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Hydrogen Sulfide Augments Neutrophil Migration through Enhancement of Adhesion Molecule Expression and Prevention of CXCR2 Internalization: Role of ATP-Sensitive Potassium Channels[1]

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In this study, we have addressed the role of H_2S in modulating neutrophil migration in either innate (LPS-challenged naive mice) or adaptive (methylated BSA (mBSA)-challenged immunized mice) immune responses. Treatment of mice with H_2S synthesis inhibitors, DL-propargylglycine (PAG) or β-cyanoalanine, reduced neutrophil migration induced by LPS or methylated BSA (mBSA) into the peritoneal cavity and by mBSA into the femur/tibial joint of immunized mice. This effect was associated with decreased leukocyte rolling, adhesion, and P-selectin and ICAM-1 expression on endothelium. Predictably, treatment of animals with the H_2S donors, NaHS or Lawesson's reagent, enhanced these parameters. Moreover, the NaHS enhancement of neutrophil migration was not observed in ICAM-1-deficient mice. Neither PAG nor NaHS treatment changed LPS-induced CD18 expression on neutrophils, nor did the LPS- and mBSA-induced release of neutrophil chemoattractant mediators TNF-α, keratinocyte-derived chemokine, and LTB_4. Furthermore, in vitro MIP-2-induced neutrophil chemotaxis was inhibited by PAG and enhanced by NaHS treatments. Accordingly, MIP-2-induced CXCR2 internalization was enhanced by PAG and inhibited by NaHS treatments. Moreover, NaHS prevented MIP-2-induced CXCR2 desensitization. The PAG and NaHS effects correlated, respectively, with the enhancement and inhibition of MIP-2-induced G protein-coupled receptor kinase 2 expression. The effects of NaHS on neutrophil migration both in vivo and in vitro, together with CXCR2 internalization and G protein-coupled receptor kinase 2 expression were prevented by the ATP-sensitive potassium (K^+_{ATP}) channel blocker, glybenclamide. Conversely, diazoxide, a K^+_{ATP} channel opener, increased neutrophil migration in vivo. Together, our data suggest that during the inflammatory response, H_2S augments neutrophil adhesion and locomotion, by a mechanism dependent on K^+_{ATP} channels. ***The Journal of Immunology,*** **2008, 181: 4287–4298.**

Neutrophil migration during an inflammatory response results mainly from the release of inflammatory mediators by resident cells, which induce the rolling and adhesion of neutrophils on endothelial cells, followed by their transmigration to the extravascular space (1–3). These events require interaction of reciprocal adhesion molecules present on neutrophils and endothelial cells. Rolling is mediated by E- and P-selectins (on endothelial cells) and L-selectin (on leukocytes) interacting with their respective carbohydrate ligand. Thereafter, adhesion and transmigration are mediated by the leukocyte β_2 integrins CD11a/CD18, CD11b/CD18, and CD11c/CD18, which interact with the immunoglobulins ICAM-1, ICAM-2, and ICAM-3, whereas the integrins VLA-4 and $\alpha_V\beta_3$, respectively, interact with VCAM-1 and PECAM-1, present mostly on endothelial cells (4). Several neutrophil chemoattractant factors such as platelet-activating factor, TNF-α, C5a, leukotriene B_4 (LTB_4),[3] and a variety of CXC chemokines have been described in the literature (5–12). Neutrophils express two major subtypes of CXC chemokine receptors, CXCR1 and CXCR2 (13, 14). Degranulation and calcium flux in response to CXCL8 are mediated by both receptors, whereas the respiratory burst and chemotaxis are mediated mainly by CXCR1 (15) and CXCR2 (16), respectively.

Recently, a gaseous mediator, H_2S, has been recognized as an important endogenous vasodilator and neuromodulator (17, 18). Moreover, its vasodilator effect seems to be mediated through opening of ATP-sensitive potassium (K^+_{ATP}) channels (17). H_2S is

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[3] Abbreviations used in this paper: LTB_4, leukotriene B_4; PAG, DL-propargylglycine; BCA, β-cyanoalanine; GRK, G protein-coupled receptor kinase; CSE, cystathionine-γ-lyase; WT, wild type; KC, keratinocyte-derived chemokine; mBSA, methylated BSA; AU, arbitrary units; FI, false immunized; i.a., intra-articular; NSAID, anti-inflammatory drug.

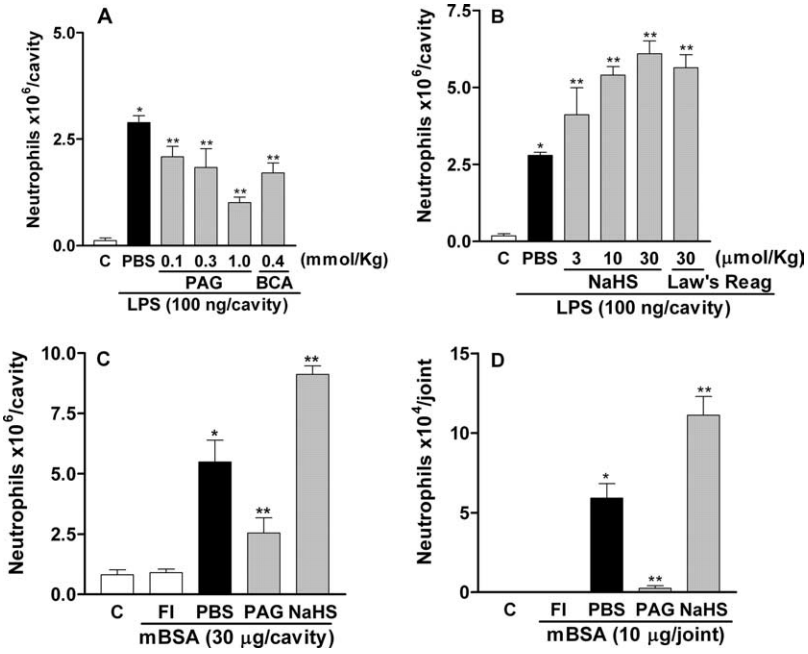

FIGURE 1. The CSE-H_2S pathway increased LPS or Ag (mBSA)-induced neutrophil migration to the peritoneal or articular cavities, respectively. *A* and *B*, Mice were pretreated (s.c.) 30 min before i.p. injection of LPS (100 ng/cavity) with PAG (0, 0.1, 0.3, and 1.0 mmol/kg; *A*) or BCA (0 and 0.4 mmol/kg; *A*) and NaHS (0, 3.0, 10, and 30 μmol/kg; *B*) or Lawesson's reagent (Reag; 0 and 30 μmol/kg; *B*). Neutrophil migration was determined 6 h after stimulus. *C* and *D*, Immunized mice were pretreated 30 min before challenge with mBSA (30 μg/cavity or 10 μg/joint) s.c. with PBS (0.2 ml), PAG (1.0 mmol/kg), and NaHS (30 μmol/kg). FI mice were treated with mBSA only. The neutrophil migration was determined 6 and 24 h later in the peritoneal and articular cavities, respectively. The first bar in all panels represents the neutrophil migration induced by PBS injected i.p. (control group; C). The values are mean $\pm$ SEM ($n = 5$) and are representative of three independent experiments. Data used for statistical analyses were from individual experiments. *, $p < 0.05$ compared with the control or FI groups; **, $p < 0.05$ compared with naive or immunized mice PBS-treated and LPS-injected or mBSA challenge, respectively (ANOVA followed by Bonferroni's *t* test).

synthesized from L-cysteine via two enzymes: cystathionine-γ-lyase (CSE) and cystathionine-β-synthetase. The literature concerning the role of the CSE-H_2S pathway in the inflammatory process is contradictory. Significant increases in H_2S production and augmentation of CSE expression were observed in rodent models of acute pancreatitis (19) and endotoxemia (20). Furthermore, the irreversible inhibition of CSE activity with DL-propargylglycine (PAG) reduced the severity of pancreatitis and endotoxic shock induced by LPS or cecal ligation and puncture, whereas sodium hydrosulfide (H_2S donor drug) increased the severity of these illnesses (19–22). By contrast, recent data have demonstrated that different H_2S donors inhibited leukocyte infiltration induced by carrageenan in the air pouch model and leukocyte adhesion in aspirin-induced gastric lesions. Predictably, β-cyanoalanine (BCA), a reversible CSE inhibitor, enhanced these parameters (23).

Given these apparent contradictory observations concerning the effects of H_2S on neutrophil migration mechanisms, we have investigated the role of endogenous and exogenous H_2S in modulating neutrophil migration in either innate or adaptive immune response. The mechanisms involved in H_2S modulation of neutrophil migration were also investigated.

Materials and Methods

Animals

Male BALB/c or C57BL/6 (wild type; WT) and ICAM-1-deficient (ICAM-$1^{-/-}$) mice weighing 20–25 g were housed in cages in a temperature- and light-controlled room and fed ad libitum. Mice with targeted disruption of the ICAM-1 gene were obtained from The Jackson Laboratory. All experiments were conducted in accordance with the ethical guidelines of the School of Medicine of Ribeirao Preto, University of São Paulo (São Paulo, Brazil).

Drugs and reagents

Escherichia coli LPS, methylated BSA (mBSA), PAG, BCA, Lawesson's reagent, glybenclamide, diazoxide, Percoll, RPMI 1640 (hereafter called RPMI), and HBSS balanced mediums were purchased from Sigma-Aldrich. NaHS was synthesized by Prof. Dr. Alberto Federman Neto. LPS, PAG, BCA, NaHS, Lawesson's reagent, and mBSA were dissolved in PBS, glybenclamide and diazoxide in PBS plus DMSO (5%).

Induction of experimental peritonitis in naive and immunized mice and arthritis in immunized mice

BALB/c mice were sensitized through s.c. injection of an emulsion containing 500 μg of mBSA, and 0.1 ml of CFA. Booster injections of mBSA dissolved in IFA were administered to animals 7 and 14 days after the first immunization. False-immunized (FI) mice were given injections without the Ag (mBSA). Twenty-one days after the initial injection, peritonitis or arthritis was induced by i.p. injections of mBSA (30 μg/cavity) or intra-articular (i.a.; 10 μg/joint), respectively. The mBSA was injected into mice pretreated with PBS (0.2 ml), PAG (1.0 mmol/kg), or NaHS (30 μmol/kg). FI mice were challenged with mBSA, and immunized mice were challenged with PBS (control groups). Mice were euthanized in a CO_2 chamber 6 h after i.p. or 24 h after i.a. treatments.

Naive mice were treated s.c. with PBS (0.2 ml), PAG (0.1, 0.3, or 1.0 mmol/kg), BCA (0.4 mmol/kg), NaHS (3.0, 10, or 30 μmol/kg), Lawesson's reagent (30 μmol/kg), glybenclamide (40 μmol/kg), or diazoxide (300 μmol/kg); 30 min later, they were injected i.p. with LPS (100 ng/cavity) or PBS (control group). At indicated times; the animals were killed to determine cytokine production; neutrophil rolling, adhesion, and migration; and adhesion molecule expression on mesentery venular endothelium.

In vivo neutrophil migration

Peritoneal cells were obtained by washing the peritoneal cavity with 3 ml of PBS containing 1 mM EDTA 6 h after LPS or mBSA injection. The articular joint cells were harvested 24 h after mBSA injection by two washings with 5 μl PBS/EDTA and then diluted into 90 μl of PBS-EDTA. Total cell counts were performed in a cell counter (Coulter A^CT; Coulter Corp.) and differential cell counts (100 cells in total) were measured after cytocentrifugation (Cytospin 3; Shandon Lipshaw) onto slides stained by the May-Grünwald-Giemsa method. The results are presented as the number of neutrophils per cavity or per joint.

Detection of cytokines and LTB_4 by ELISA

TNF-α and keratinocyte-derived chemokine (KC or CXCL1) levels were detected in mouse peritoneal exudates by ELISA, 1 h after treatment or in joint tissue homogenates 6 h after treatment (24). The concentrations of LTB_4 in mouse peritoneal exudates or joint tissue homogenates were detected by the LTB_4 enzyme immunoassay kit (Cayman Chemical), according to the manufacturer's instructions. The results were expressed as picograms per milliliter.

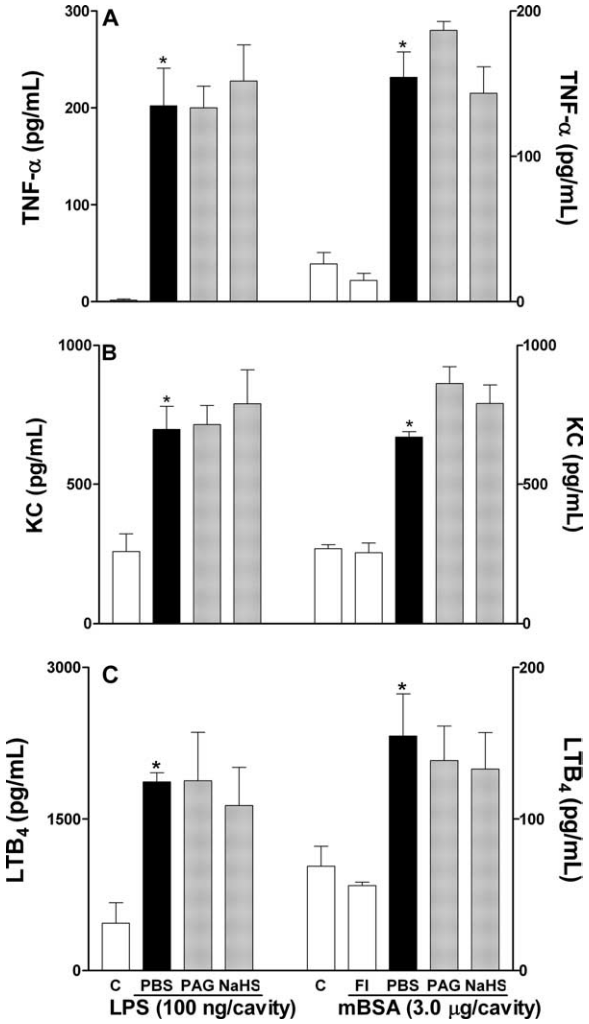

FIGURE 2. Modulation of the CSE-H_2S pathway did not affect TNF-α, KC, and LTB_4 concentrations in peritoneal exudate and joint tissue during immune response. Animals were pretreated (s.c.) with PBS (0.2 ml), PAG (1.0 mmol/kg), or NaHS (30 μmol/kg) 30 min before i.p injection with LPS (100 ng/cavity), in naive mice (*left*) or i.a. with mBSA (10 μg/joint) in immunized or FI mice (*right*). The concentrations of TNF-α (*A*), KC (*B*), and LTB_4 (*C*) were determined 1 h after LPS administration or 6 h after mBSA challenge, see *Materials and Methods*. The bars named C in all panels represent the concentration of the mediators induced by PBS injection (control groups). All values are mean ± SEM ($n = 5$) and are representative of three independent experiments. The data used for statistical analyses were from individual experiments. *, $p < 0.05$ compared with control or FI groups (ANOVA followed by Bonferroni's *t* test).

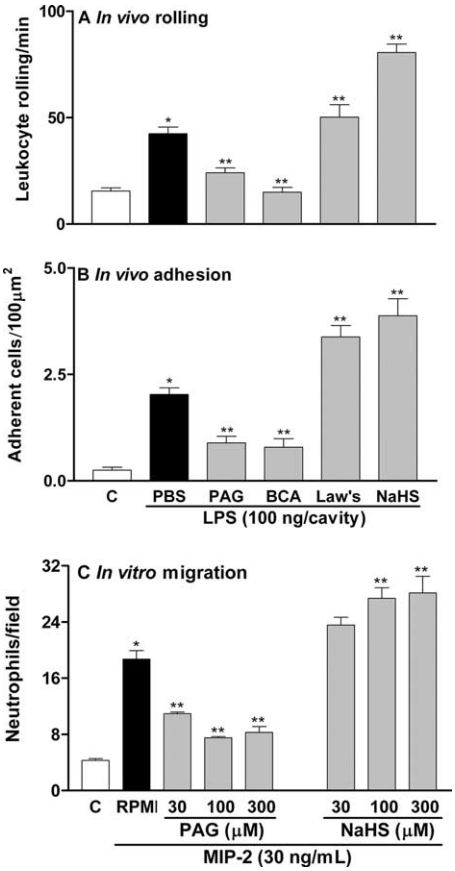

FIGURE 3. The CSE-H_2S pathway improved LPS-induced leukocyte rolling and adhesion and MIP-2-mediated neutrophil chemotaxis in vitro. Mice were pretreated (s.c.) with PBS (0.2 ml), PAG (1.0 mmol/kg), BCA (0.4 mmol/kg), NaHS (30 μmol/kg), or Lawesson's reagent (Law's; 30 μmol/kg), and 30 min later LPS (100 ng/cavity) was administered i.p. Leukocyte rolling (*A*) and adhesion (*B*) were evaluated by intravital microscopy in the mesentery venules 2 and 4 h after LPS injection, respectively. The first bar in *A* and *B* represents the neutrophil rolling and adhesion induced by PBS injected i.p., respectively (control group C). *C*, Neutrophils were incubated with PAG (30, 100 or 300 μM) or NaHS (30, 100, or 300 μM) 30 min before measuring chemotaxis induced by MIP-2 (30 ng/ml). The first bar in *C* represents the neutrophil chemotaxis induced by RPMI (control group). The values are mean ± SEM ($n = 5$) and are representative of three independent experiments. The data used for statistical analyses were from individual experiments. *, $p < 0.05$ compared with control group; **, $p < 0.05$ compared with responses induced by LPS or MIP-2 (ANOVA followed by Bonferroni's *t* test).

Determination of leukocyte rolling and adhesion to the mesenteric microcirculation by intravital microscopy

Leukocyte rolling and adhesion were examined 2 or 4 h after inflammatory challenge, respectively, and measured as previously described (25, 26). Briefly, animals were anesthetized, and the mesenteric tissue was exposed for microscopic in situ examination. Postcapillary venules, with a diameter of 8–12 μm, were chosen, and the interaction of leukocytes with the luminal surface of the venular endothelium was evaluated by counting the number of rolling leukocytes after 10 min. A leukocyte was considered to be adherent to the venular endothelium if it remained stationary for >30 s. Cells were counted in the recorded image using five different fields for each animal to avoid variability due to sampling. Data were then averaged for each animal (27).

Mouse neutrophil isolation

Neutrophils from bone marrow were isolated according to the procedure described previously (28), with some modifications. Briefly, mice were killed, the femurs and tibias from both hind limbs were removed and freed of soft tissue, and the extreme distal tip of each was dissected. After dispersal of cell clumps and removal of debris, the remaining bone marrow cells were suspended in 2 ml of HBSS and fractionated on a Percoll gradient. The enriched neutrophil fraction was recovered at the interface of 65% and 72% Percoll.

Chemotaxis assay

Chemotaxis was evaluated according to the procedure described in Ref. 29. A 48-well microchemotaxis plate (Neuro Probe) in which the chambers were separated by polyvinylpyrrolidone-free polycarbonate membrane (5-μm pore size) was used. Chemoattractant, 28 μl of RPMI containing 0.01% BSA (control group) or MIP-2/CXCL2 (30 ng/ml) in RPMI were placed in the lower chamber. Purified neutrophil suspension (50 μl of 10^6 cells/ml) pretreated for 30 min with PAG (30, 100, or 300 μM), NaHS (30, 100 or 300 μM), glybenclamide (100 μM), MIP-2 (100 ng/ml), or NaHS (30 μM) plus MIP-2 was then placed in the upper chamber. Chambers were incubated at 37°C with 5% CO_2 for 1 h. The results are expressed as the

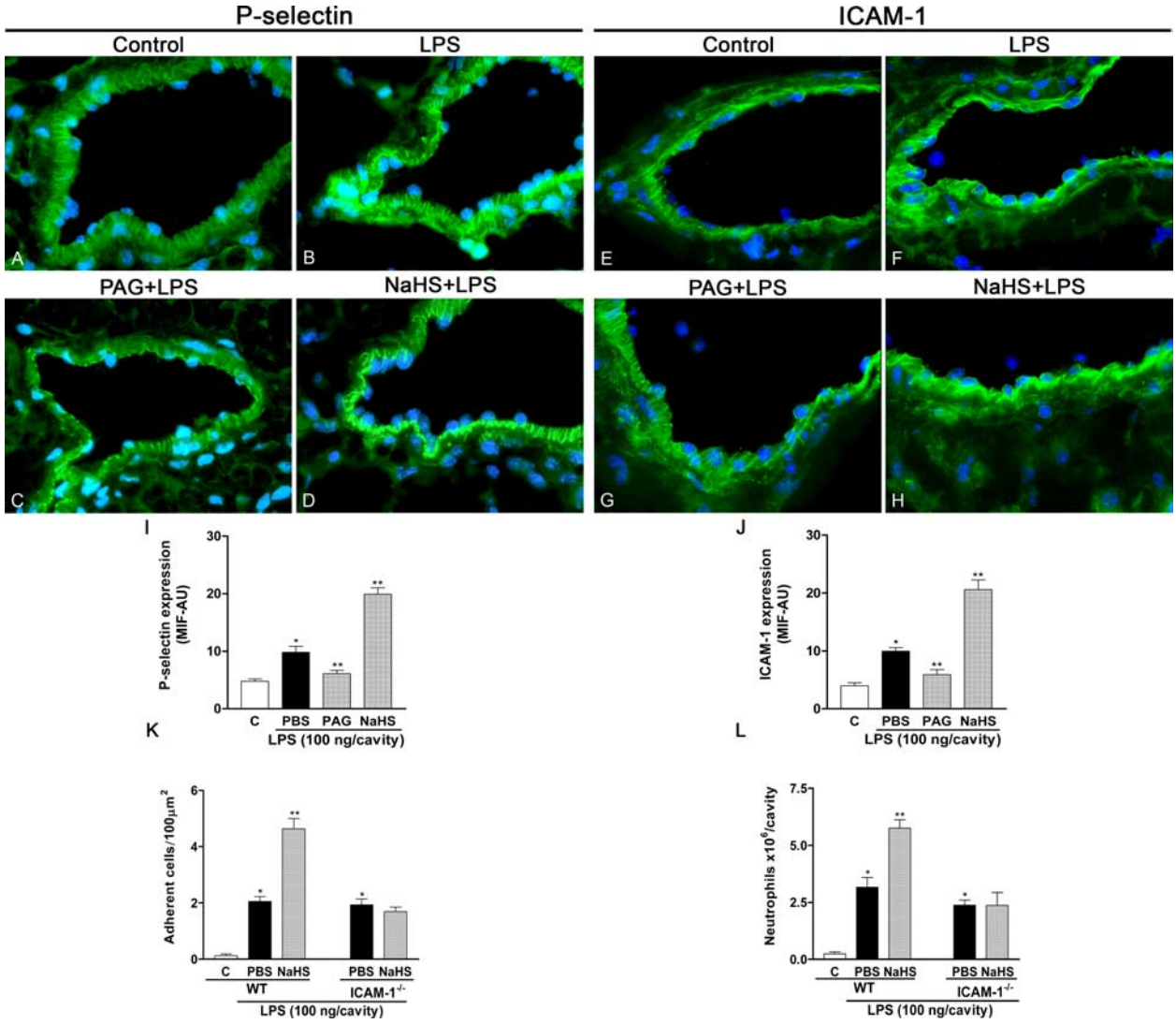

FIGURE 4. The CSE-H_2S pathway enhanced LPS-induced P-selectin and ICAM-1 expression on mesenteric vessels, and H_2S donor treatment did not enhance leukocyte adhesion and neutrophil migration induced by LPS in ICAM-1-deficient mice. Immunofluorescence staining for P-selectin (*A–D*) and ICAM-1 (*E–H*) on mesenteric vessels from PBS i.p.-injected animals (*A* and *E*; control groups C). LPS (100 ng/cavity)-challenged animals are shown in *B–D* and *F–H*. Pretreatments s.c. 30 min before LPS with PBS (*B* and *F*), PAG 1.0 mmol/kg (*C* and *G*), and NaHS 30 μmol/kg (*D* and *H*). These are representative images of two independent experiments taken from four fields per histological section from three mice per group. Quantitative analysis of immunofluorescence staining (MIF, mean intensity fluorescence) in arbitrary units (AU) for P-selectin (*I*) and ICAM-1 (*J*) on venular endothelium of mice after indicated treatments. The analysis was performed in four fields per histological section from each individual mouse. Data represent the means ± SEM ($n = 3$). The area of stained vessels was determined by ImageJ analysis. *K* and *L*, The WT or ICAM-$1^{-/-}$ mice were treated with PBS (0.2 ml s.c.) or NaHS (30 μmol/kg s.c.) and 30 min after they were injected i.p. with LPS (100 ng/cavity). Leukocyte adhesion (*K*) was determined by intravital microscopy in the mesentery 4 h after LPS. The neutrophil migration (*L*) was evaluated 6 h after LPS. The first bar represents neutrophil adhesion (*K*) and migration (*L*) induced by PBS injection (control group). The values are means ± SEM ($n = 5$) and are representative of three separate experiments. The data used for statistical analyses were from the individual experiments. *, $p < 0.05$ compared with control group; **, $p < 0.05$ compared with PBS treated LPS-injected group (ANOVA followed by Bonferroni's *t* test).

mean number of neutrophils per field and are representative of triplicate measurements from three separate experiments.

Flow cytometric analysis of CD18 and CXCR2 expression on neutrophils

The neutrophil suspensions were treated for 30 min with PAG (300 μM), NaHS (300 μM), glybenclamide (100 μM), or NaHS plus glybenclamide. Cells were then incubated with RPMI (control), with MIP-2 (10 or 30 ng/ml) for 1 h, or with LPS (1 μg/ml) for 3 h. The expression of CD18 and CXCR2 was measured according to the procedure described in (30). Briefly, neutrophil suspensions (0.5×10^6) were incubated with anti-CD16/CD32 mAbs (Fc block, clone 2.4G2; BD Pharmingen), followed by 30 min of incubation at 4°C with fluorescent-labeled Abs: PE-conjugated anti-CXCR2 mAb (1/50; R&D Systems), PE-conjugated anti-CD18 mAb (1/100; BD Biosciences) and PerCP-conjugated anti-Gr-1 mAb (1/200; BD Biosciences). The cells were then washed, fixed in 2% formaldehyde, and analyzed by flow cytometry in a FACSort flow cytometer using CellQuest software (BD Biosciences). Neutrophils were identified by their light scatter properties and expression of Gr-1 in granulocytes cells, and the expressions of CXCR2 or CD18 were determined in this population.

Immunofluorescence assay for P-selectin, ICAM-1/CD54, or G protein-coupled receptor kinase 2 (GRK2)

An immunofluorescence assay was used to analyze the level of P-selectin or ICAM-1 positivity on mouse mesentery venules, 2 h after LPS injection, as previously described (31). The expression of GRK2 on neutrophils was also determined by immunofluorescence (32). For this, neutrophils from mice were treated for 30 min with PAG (300 μM), NaHS (300 μM), glybenclamide (100 μM), or NaHS plus glybenclamide and subsequently stimulated with RPMI or MIP-2 (30 ng/ml) for 1 h. Following this, slices

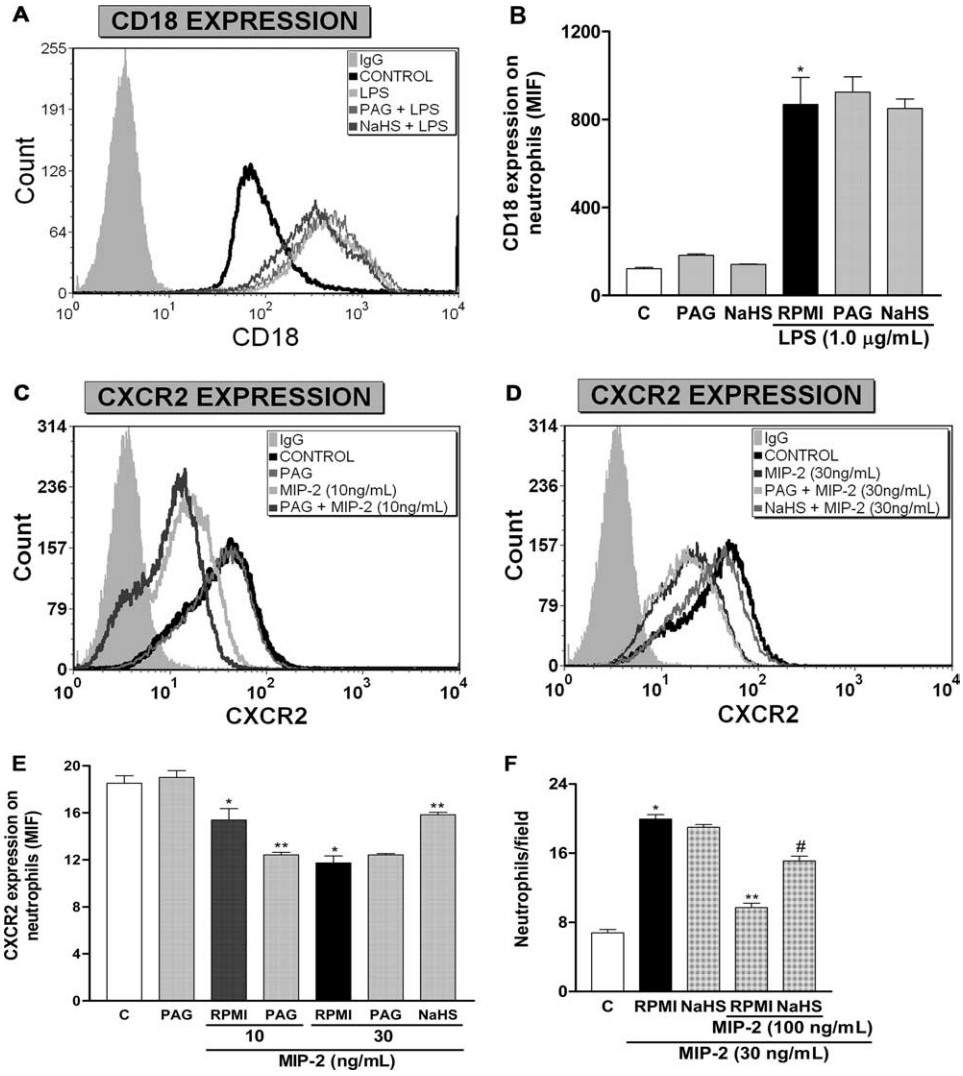

FIGURE 5. H_2S prevented MIP-2-induced neutrophil CXCR2 desensitization and internalization but had no effect on CD18 expression on neutrophils. *A*, Histograms represent neutrophil CD18 expression index (percent of gated) measured by FACSort flow cytometry 3 h after LPS or RPMI stimulations. Neutrophils were treated with RPMI (control), PAG (300 μM), or NaHS (300 μM) 30 min before LPS (1.0 μg/ml) or RPMI stimulation. *B*, Quantitative analysis of fluorescence staining for CD18 on mouse neutrophils after indicated treatments. *C* and *D*, Histograms represent neutrophil CXCR2 expression index (percent of gated) measured by FACSort flow cytometry 1 h after MIP-2 or RPMI stimulation. Neutrophils were incubated with RPMI (control), PAG (300 μM), or NaHS (300 μM), 30 min before MIP-2 (10 or 30 ng/ml) or RPMI stimulation. *E*, Quantitative analysis of fluorescence staining (MIF, mean intensity fluorescence) for CXCR2 on mouse neutrophils after indicated treatments. Values represent the percentage of neutrophils staining positive, are means ± SEM ($n = 3$), and are representative of two separate experiments. *F*, Neutrophils were incubated with RPMI, MIP-2 (100 ng/ml), NaHS (30 μM), or NaHS plus MIP-2 30 min before measuring chemotaxis induced by MIP-2 (30 ng/ml) or RPMI (control group). The values are mean ± SEM ($n = 5$) and are representative of three independent experiments. The data used for statistical analyses were from individual experiments. *, $p < 0.05$ compared with control group; **, $p < 0.05$ compared with RPMI treatment; #, $p < 0.05$ compared with RPMI + MIP-2 group (ANOVA followed by Bonferroni's *t* test).

were prepared using 5.0×10^4 neutrophils (32). Five-micrometer-thick frozen mesentery tissue sections or neutrophil slices were fixed with paraformaldehyde (4%) in a wet chamber at room temperature. The slides were incubated with PBS containing 1% BSA (PBS-BSA), and then slices were incubated for 1 h with FITC-conjugated anti-mouse CD54-ICAM-1 or P-selectin mAb (1/200; BD Pharmingen) in PBS-BSA. The neutrophils slices were then incubated with rabbit anti-mouse GRK2 mAb (1/200; Santa Cruz Biotechnology) overnight, and then with red-fluorescence compost Alexa Fluor 594 (goat anti-rabbit; 1/400; Invitrogen). Subsequently, slides were mounted using 4′,6′-diamidino-2-phenylindole (Vector Laboratories) and sealed with enamel. The results of qualitative analysis are expressed as fluorescence intensity of stained venules (×100) or neutrophils (×40) present in the fluorescence microscopic field.

Assay of neutrophil H_2S synthesis

H_2S biosynthesis was measured as described previously (33). Briefly, after stimulation with CXCL8 (100 ng/ml) for 1 h, human neutrophils (4×10^6 cells per sample) were homogenized in potassium phosphate buffer (pH 7.4). The assay mixture containing neutrophil extract (100 μl), L-cysteine (10 mM), pyridoxal 5′-phosphate (2 mM), and PBS or PAG (25 mM) was incubated for 2 h at 37°C. Then, zinc acetate (a H_2S trap) was added, followed by centrifugation. *N*,*N*-Dimethyl-*p*-phenylenediamine sulfate followed by $FeCl_3$ was then added to 100 μl of the supernatant, and optical density was measured at 670 nm. The H_2S concentration of each sample was calculated against a calibration curve of NaHS (100–0.1 μg/ml).

Statistical analysis

Data are reported as means ± SEM and are representative of two or three different experiments. The means from different treatments in individual experiments were compared by ANOVA. When significant differences were identified, individual comparisons were subsequently made using Bonferroni's *t* test for unpaired values. The level of significance was set at $p < 0.05$.

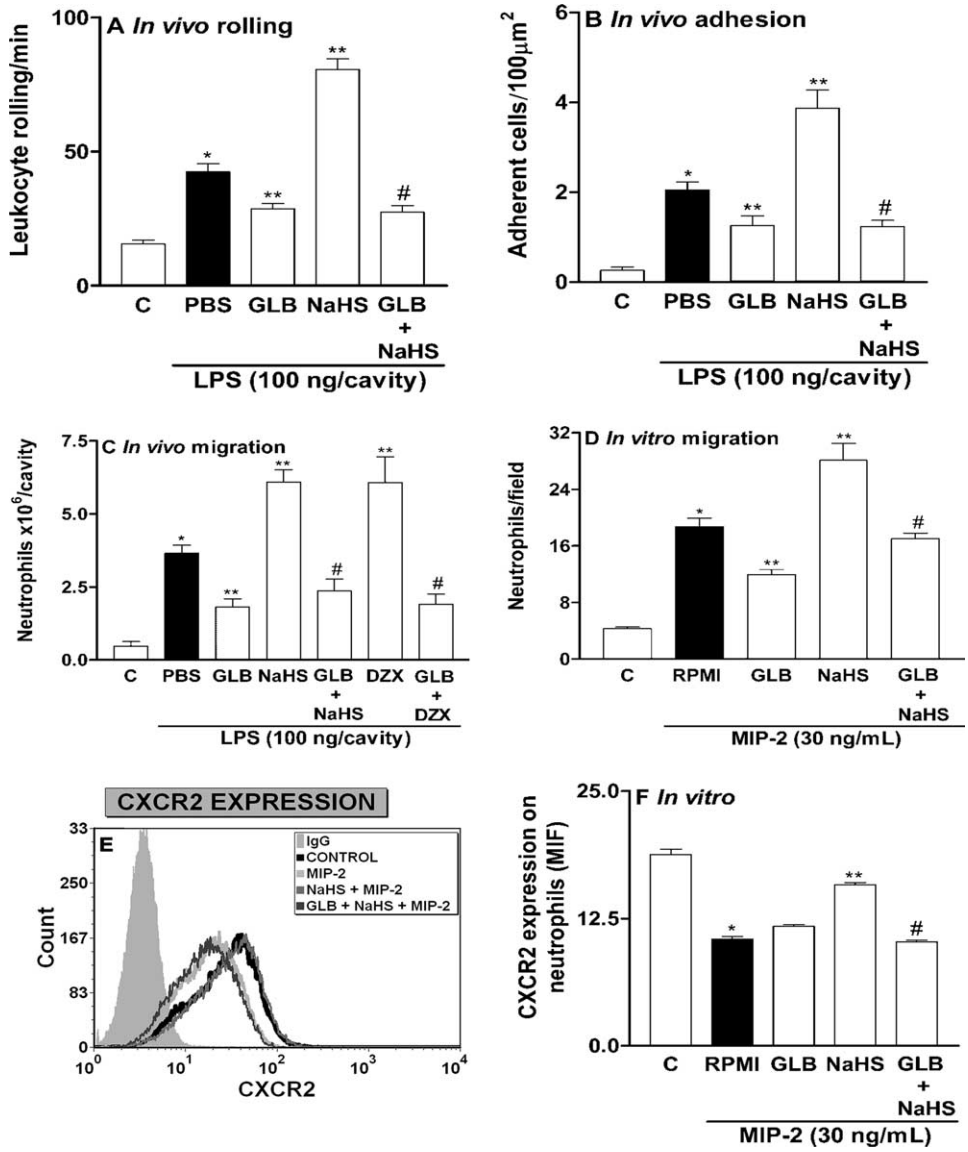

FIGURE 6. The CSE-H_2S pathway improved LPS-induced neutrophil rolling, adhesion, migration in vivo, and chemotaxis in vitro and prevents MIP-2-induced internalization of CXCR2 receptor through activation of K^+_{ATP} channels. *A* and *B*, Mice were pretreated (s.c.) with PBS (0.2 ml), NaHS (30 μmol/kg), glybenclamide (GLB; 40 μmol/kg), or s.c. with glybenclamide (40 μmol/kg, 15 min before NaHS); 30 min later LPS (100 ng/cavity) was administered i.p. Leukocyte rolling (*A*) and adhesion (*B*) was evaluated by intravital microscopy in the mesenterium 2 and 4 h after LPS injection, respectively. *C*, Mice were pretreated (s.c.) with PBS (0.2 ml), NaHS (30 μmol/kg), glybenclamide (40 μmol/kg), diazoxide (DZX; 300 μmol/kg), or glybenclamide (40 μmol/kg, 15 min before NaHS) or glybenclamide (40 μmol/kg, 15 min before diazoxide), and 30 min later they were injected i.p. with LPS (100 ng/cavity). Neutrophil migration was determined 6 h after stimulus. The first bar in *A, B*, and *C* represents the neutrophil rolling, adhesion, and migration induced by PBS, respectively (control group). The values are mean ± SEM ($n = 5$) and are representative of three independent experiments. The data used for statistical analyses were from individual experiments. *D*, Neutrophils were incubated with NaHS (300 μM), glybenclamide (100 μM) or (100 μM; 15 min before NaHS) 30 min before measuring chemotaxis induced by MIP-2 (30 ng/ml). The first bar represents the neutrophil chemotaxis induced by RPMI (*C*). *E*, Histograms represent neutrophil CXCR2 expression index (person of gated). Neutrophils were incubated with RPMI (control), NaHS (300 μM) or glybenclamide (100 μM) plus NaHS (300 μM), 15 min before MIP-2 (30 ng/ml) stimulation. Neutrophil CXCR2 expression index (percent of gated) was measured by FACSort flow cytometry 1 h after MIP-2 or RPMI (control group) stimulation. *F*, Quantitative analysis of fluorescence staining (MIF, mean intensity fluorescence) for CXCR2 on mouse neutrophils after indicated treatments. The first bar represents CXCR2 expression induced by RPMI (control group). Values represent the percentage positive staining neutrophils and are expressed as the mean ± SEM and are representative of two separate experiments. *, $p < 0.05$ compared with control group; **, $p < 0.05$ compared with responses induced by LPS + PBS or MIP-2 + RPMI; #, $p < 0.05$ compared with LPS + NaHS or MIP-2 + NaHS groups (ANOVA followed by Bonferroni's *t* test).

Results

H_2S augmented LPS and mBSA induced neutrophil migration to the peritoneum or femur/tibial joint

Pretreatment of mice with the CSE inhibitors PAG or BCA resulted in a significant reduction of neutrophil migration to the peritoneal cavity triggered by LPS (Fig. 1*A*). On the other hand, pretreatment with H_2S donors, NaHS, or Lawesson's reagent enhanced the LPS-induced neutrophil migration. The effects of PAG and NaHS were dose dependent (Fig. 1*B*). With the purpose of elucidating whether H_2S was also involved in neutrophil recruitment during adaptive immune inflammation, we investigated if either inhibition of H_2S synthesis by PAG or a H_2S donor (NaHS) modulated neutrophil migration in immune inflammation induced by mBSA in previously immunized mice. The administration of mBSA to immunized mice induced neutrophil migration to the peritoneal cavity, measured at 6 h, and this response was

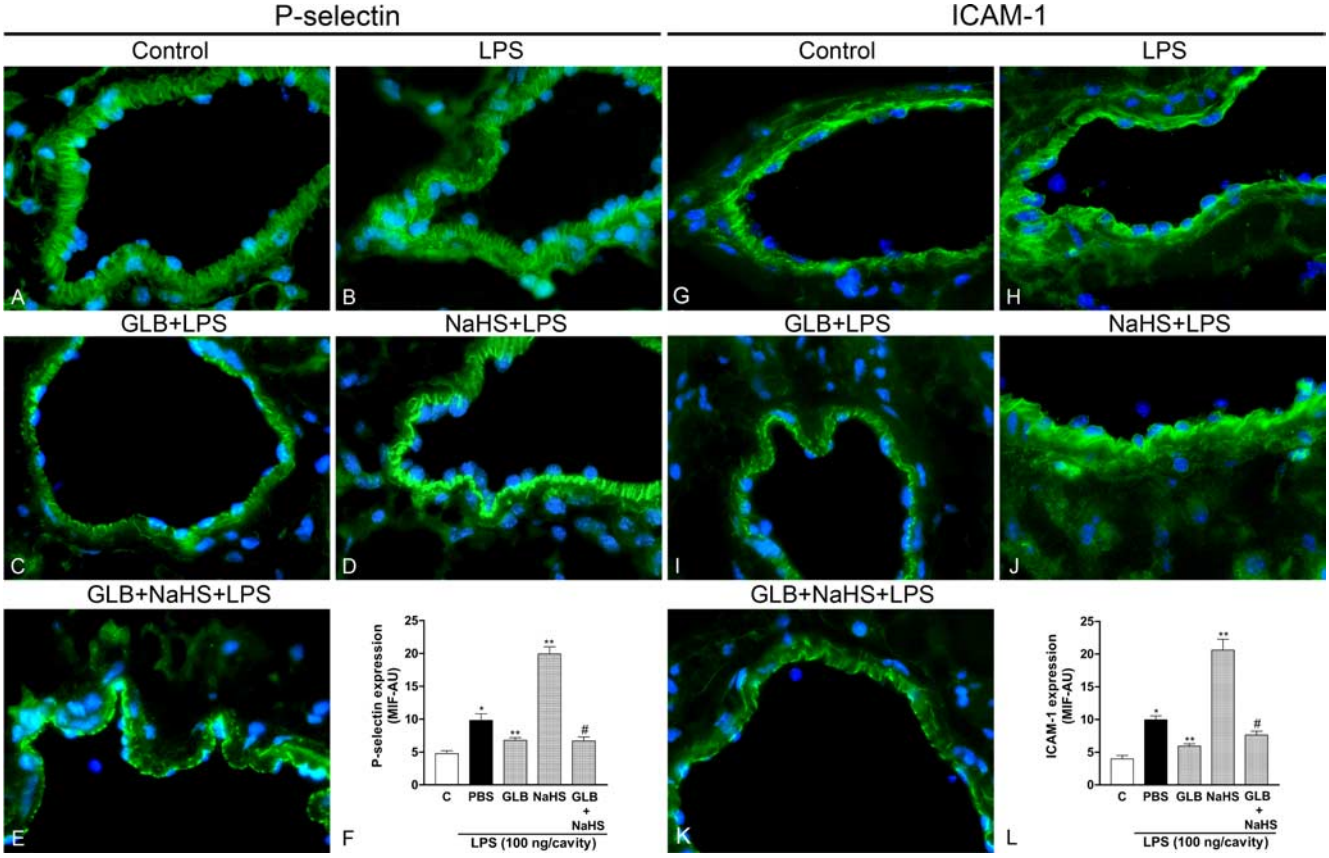

FIGURE 7. The CSE-H_2S pathway increased LPS-induced P-selectin and ICAM-1 expression on mesenteric vessels endothelial cells through activation of K^+_{ATP} channels. Immunofluorescence staining for P-selectin (*A–E*) and ICAM-1 (*G–K*) on mesenteric vessels from PBS i.p.-injected animals (*A* and *G*; control groups). LPS (100 ng/cavity)-challenged animals are shown in *B–E* and *H–K*. Pretreatments s.c. 30 min before LPS with PBS (*B* and *H*), glybenclamide (GLB; 40 μmol/kg; *C* and *I*), NaHS 30 μmol/kg, (*D* and *J*), and glybenclamide plus NaHS (*E* and *K*). The images are representative of two independent experiments taken from four fields per histological section from three mice per experiment. Quantitative analysis of immunofluorescence staining (MIF, mean intensity fluorescence) in arbitrary units (AU) for P-selectin (*F*) and ICAM-1 (*L*) on venular endothelium of mice after indicated treatments. The analysis was performed in four fields per histological section from each individual mouse. The data represent the means ± SEM ($n = 3$). The area of stained vessels was determined by ImageJ analysis. *, $p < 0.05$ compared with control group; **, $p < 0.05$ compared with LPS + PBS groups; #, $p < 0.05$ compared with LPS plus NaHS groups (ANOVA followed by Bonferroni's *t* test).

inhibited by PAG and potentiated by NaHS (Fig. 1*C*). PBS administered i.p. to naive or immunized mice (control group) or mBSA to FI mice did not induce neutrophil migration. Moreover, i.a. injection of mBSA also induced neutrophil migration to the articular joints 24 h after treatment (Fig. 1*D*). Pretreatment of the immunized mice with PAG resulted in complete ablation and with NaHS enhancement of this neutrophil migration into articular cavities (Fig. 1*D*). These results suggest that the CSE-H_2S pathway augments neutrophil migration in both innate and adaptive immune responses in different inflammatory sites.

H_2S did not modulate the production of neutrophil chemotactic mediators

We investigated whether PAG or NaHS modulated the release of TNF-α, KC or LTB_4, chemotactic mediators of LPS- or mBSA-induced neutrophil migration. As shown in Fig. 2, mice pretreated with PAG or NaHS and injected with LPS or mBSA presented similar peritoneal exudate or joint tissue levels of TNF-α (Fig. 2*A*), KC (Fig. 2*B*), and LTB_4 (Fig. 2*C*), compared with mice pretreated with PBS injected with LPS or mBSA. PBS administered i.p. to naive or immunized mice (control group) or mBSA to FI mice did not induce TNF-α, KC, or LTB_4 release. The level of these mediators was determined 1 or 6 h after LPS and mBSA, respectively. These data suggest that the effect of the CSE-H_2S pathway on neutrophil migration is not associated with any modulation of synthesis or release of chemotactic mediators.

H_2S improved leukocyte rolling and adhesion induced by LPS in naive mice

To clarify the mechanisms by which H_2S increased neutrophil migration to the inflammatory site, we examined whether it modulated leukocyte rolling and adhesion in vivo. The pretreatment of mice with PAG or BCA significantly decreased leukocyte rolling and adhesion on the endothelium, while treatment with NaHS or Lawesson's reagent significantly enhanced LPS-induced leukocyte rolling (Fig. 3*A*) and cell adhesion (Fig. 3*B*). The i.p. injection of PBS in mice did not induce significant leukocyte rolling or adhesion on endothelial cells (Fig. 3, *A* and *B*; control group), and neither PAG nor NaHS treatments had any influence on these parameters (data not shown).

H_2S increased MIP-2 induced neutrophil chemotaxis

Next, we evaluated the effects of PAG or NaHS on neutrophil chemotaxis in vitro. Pretreatment of neutrophils with PAG dose dependently inhibited, whereas NaHS dose dependently enhanced MIP-2-induced murine neutrophil chemotaxis (Fig. 3*C*). In addition, H_2S also increased CXCL8-induced human neutrophil chemotaxis in vitro (40%, $n = 5$, $p < 0.05$, *t* test; data not shown in Fig. 3). Supporting a role for H_2S in neutrophil chemotaxis, CSE activity, evaluated by

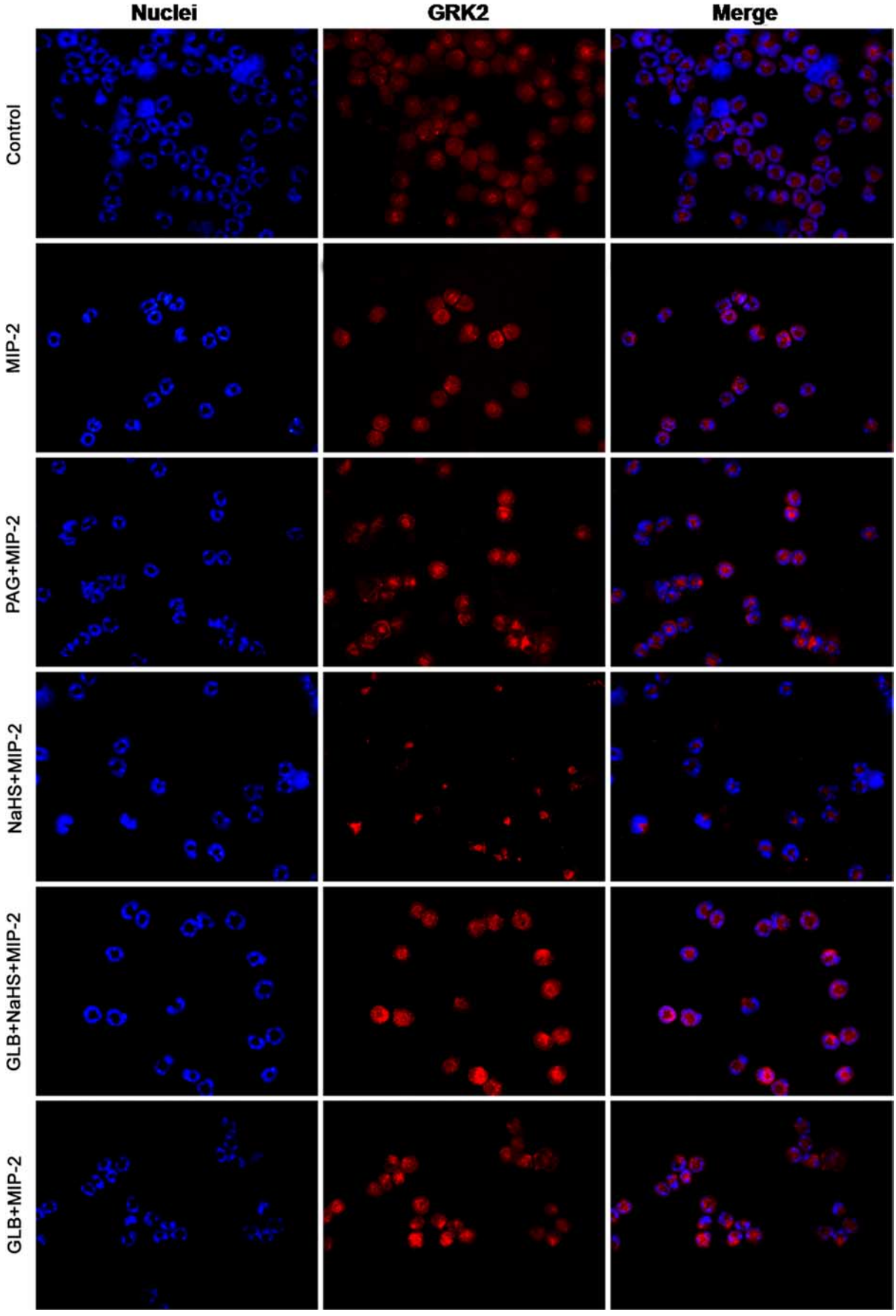

FIGURE 8. The CSE-H_2S pathway reduced MIP-2-induced GRK2 expression in neutrophils through activation of K^+_{ATP} channels. Neutrophils incubated with RPMI (control and MIP-2 groups), PAG (300 μM; PAG + MIP-2), NaHS (300 μM; NaHS + MIP-2), glybenclamide (GLB; 100 μM) plus NaHS (GLB + NaHS + MIP-2) and glybenclamide (glybenclamide + MIP-2). After 30 min, RPMI or MIP-2 (30 ng/ml) was added to

H_2S synthesis, using the CSE substrate L-cysteine (33), was increased in human neutrophils stimulated with CXCL8 (HBSS incubated, 1.2 ± 0.6 $\mu g/10^6$ cells; CXCL8 (100 ng/ml)-stimulated neutrophils, 2.5 ± 0.3 $\mu g/10^6$ cells, $n = 5$, $p < 0.05$, t test). These results suggest that the CSE-H_2S pathway is activated during the inflammatory process and also regulates neutrophil locomotion.

H_2S augmented LPS-mediated P-selectin and ICAM-1 expression on venular endothelial cells

In clarifying the mechanisms by which H_2S increased neutrophil recruitment in vivo, we observed that LPS induced a significant augmentation of P-selectin and ICAM-1 expression on mouse mesenteric vessel endothelium compared with that in PBS-treated animals (Fig. 4, *A, B, E,* and *F,* respectively). However, the pretreatment of mice with PAG significantly inhibited LPS-induced P-selectin and ICAM-1 expression (Fig. 4, *C* and *G,* respectively). In contrast, the pretreatment of mice with NaHS significantly enhanced the LPS-induced P-selectin and ICAM-1 expression on endothelial cells (Fig. 4, *D* and *H,* respectively). We did not observe ICAM-1 expression on vessel endothelial cells in ICAM-$1^{-/-}$ mice challenged with LPS. Also, fluorescence was not observed when specific Abs were substituted by isotype control IgG2-FITC (data not shown). Quantitative image analysis of the immunofluorescence staining for P-selectin or ICAM-1 on vessel endothelial cells of mouse mesentery, performed using ImageJ software (or analysis), confirmed that the expression of P-selectin (Fig. 4*I*) and ICAM-1 (Fig. 4*J*) was significantly reduced when H_2S synthesis was inhibited by PAG. We also established that the expression of P-selectin (Fig. 4*I*) or ICAM-1 (Fig. 4*J*) was enhanced by NaHS treatment.

ICAM-1 is involved in the H_2S-mediated increase in neutrophil adhesion and migration induced by LPS

Compared with PBS administration, an injection of LPS (100 ng/cavity i.p.) induced significant leukocyte adhesion to venular endothelium (Fig. 4*K*) and neutrophil migration in ICAM-$1^{-/-}$ mice (Fig. 4*L*), which was similar to that induced in WT mice. The pretreatment of the WT mice with NaHS significantly enhanced the neutrophil migration triggered by injection of LPS. By contrast, NaHS did not increase the neutrophil migration induced by the same stimulus in ICAM-$1^{-/-}$ mice.

H_2S prevented MIP-2-induced CXCR2 receptor internalization on neutrophils but did not interfere with CD18 expression on neutrophils

In contrast with the above observations in P-selectin or ICAM-1 expression on endothelium venules, neither PAG nor NaHS influenced LPS enhanced CD18 expression on neutrophils (Fig. 5*A*). Quantitative image analysis of the staining for CD18 on neutrophils confirmed that the expression of CD18 was not changed when H_2S synthesis was inhibited by PAG or in the presence of NaHS (Fig. 5*B*). To elucidate the mechanisms by which H_2S increases neutrophil chemotaxis in vitro, we examined whether it prevented internalization of the chemotactic receptor (CXCR2) on neutrophils activated by the chemotactic mediator MIP-2. MIP-2 at both doses (10 and 30 ng/ml) induced a significant internalization of CXCR2 in neutrophils (evaluated by CXCR2 membrane expression). Inhibition of H_2S production by incubation of neutrophils with PAG improved the CXCR2 internalization induced by MIP-2 at the dose of 10 ng/ml, but not at the dose of 30 ng/ml. Moreover, H_2S donor (NaHS) prevented the internalization of the CXCR2 receptor induced by MIP-2 at a dose of 30 ng/ml (Fig. 5, *C–E*). These results suggest that H_2S prevents CXCR2 internalization but does not interfere with CD18 expression on neutrophils during an inflammatory response.

H_2S prevented MIP-2-induced CXCR2 receptor desensitization during neutrophil chemotaxis

We further examined whether H_2S could prevent the desensitization of CXCR2 on neutrophils when activated by the chemotactic mediator MIP-2. MIP-2 resulted in a significant reduction of neutrophil chemotaxis induced by the same CXCR2 agonist (receptor desensitization), a phenomenon that was prevented by treatment with NaHS (Fig. 5*F*).

Participation of K^+_{ATP} channels in the in vivo enhancement of LPS-induced neutrophil rolling, adhesion, and migration by H_2S

Next, we investigated whether activation of the K^+_{ATP} channel was involved in the effects of H_2S on neutrophil migration to the inflammatory site. The pretreatment of mice with a K^+_{ATP} channels blocker (glybenclamide) significantly decreased leukocyte rolling (Fig. 6*A*) and adhesion (Fig. 6*B*) on endothelium, and neutrophil migration in vivo (Fig. 6*C*) induced by LPS. In contrast, diazoxide, a K^+_{ATP} channel opener, enhanced the LPS-induced neutrophil recruitment in a manner similar to that observed with NaHS (Fig. 6*C*). Because systemic administration of glybenclamide resulted in an inhibitory effect on neutrophil migration, we sought to investigate whether in the presence of glybenclamide, the administration of NaHS or diazoxide would change LPS-induced neutrophil accumulation in the peritoneal cavity. The enhancement effect of NaHS or diazoxide on LPS-induced neutrophil rolling (Fig. 6*A*), adhesion (Fig. 6*B*), or trafficking (Fig. 6*C*) was significantly prevented by treatment of the animals with glybenclamide.

The role of K^+_{ATP} channels in H_2S enhancement of MIP-2-induced in vitro neutrophil chemotaxis and CXCR2 internalization

We observed that the enhancement of MIP-2-induced in vitro neutrophil chemotaxis by NaHS was prevented by incubation of neutrophils with glybenclamide (Fig. 6*D*). In addition, glybenclamide alone reduced in vitro neutrophil chemotaxis induced by MIP-2 (Fig. 6*D*). Furthermore, although, glybenclamide did not change CXCR2 internalization, it reversed the effect of NaHS on neutrophil CXCR2 internalization (Figs. 6, *E* and *F*). Quantitative image analysis of the staining for CXCR2 on neutrophils, confirmed that K^+_{ATP} channel blockade prevented the H_2S effects on CXCR2 internalization induced by MIP-2 (Fig. 6*F*). These results suggest that H_2S modulates neutrophil recruitment and prevents CXCR2 internalization by a mechanism dependent on K^+_{ATP} channels.

Participation of ATP-sensitive potassium channels in the H_2S enhancement of LPS-induced P-selectin and ICAM-1 expression on venular endothelium

The pretreatment of mice with a K^+_{ATP} channel blocker (glybenclamide) significantly decreased P-selectin (Fig. 7*C*) and ICAM-1

the cultures; 1 h later, GRK2 expression was evaluated by immunofluorescence. First column, blue staining by 4′,6′-diamidino-2-phenylindole represents neutrophil nuclei; second column, red staining represents GRK2 expression in neutrophil cytoplasm; third column, an overlay of nuclei and GRK2 expression. These qualitative analyses are representative images of three independent experiments taken from four fields per lamina prepared with 5×10^4 neutrophils (×40).

(Fig. 7*I*) expression on endothelium induced by LPS compared with no treatment (Fig. 7, *A, B, G,* and *H*). Moreover, the enhancement effect of NaHS on LPS-induced P-selectin (Fig. 7*D*) or ICAM-1 (Fig. 7*J*) was significantly prevented by treatment of animals with glybenclamide (Fig. 7, *E* and *K,* respectively). Quantitative image analysis of the immunofluorescence staining on vessel endothelial cells of mouse mesentery, performed using ImageJ software, confirmed that K^+_{ATP} channel blockage prevented the H_2S effects on P-selectin (Fig. 7*F*) and ICAM-1 (Fig. 7*L*) expression.

H_2S reduced MIP-2-induced GRK2 expression on neutrophils through activation of K^+_{ATP} channels

Clarifying the mechanisms by which H_2S prevents CXCR2 internalization on neutrophils, we observed that MIP-2 induced a significant augmentation of GRK2 expression on neutrophils. The pretreatment of neutrophils with PAG enhanced, whereas NaHS reduced, MIP-2-induced GRK2 expression. Moreover, glybenclamide also improved the MIP-2-induced GRK2 expression and prevented the inhibitory effect of NaHS upon GRK2 expression (Fig. 8). Quantitative image analysis of the immunofluorescence staining for GRK2 on neutrophils, performed using ImageJ, confirmed the results described above (data not shown). These results suggest that H_2S prevents CXCR2 internalization via inhibition of GRK2 expression by a K^+_{ATP} channel dependent mechanism.

Discussion

In addition to endogenous NO (27, 34) and carbon monoxide (35, 36), H_2S, another endogenously occurring gas, plays an important role in the modulation of the acute inflammatory processes (37). It was observed in this study that H_2S produced during innate and adaptive inflammatory response enhanced neutrophil migration by increasing their rolling, adhesion, and locomotion. These effects are dependent on K^+_{ATP} channels.

Treatment with PAG or BCA decreased whereas NaHS or Lawesson's reagent augmented neutrophil migration during an innate or immune response. It is important that these effects of H_2S synthesis inhibitors appear not to be a consequence of blood pressure changes, given that at the doses used PAG or BCA did not significantly change blood pressure (38). In agreement with our results, it has been reported in the literature, that the inhibition of endogenous H_2S formation by treatment of animals with PAG reduced leukocyte recruitment and consequently ameliorated either local or systemic organ injuries in either caerulein-induced rat pancreatitis or acute mouse endotoxemia models (19, 20, 39). Moreover, PAG reduced edema formation and neutrophil migration in the rat paw challenged with carrageenan (37). Furthermore, our group has recently shown that H_2S contributed to development of inflammatory nociception by a mechanism dependent on augmentation of neutrophil migration (40). In contrast to these findings, there are additional studies showing that H_2S has anti-inflammatory effects. For instance, recent studies demonstrated that different H_2S donors inhibit leukocyte infiltration in carrageenan-injected air pouch or hindpaw (23, 41), gastric injury induced by anti-inflammatory nonsteroidal anti-inflammatory drugs (NSAID; Ref. 42), colitis induced by trinitrobenzenesulfonic acid (43) and into lung during caerulein-induced pancreatitis and systemic inflammation induced by high doses of LPS (44, 45). The explanation for these apparent anomalies may be severalfold. Firstly, the differences in experimental models employed. Whereas we investigated neutrophil migration into peritoneal cavity and femur/tibial joint induced by low doses of LPS and Ag (mBSA), respectively, these others studies used either an air pouch or colitis models, pancreatitis-, endotoxemic shock-induced lung injury, NSAIDs induced gastric mucosal injury or hindpaw inflammation (23, 41–45). In particular with NSAIDs-induced gastric injury, it is important that the anti-inflammatory action of H_2S could be a consequence of an increase in gastric mucosal blood flow. Mucosal blood flow is essential to maintain gastric mucosal integrity; hence increased blood flow would reduce the risk of lesion by NSAID treatment. Thus, an increase in gastric blood flow resulting from H_2S donation could primarily prevent any initial mucosal lesion by reducing the production of local inflammatory mediators, consequently reducing leukocyte to endothelium adhesion, an event essential to the progression of gastric tissue lesion (46). Reinforcing this explanation, NO synthase inhibitors that improve neutrophil migration induced by different stimuli (27) have been shown to enhanced the NSAID-induced gastric lesion via a reduction of mucosal blood flow (47, 48).

The recruitment of neutrophils to the inflammatory focus is a multifactorial event that is dependent on the release of inflammatory chemotactic mediators, such as TNF-α, chemokines, and LTB_4 (5). These mediators result in endothelial cell activation which consequently become susceptible to neutrophil interaction, permitting the following sequential events: rolling, firm adhesion, transmigration, and locomotion (4). Our data demonstrate that the effect of H_2S on neutrophil migration is not due to increased production of chemotactic mediators, because neither the inhibition of H_2S production nor the administration of a H_2S donor influenced LPS or mBSA-induced TNF-α, KC, and LTB_4 release, all recognized neutrophil chemotactic mediators in these described models (5, 8, 10). However, H_2S did increase the expression of P-selectin and ICAM-1 on venular endothelial cells and consequently augment neutrophil/endothelium rolling and adhesion processes, an effect that will contribute to the enhanced migration of neutrophils. In fact, the enhancement of leukocyte adhesion and transmigration by treatment of the animals with a H_2S donor was not observed in ICAM-1-deficient mice. In further agreement, H_2S enhanced neutrophil trafficking in cecal ligation and puncture-induced sepsis has been demonstrated, contemporaneously with augmented expression of ICAMs and selectins on lung and liver endothelium (22).

An important feature of G protein-coupled receptors, including chemokine receptors, is their rapid internalization from the plasma membrane into the endosomal cell compartments and their desensitization, observed after persistent agonist stimulation. Receptor activation induces an increase in the expression of GRKs, which phosphorylates G protein-coupled receptors to signal receptor desensitization and internalization (49). Both CXCR1 and CXCR2 are down-regulated in this manner after their stimulation with CXCL8 (50). These events appear to be responsible for the down-regulation of chemokine effects, including chemotaxis (51). Recently, it was demonstrated that NaHS augmented the expression of CXCR2 on neutrophils and thus facilitated MIP-2-directed migration of these cells (22). In this study, it was observed that H_2S improved neutrophil chemotaxis induced by MIP-2. Moreover, H_2S also prevented MIP-2-induced CXCR2 internalization and desensitization as well as MIP-2 induced GRK2 expression. The lack of effect of PAG on CXCR2 internalization induced by MIP-2 at a dose of 30 ng/ml could be explained by the fact that this dose of MIP-2 is too high and was not modulated by the amount of endogenously released H_2S. Supporting this hypothesis, a pharmacological dose of H_2S donor was effective in preventing the internalization of CXCR2 induced at this dose of MIP-2. Together, these data strongly suggest that, besides the direct effect on neutrophil-endothelium interaction, H_2S also augments neutrophil locomotion by a mechanism that is dependent on the inhibition of chemokine-induced GRK expression, leading to the prevention of CXCR2 desensitization and internalization.

We have demonstrated in this study that activation of the potassium channel is the mechanism by which H_2S results in enhanced P-selectin and ICAM-1 expression, leading to neutrophil rolling and adhesion, and ultimately resulting in neutrophil chemotaxis. Additionally, these fundamental inflammatory events, regulated by GRK2 up-regulation and CXCR2 internalization, are modulated by the same H_2S-K^+_{ATP} channel partnership. This corresponds with previous observations that activation of the K^+_{ATP} channel also mediates the vasodilatory effect of H_2S (17). Consistently, diazoxide and minoxidil, K^+_{ATP} channel openers, are reported to mimick the effect of a H_2S donors on neutrophil migration both in vivo and in vitro (this study and Ref. 29). Similarly, gliclazide, another K^+_{ATP} channel blocker, inhibited neutrophil adhesion, migration as well as the expression of adhesion molecules on endothelial cells induced by high glucose concentrations or insulin (52–54). However, it is important to reinforce the finding that glybenclamide does not affect the activity of CSE and cystathionine-β-synthetase, enzymes involved in H_2S production. In fact, it has been demonstrated that the glybenclamide treatment did not change the rise in plasma H_2S in animals submitted to hemorrhagic shock, and it did not interfere with the augmented biosynthesis of H_2S from cysteine in liver (38). Reinforcing that the CSE-H_2S/K^+_{ATP} pathway was activated during the inflammatory process and thus resulted in increased neutrophil migration, we additionally observed that the incubation of neutrophils with CXCL8 enhanced H_2S production. Moreover, glybenclamide treatment mimicked the investigated action of PAG and prevented the effects of H_2S on in vivo and in vitro neutrophil migration, as well as on CXCR2 internalization and GRK2 expression.

Consistent with the modulation of neutrophil function by agents that interfere with K^+_{ATP} channel activity, it had been shown that the response of neutrophils to inflammatory mediators is preceded or accompanied by membrane electrical depolarization and subsequent repolarization (55, 56). These changes increased the release of Ca^{2+} from intracellular stores and stimulated the uptake of extracellular Ca^{2+} ions (57); events that are thought to be primary steps in neutrophil migration and have been correlated with adhesion and locomotion (58, 59). Subsequent studies have shown that the initial increase in intracellular Ca^{2+} concentration in response to fMLP occurs together with membrane hyperpolarization and is dependent on an intact potassium gradient and coincident with cytoplasmic acidification (60). It is important to point out that there is evidence that diazoxide, a K^+_{ATP} channel opener, can attenuate endothelium P-selectin expression and neutrophil adhesion during ischemia-reperfusion (61). Differences in the experimental models could explain these contradictions.

In summary, our results demonstrate a role for the CSE-H_2S pathway as a modulator of key inflammatory events occurring at the interface of leukocytes and the vascular endothelium, during both innate and adaptive immune responses. Furthermore, the proinflammatory effects of H_2S appear to be mediated via activation of K^+_{ATP} channels. Together, these results identify H_2S synthesis and K^+_{ATP} channels as potential targets for novel anti-inflammatory therapies.

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Disclosures

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