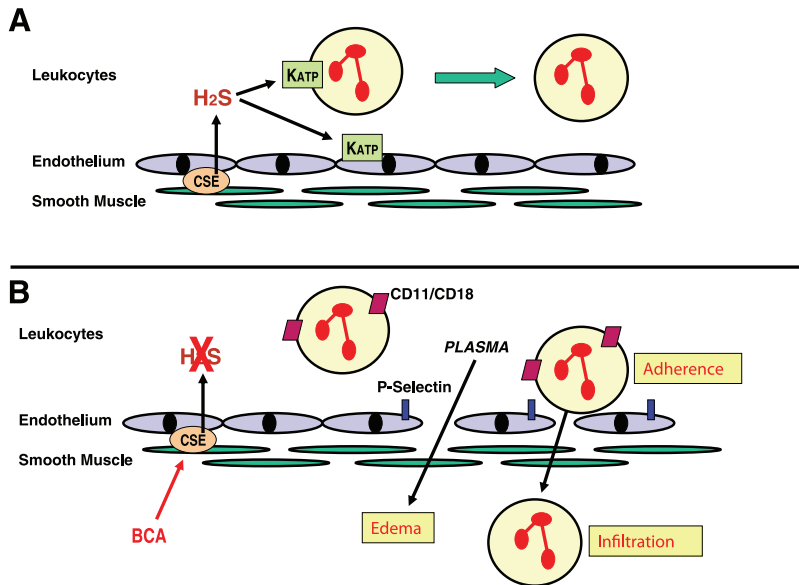
$H_2S$  is an important modulator of vascular tone and acts as a neuromodulator. The present study demonstrates that  $H_2S$  also plays important roles in inflammation (Fig. 3). Several  $H_2S$  donors were shown to suppress leukocyte adherence to the vascular endothelium and to reduce leukocyte infiltration 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  $\mu$ mol/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  $\gamma$ -lyase ( $\beta$ -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  $\mu$ mol/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  $\pm$  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- $\gamma$ -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  $\beta$ -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  $\mu$ M 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  $\beta$ -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 ( $\beta$ -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]