A variety of stimuli, including monosodium urate (MSU) crystals, activate the NLRP3 inflammasome, and this activation involves several molecular mechanisms including xanthine oxidase (XO) up-regulation and mitochondrial dysfunction. Upon oligomerization of apoptosis-associated speck-like protein containing a CARD (ASC), caspase-1 becomes active and cleaves the proinflammatory cytokine IL-1β into its active secreted form. Hydrogen sulfide (H₂S), a gasotransmitter mainly produced by cystathionine-γ-lyase (CSE) in macrophages, could modulate inflammation. Here, we sought to investigate the effects of exogenous and endogenous H₂S on NLRP3 inflammasome activation in vitro and in vivo. Primed bone marrow–derived macrophages (BMDM) isolated from wildtype (wt) or CSE-deficient mice and human macrophages (THP1 cells and primary macrophages), were stimulated with MSU crystals in the presence or absence of a H₂S donor, sodium thiosulfate (STS) or GYY4137 (GYY). In murine and human macrophages in vitro, both STS and GYY inhibited MSU crystal–induced IL-1β secretion in a dose-dependent manner. Moreover, the H₂S donors inhibited MSU crystal–induced XO/caspase-1 activities, mitochondrial reactive oxygen species (ROS) generation, and ASC oligomerization. Accordingly, IL-1β secretion and XO/caspase-1 activities were higher in CSE-deficient BMDMs than in wt BMDMs. For in vivo studies, we experimentally induced peritonitis by intraperitoneal injection of MSU crystals into mice. GYY pretreatment ameliorated inflammation, evidenced by decreased IL-6/monocyte chemoattractant protein-1 (MCP-1) released into peritoneal lavages. Taken together, our results suggest that both exogenous (via H₂S donors) and endogenous (via CSE) H₂S production may represent approaches for managing, for example, acute gout or other inflammation conditions.

Hydrogen sulfide (H₂S) has been recognized as the third gasotransmitter next to nitric oxide (NO) and carbon monoxide (CO). It can diffuse freely in intra-/intercellular compartiments, act as an autocrine or paracrine signal, and mediate various physiological functions, including cytoprotection, neuromodulation, ischemic responses, and oxygen sensing (1). H₂S is produced in mammalian cells mostly as a product of the sulfur-containing amino acid (methionine and cysteine) metabolism. Two pyridoxal 5′-phosphate–dependent transulfuration enzymes, cystathionine-γ-lyase (CSE)² and cystathionine-β-synthase (CBS) are mainly involved in H₂S production using l-cysteine and homocysteine as substrates, respectively. Two other enzymes, cysteine aminotransferase and 3-mercaptoppyruvate sulfurtransferase (3-MST), could cooperate to generate H₂S; cysteine aminotransferase converts cysteine into 3-mercaptoppyruvate and 3-MST transfers its sulfur to other sulfur acceptors, thereby generating H₂S (2).

The inflammatory role of H₂S remains controversial because both pro-inflammatory and anti-inflammatory effects have been reported. For instance, an increased circulatory H₂S level has been associated with inflammation in acute pancreatitis and the treatment with propargylglycine (a CSE inhibitor) reduced the severity of the disease (3). In the murine model of cecal ligation and puncture-induced sepsis, plasma levels of H₂S, H₂S-synthesizing activity, and CSE expression were all increased, and propargylglycine treatment protected against sepsis, whereas NaHS further aggravated the inflammation (4). On the contrary, an anti-inflammatory role of H₂S has been described under different conditions, and H₂S-releasing drugs have been proposed as therapeutic tools (5). H₂S decreased the expression of endothelial adhesion molecules, leukocyte adherence, and cell recruitment in an air pouch model of inflammation (6). H₂S donors also reduced the expression of pro-inflammatory cytokines induced by LPS in RAW 264.7 cells (7). In a more recent publication (8), a new slow H₂S-releasing molecule...
administration reduced IL-1 levels in LPS-treated mice. Such contrasting H$_2$S effects can be mediated by different mechanisms such as interactions with ion channels, protein S-sulfhydration, or modulation of the second messengers like calcium or cAMP (9–11). Indeed, H$_2$S activates the nuclear factor erythroid 2-related factor 2 (Nrf2) antioxidant signaling pathway and thus up-regulates the expression of heme oxygenase-1 (HO-1) (12).

Inflammatory processes are mediated by protein complexes called inflammasomes. Several types of inflammasomes have been characterized by different sensor proteins: NLRP1, NLRP3, NLRC4, and AIM2 inflammasomes. NLRP3 inflammasome is formed by the NLRP3 sensor molecule, the adaptor protein ASC, and the effector caspase-1. Different stimuli can activate this inflammasome including both pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). The first signal, alternatively called priming, required for inflammasome activation is mediated by pro-inflammatory pathways such as the Toll-like receptor–mediated nuclear factor (NF)-κB activation. This signal drives NLRP3 up-regulation and pro-IL-1β transcription/translation. The second signal driven by different PAMPs like nigericin, and DAMPs, such as monosodium urate (MSU), promotes inflammasome assembly leading to the activation of caspase-1 that can catalyze the maturation of pro-IL-1β to active IL-1β (13).

Besides their different biological properties, NLRP3 inflammasome activators mediate inflammasome assembly through two main mechanisms: reactive oxygen species (ROS) production and potassium efflux (14–16). In particular, both PAMPs and DAMPs trigger ROS generation, which in turn induces the assembly of the NLRP3 inflammasome. In this context, we previously demonstrated that xanthine oxidase (XO)-derived mitochondrial ROS are the trigger for IL-1β release, and that XO blockade results in impaired IL-1β/caspase-1 secretion (17). The tight regulation of NLRP3 inflammasome activation is important to avoid potential damage. Deregulated inflammasome activation is linked to autoimmune and autoinflammatory diseases, such as gout (18). Gout is the most common form of inflammatory arthritis and its prevalence is more than 2% worldwide (19). Gout is caused by precipitation of MSU in articular joints of patients with hyperuricemia and is characterized by abrupt, self-limiting attacks of joint inflammation. During the first phase of a gout attack, MSU crystals precipitate within the joint, stimulate Toll-like receptors expressed by resident monocytes, and induce oligomerization and activation of the NLRP3 inflammasome, thereby activating caspase-1 activity and pro-IL-1β maturation to IL-1β (20, 21). Beside classical therapeutics using nonsteroidal anti-inflammatory drugs, glucocorticosteroids or colchicines (22), anti-IL-1 therapies have been developed but not well adopted due to some safety issues (23). Since existing therapies to treat gout still generate severe side effects or are not effective enough in all patient subtypes, finding new regulators of NLRP3 inflammasome activation is of major importance.

Endogenous regulators of NLRP3 inflammasome activation in macrophages, including double-stranded RNA–dependent protein kinase (PKR), guanylate-binding protein 5 (GBP5), and Nek7, have been reported (24). In addition, some natural products have been identified as regulators of NLRP3 inflammasome in macrophages, such as Aloë vera, red ginseng (25), curcumin, (26), sulforaphane present in cruciferous vegetables (25), and propolis. Interestingly, molecular mechanisms of sulforaphane and propolis involve cysteine modification of target proteins (27) and H$_2$S release (28), respectively. In this study, we demonstrate that H$_2$S is capable of inhibiting NLRP3 inflammasome by reducing XO activity, mitochondrial ROS production, ASC oligomerization, and caspase-1 activity.

**Results**

H$_2$S donors inhibit NLRP3 inflammasome activation in MSU or nigericin-stimulated macrophages

Two H$_2$S donors, GYY4137 (GYY) and STS, have been used to elevate intracellular H$_2$S levels. GYY action involves slow H$_2$S releasing to extracellular solutions followed by passive diffusion into the cells without transporters, whereas STS needs specific transporters to get into the cells in which it is then converted to H$_2$S. To evaluate H$_2$S impacts on inflammasome activation, we treated macrophages with either GYY or STS, and measured IL-1β secretion upon exposure to NLRP3 agonists. In primed murine and human macrophages (BMDM and THP1 cells, respectively), GYY inhibited MSU-induced IL-1β secretion in dose-dependent manners (Fig. 1, A and C). By contrast STS was effective in THP1 cells (Fig. 1B) but not in BMDM (Fig. 1A), which could be accounted for by specific expression of the STS transporter (SLC13α4) in THP1 cells. The RT-PCR C$_i$ values of SLC13α4 are 26.9 for THP1, whereas >40 (i.e. undetectable) for BMDM. Both GYY and STS inhibited MSU-induced IL-1β secretion in primary human macrophages (Fig. 1D).

To further test whether the decreased IL-1β secretion was caused by inhibitory actions of H$_2$S on caspase-1 activation and IL-1β processing, we analyzed caspase-1/IL-1β protein expression by Western blot. In line with the IL-1β ELISA results, H$_2$S donors inhibited both caspase-1 processing and IL-1β maturation that were induced by NLRP3 agonists (MSU crystals and nigericin), as evidenced by reduced active p20 caspase-1 and active p17 IL-1β levels in cell supernatants, respectively (Fig. 1E). Once again, GYY was effective for such protein level changes in all these cell types, whereas STS only in human macrophages (THP1 and primary cells, Fig. 1E).

To rule out the possibility that the observed inhibitory effects of H$_2$S donors could be due to increased cytotoxicity or decreased cell proliferation, we measured lactate dehydrogenase (LDH) release and MTT conversion. MSU increased cytotoxicity by 30% in both THP1 and BMDM cells, and H$_2$S donors co-treatment did not cause additional changes (Fig. S1, A and B). Cell proliferation assessed by MTT was also equivalent between MSU-treated cells and MSU/H$_2$S–treated cells (Fig. S1, C and D). Therefore, the inhibitory effects of H$_2$S donors could not be attributed to their effects on cell death or proliferation.
H₂S inhibits NLRP3 inflammasome

Figure 1. H₂S donors inhibited NLRP3 inflammasome activation induced by macrophage stimulation with MSU crystals and nigericin. IL-1β concentration determined by ELISA in cell supernatants. A, BMDM primed overnight with 100 ng/ml of Pam3Cys were stimulated for 6 h with 500 μg/ml of MSU, 25 mM STS, or increasing doses of GYY as indicated. B, THP1 primed overnight with 100 ng/ml of phorbol 12-myristate 13-acetate (PMA) were stimulated with 500 μg/ml of MSU in the presence of the indicated concentrations of STS. C, THP1 primed overnight with 100 ng/ml of PMA were stimulated with 500 μg/ml of MSU in the presence or absence of 25 mM STS or the indicated concentrations of GYY. D, primary human macrophages primed overnight with 100 ng/ml LPS were stimulated with 500 μg/ml of MSU in the presence or absence of 25 mM STS or 100 μM GYY for 6 h. E, cell extracts (XT) and supernatants (SN) were analyzed by immunoblots using anti-IL-1β and Caspase-1 antibodies. Equal amounts of protein loading were assessed by tubulin immunoblot. As an additional control of NLRP3 inflammasome activation, 10 μM nigericin was added 1 h before the end of the 6-h incubation period on cells previously treated or not with H₂S donors.

H₂S donors inhibit xanthine oxidase activity and mitochondrial ROS generation induced by NLRP3 inflammasome activators

We next investigated how H₂S inhibits NLRP3 inflammasome. First, H₂S did not influence phagocytosis of MSU crystals in THP1 cells (Fig. 2A). Second, H₂S is known to increase miR-21 expression and attenuate inflammasome formation/caspase-1 activation via miR-21-dependent mechanisms in cardiomyocytes (29); thus the effect of STS on nigericin-induced IL-1β secretion from THP1 cells was examined in the presence or absence of actinomycin D, a RNA synthesis inhibitor (Fig. 2B). STS inhibited IL-1β secretion even in the presence of actinomycin D, which ruled out the involvement of microRNA.

The third mechanism for the inhibitory H₂S actions on NLRP3 inflammasome could be regulation of XO, the oxidized form of xanthine dehydrogenase. We previously demonstrated that treatment with febuxostat (a XO-inhibitor) diminished MSU-induced mitochondrial ROS generation, NLRP3 inflammasome activation, and IL-1β secretion (17). Thus, we hypothesized that H₂S donors could have similar effects. MSU-induced XO activity was decreased by GYY in BMDM and STS in THP1 cells (Fig. 2C, left and center). Accordingly, different H₂S donors (NaHS, STS, and GYY) could inhibit enzyme activity of purified XO (Fig. 2C, right).

XO can generate mitochondrial ROS, and we and others reported that mitochondrial ROS contribute to the activation of NLRP3 inflammasome (17, 30, 31). We next measured mitochondrial ROS production in both BMDM and THP1 cells that were incubated with MSU crystals in the presence or absence of H₂S donors. In line with the observed inhibition of IL-1β secretion and XO activity, GYY but not STS efficiently reduced mitochondrial ROS production in BMDM, whereas both GYY and STS significantly inhibited mitochondrial ROS generation in THP1 cells (Fig. 2D). Taken together, these results indicate that H₂S inhibits both MSU crystal–induced XO activity and subsequent XO-generated mitochondrial ROS.

H₂S donors inhibit ASC oligomerization and caspase-1 activity induced by NLRP3 inflammasome activators

Activation of NLRP3 inflammasome results in the recruitment of ASC followed by the formation of high-molecular weight ASC oligomers, which in turn recruits caspase-1. Indeed, stimulation of THP1 cells with MSU crystals led to the formation of ASC dimers as well as ASC oligomers as detected in the cell pellets (Fig. 2E, upper panel). However, treatment of cells with STS markedly suppressed the ASC oligomerization. Such STS effects were not due to decreased total ASC levels, because STS did not affect ASC expression in cell lysates (Fig. 2E, middle panel). Furthermore, as expected, the levels of active caspase-1 (p20) and IL-1β (p17) upon MSU stimulation were markedly lower in STS-treated cell supernatants (Fig. 2E, lower two panels).
Another possible target of \( H_2S \) donors for NLRP3 inflammasome inhibition is caspase-1 itself. Caspase-1 has a crucial cysteine in its active site (Cys\(^{163} \)) and S-sulfhydration of this and/or other cysteines could modulate caspase-1 activity. Both MSU and nigericin induced caspase-1 activation in murine BMDM and human THP1 cells, and the activation was strongly suppressed by \( H_2S \) donors in both cell types (Fig. 3A). When supernatants of nigericin-treated or untreated THP1 cells were incubated with GYY, caspase-1 inhibition was comparable with the one obtained with Ac-YVAD-CHO, a caspase inhibitor (Fig. 3B). These results indicate that \( H_2S \) donors could target directly active caspase-1 present in supernatants.

To interrogate this in another system, we tested the effect of \( H_2S \) on a cell-free assay in which caspase-1 has been activated upon disruption of cell integrity (32). As expected, active p20 caspase-1 was detected upon incubation of cell lysate at 37 °C, and the specific caspase-1 inhibitor Z-YVAD-fmk blocked active caspase-1 generation. Incubation of cell lysates with STS also strongly inhibited caspase-1 activation; no caspase-1 p20 protein was detectable (Fig. 3C, left panel). In addition, caspase-1 activation was inhibited by STS as well as two unrelated \( H_2S \) donors (NaHS and GYY); their effects were comparable with Z-YVAD-fmk (Fig. 3C, right panel).

Caspase-8 has been recently shown to contribute to the canonical NLRP3 inflammasome signaling (33). The experiment that uncovered NLRP3-ASC-caspase-8-dependent IL-1β processing required prolonged stimulation of caspase-1-deficient cells. Indeed, prolonged nigericin and MSU stimulation of caspase-1-deficient BMDM led to significant increases in IL-1β secretion although the levels were 20–30 times lower than those obtained in caspase-1-expressing BMDM (Fig. S2, B, C, E, and F). As expected, the cytotoxicity (assessed by LDH) upon nigericin stimulation was lower in caspase-1-deficient BMDM than in wt BMDM (Fig. S2A) but was not significantly different in caspase-1 KO BMDM stimulated with MSU (Fig. S2D).

Interestingly, GYY inhibited IL-1β secretion in both wt and caspase-1-deficient BMDM, suggesting that GYY may block caspase-8 activity (Fig. S2, B, C, E, and F). Indeed, caspase-8 activity was induced by nigericin in caspase-1-deficient BMDM and GYY almost completely inhibited this induction (Fig. S2G). Consistently, ASC oligomerization was also found in caspase-1-deficient BMDM and it was inhibited by GYY treatment (Fig. S2H). All these results suggest that both caspase-1 and caspase-8 are the \( H_2S \) targets.

To assess if the inhibitory effects were specific for the inflammatory caspases-1/8 or can affect other caspases involved in the initiation and execution of apoptosis such as caspase-9 and caspase-3, respectively, we used staurosporine stimulation, a well known inducer of apoptosis (34). We found that indeed caspase-9 and -3 were induced upon staurosporine stimulation

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**Figure 2. Mechanisms involved in \( H_2S \) inhibition of NLRP3 inflammasome activation in macrophages.** A, phagocytosis assay: THP1 primed overnight with 100 ng/ml of PMA were incubated with 500 \( \mu \)g/ml of MSU in the presence or absence of 25 mM STS or 100 \( \mu \)M NaHS for 4 h. FACS analysis was performed using MSU positive cells with high cell granularity and size. B, IL-1β concentration was determined by ELISA in THP1 supernatants. Primed THP1 were preincubated for 30 min with 10 \( \mu \)g/ml of actinomycin D and 10 \( \mu \)M nigericin was added 1 h before the end of the 6-h incubation period on cells previously treated or not with 25 mM STS. C, XO activity in primed BMDM or primed THP1 stimulated with 500 \( \mu \)g/ml of MSU for 2 h in the presence or absence of 100 \( \mu \)M GYY or 25 mM STS, respectively. XO activity in XO purified from buttermilk preincubated or not for 1 h with 100 \( \mu \)M GYY or 25 mM STS. D, mitochondrial ROS in primed BMDM (overnight with 100 ng/ml of Pam3Cys) or primed THP1 (overnight with 100 ng/ml of phorbol 12-myristate 13-acetate) stimulated with 500 \( \mu \)g/ml of MSU for 1 h in the presence or absence of 100 \( \mu \)M GYY or 25 mM STS. E, ASC oligomerization was visualized by ASC Western blot analysis on pellets. ASC levels were determined in cell lysates and active caspase p20 and active IL-1β p17 in the corresponding supernatants by specific Western blots.
of cells (Fig. S3). GYY, a H₂S donor, did not block caspase-3 or caspase-9 activity in both BMDM and THP1 cells (Fig. S3, A and B). Taken together, we showed that H₂S donors specifically inhibited caspase-1/8 of NLRP3 inflammasome, without inhibiting caspase-3 and -9.

**H₂S donors inhibit NLRP3 inflammasome activation in both Nrf2-dependent and -independent pathways**

It has been demonstrated that H₂S activates Nrf2 by inactivating Keap1 by S-sulfhydration (35–37), and that MSU also promotes Nrf2 signaling and thus up-regulates HO-1 expression (12). Accordingly, in STS-stimulated THP1 cells, we found increased Nrf2 and HO-1 expression in the nuclear and cytoplasmic fractions, respectively (Fig. S4 A, upper panels). In addition, mRNA expression of the two direct targets of Nrf2, HO-1 and NADH-quinone oxidoreductase (NQO), was induced by MSU, GYY, and tert-butylhydroquinone (TBHQ, a well known Nrf2 activator (38)) in BMDM cells (Fig. S4 A, lower graphs). In line with Nrf2-dependent mechanisms for NLRP3 inflammasome inhibition by H₂S, GYY and TBHQ decreased IL-1β secretion to a similar extent (Fig. S4B).

**Figure 3. H₂S donors inhibited caspase-1 activity induced by NLRP3 inflammasome activators.**

A, caspase-1 activity assay: caspase-1 activity in supernatants of primed BMDM or THP1 cells incubated for 6 h with MSU crystals or 1 h with nigericin in the presence or absence of H₂S donors (25 mM STS or 100 μM GYY). B, GYY or inhibitor of caspase-1(Ac-YVAD-CHO) were added in supernatants containing active caspase-1 (from nigericin-stimulated cells). C, cell-free caspase-1 activity assay. Cell lysates of THP1 cells were incubated at 37°C during 45 min in the presence or absence of 10 μM Z-YVAD-fmk or H₂S donors as indicated: 25 mM STS, 100 μM GYY, or 100 μM NaHS. Negative control (first lane) was frozen as soon as the lysate was taken. The immunoblot was performed to detect caspase-1.
H₂S inhibits NLRP3 inflammasome

It has been reported that MSU-induced IL-1β secretion and NLRP3 inflammasome activation are inhibited by the knock-down of Nrf2 or by a HO-1 inhibitor (39). In agreement, MSU or nigericin-induced IL-1β secretion was lower in Nrf2-deficient BMDM than in wt BMDM, as well as in wt BMDM treated with the HO-1 inhibitor than in non-treated respectives (Fig. S4, C and D). In addition, GYY decreased IL-1β levels in wt and Nrf-2-deficient BMDM to a similar extent (Fig. S4E). Finally, even in the presence of the HO-1 inhibitor, GYY was still effective in inhibiting IL-1β secretion (Fig. S4F). Thus we conclude that H₂S donors could inhibit NLRP3 inflammasome activation in both Nrf2-dependent and Nrf2-independent pathways.

Endogenous H₂S production modulates NLRP3 inflammasome activation

We first assessed that CSE is the prominent H₂S-producing enzyme expressed in BMDM; CSE mRNA levels are 17- and 4-fold higher than those of CBS and 3-MST, respectively (data not shown). Because CSE-produced endogenous H₂S could modulate NLRP3 inflammasome, we stimulated primed BMDM from wt or CSE-deficient mice with MSU or nigericin. We found higher IL-1β secretion in CSE-deficient BMDM compared with wt BMDM by ELISA (Fig. 4A) and Western blots (Fig. 4B). Of particular notes, GYY treatment inhibited inflammasome activation similarly in wt and CSE-deficient BMDM (Fig. 4, A and B), indicating that CSE deficiency may influence NLRP3 activation most exclusively via H₂S generation within the cells. As we showed before that H₂S donors inhibited both MSU-induced XO activity and mitochondrial ROS generation, we hypothesized that, conversely, lowering endogenous H₂S levels could lead to opposite effects. Indeed, we found that XO activity was constitutively higher in CSE-deficient BMDM (Fig. 4C) and mitochondrial ROS generation upon MSU stimulation was slightly but significantly higher in CSE-deficient BMDM (Fig. 4D). Caspase-1 activity was also significantly increased under basal or stimulated (by MSU or nigericin) conditions in CSE-deficient cells (Fig. 4E). H₂S donors inhibited similarly caspase-1 activity from wt or CSE-deficient BMDM cells (Fig. 4E). Finally, ASC oligomerization was increased in MSU-stimulated CSE-deficient cells compared with wt cells (Fig. 4F). Taken together, these results indicate that endogenous H₂S generated by CSE could regulate NLRP3 inflammasome activation.

H₂S donors inhibit cytokine production during murine peritonitis

We further investigate the H₂S effects on inflammasome in vivo using a MSU-induced peritonitis model. This model is characterized by IL-1β and NLRP3 inflammasome–dependent migration of neutrophils into the peritoneal cavity (40). MSU
induced cell recruitment into the peritoneal cavity as expected but, surprisingly, GYY pretreatment failed to inhibit this leukocyte infiltrate (Fig. 5B). We could not detect significant secretion of IL-1β levels in peritoneal fluids in MSU-injected mice (Fig. 5C). Given the difficulties in detecting circulating IL-1β in mice (41), we tried another approach to evaluate inflammation in this model. Because IL-1β secretion could induce other cytokines such as IL-6 (42), we established in BMDM that IL-1β down-modulation was impacted on MSU-induced IL-6 and MCP-1 production (Fig. 5A and not shown). MSU injection led to significantly increased levels of these two pro-inflammatory proteins and pretreatment with H2S donors inhibited both productions (Fig. 5C). These findings indicate that H2S can modulate inflammatory signals in MSU-mediated acute peritonitis, although, in these experimental settings, H2S cannot robustly dampen neutrophil recruitment.

**Discussion**

The anti-inflammatory effect of H2S have been described, in particular on IL-6, TNF, and IL-1β production from both in vitro and in vivo studies (8, 11, 43). The molecular mechanisms underlying the inhibition of these cytokines in LPS-stimulated RAW 264.7 macrophages involve NF-κB inhibition and reduced transcription of IL-6, TNF, and IL-1β genes (8). In LPS-challenged THP1 cells, pre-treatment with NaHS inhibits IL-6 and TNF production. H2S may modulate histone modifications and thereby chromatin openness at the IL-6 and TNF-α promoters (44).

In vitro, IL-1β secretion requires two steps: the first is a priming step (which includes proIL-1β mRNA induction/translation), the second step is the maturation of pro-IL-1β to active IL-1β by inflammasome activation. By incubating already primed macrophages with NLRP3 inflammasome activators in the presence or absence of H2S donors, we gained insights into H2S donor effects on the second step. H2S donors, such as sulfide salts and GYY, consistently inhibited NLRP3 inflammasome activation in both BMDM and human macrophages. By contrast, STS was only active in human macrophages (not BMDM) that expressed the thiosulfate transporter SLC13a4 (45).
It was demonstrated before that some NLRP3 inflammasome activators could promote IL-1β secretion by triggering necrosis, independently of inflammasome activation (46). However, MSU-induced cytotoxicity was not affected by H2S in our experiments (Fig. S1), therefore excluding the possibility that the inhibitory effects of H2S on IL-1β secretion could be accounted for by decreased MSU-induced cytotoxicity. Additionally, the fact that H2S does not affect MSU-induced cell death (although H2S inhibits caspase-1 and -8 activity) is consistent with previously published observations in which MSU-induced cell death occurs in a NLRP3/caspase-1–independent, but cathepsins-dependent way (47).

NLRP3 inflammasome activation involves XO activity (17). We found that cellular XO activity, and subsequent XO-generated mitochondrial ROS production were both inhibited by H2S. In accordance, H2S donors blocked the activity of purified XO protein. The effect of H2S on XO activity could be exerted at different levels. First, H2S can act on the redox state of the xanthine oxidoreductase enzyme, which exists in the reduced form as xanthine dehydrogenase (XDH) and in the oxidized form as XO. The conversion of XDH into XO requires oxidative stress conditions such as increased intracellular ROS (48), and H2S can inhibit this conversion by its ROS scavenging capacity (49). H2S is also a powerful reductant (50) that can reduce XO into XDH, ultimately decreasing the amount of XO activity in the cells. Another possibility is the modification of the XO active site structure. The active site of XO contains a molybdenum center characterized by a square-pyramidal coordination geometry with an apical Mo = O, three sulfurs, and an oxygen in the equatorial plane (51). H2S could affect any of these sulfurs that are important for the enzymatic activity.

A disulfide bond is required for NLRP3 activation and subsequent ASC oligomerization. It has been proposed that regulation of disulfide bond formation by ROS is a critical point for NLRP3 inflammasome activation. Particularly, it has been speculated that NLRP3 is autoinhibited and, upon activation by ROS, NLRP3 became activated by forming a disulfide bond and changing its conformation (52). It is possible that H2S donors affect this disulfide bond, either directly by persulfhydration of NLRP3, or indirectly by decreased ROS generation. In the same context, Nek7 is required for NLRP3 inflammasome activation induced by different NLRP3 stimuli, including ATP, nigericin, MSU crystals, and alumn. In in vivo models of intraperitoneal MSU crystal injection, Nek7 was necessary to induce peritoneal IL-1β. Phosphorylated Nek7 interacts directly with NLRP3 to promote inflammasome assembly (53). Another possibility is that H2S could affect Nek7 interaction with NLRP3, thereby inhibiting inflammasome assembly.

ASC oligomerization requires ASC phosphorylation in more than one site, and this phosphorylation occurs after activation of Syk and Jnk pathways. In this context, it was demonstrated that NLRP3 and AIM2 inflammasomes but not the NLRC4 inflammasome require Syk and Jnk for formation of ASC specks and full inflammasome activity (54). Interestingly, a H2S donor inhibited Syk phosphorylation and degranulation of mast cells induced by antigenic and non-antigenic stimuli (55).

Caspases are cysteine proteases synthesized as a single-chainzymogen and activated by intrachain cleavage mediated by a specific initiator caspase (56). We found that H2S donors strongly inhibit caspase-1 activity in two different assays, and as efficiently as a caspase-1 inhibitor. Moreover, the H2S donor GYY inhibited caspase-8 activity. In a different cellular setting, H2S donors dose-dependently inhibited activity of recombinant caspase-3. Of note, the inhibitory effects of H2S were abolished by DTT, suggesting that the inhibition of caspase-3 was mediated by the reversible modification of protein thiols including persulfidation (45). We anticipated that this inhibitory effect of H2S could be mediated by persulfidation of crucial cysteine residues in caspases, especially the conserved cysteine residue of the active site. Future experiments will be aimed to identify persulfidated residues of caspase-1 and -8 in cells treated or not with H2S donors.

ASC oligomerization and caspase-1 activation are common steps in both NLRP3 and AIM2 inflammasome activation. As H2S impacted both ASC oligomerization and caspase-1 activity it is therefore anticipated that AIM2 activation would be as well impaired. Indeed, in BMDM, AIM2 inflammasome activation by a dsDNA, such as p(dA:dT), was inhibited by the H2S donor NaHS, as shown by decreased active IL-1β (p17) and caspase-1 (p20) in cell supernatants (Fig. S5A). In THP1, a similar effect was obtained; STS decreased IL-1β in supernatants upon AIM2 activation (Fig. S5B). Those results highlighted H2S as a negative regulator not only of NLRP3 inflammasome but also of AIM2 inflammasome and maybe of other caspase-1 containing inflammasomes such as NLRC4 and NLPR1.

Recently, anti-inflammatory properties have been described for caffeic acid phenethyl ester (CAPE), a compound present in propolis. CAPE is able to block inflammasome assembly by disrupting the NLRP3–ASC interaction (57). Interestingly, CAPE could induce CSE expression (28), suggesting that its effects are mediated by a cellular H2S level increase. Another interesting compound is sulforaphane, an isothiocyanate that reacts with sulfhydryl groups in protein cysteine residues. Sulforaphane inhibited IL-1β secretion via NLRP1, NLRP3, NLRC4, and AIM2 inflammasomes in BMDM, all in an Nrf2-dependent fashion, by reacting with cysteine residues of Keap1. Sulforaphane inhibited MSU-induced IL-1β secretion, suggesting that it may also inhibit NLRP3 inflammasome in vivo (58). All these results support our data on the inhibition of NLRP3 inflammasome activation by the gasotransmitter H2S.

Finally, we evaluated the anti-inflammatory potential of the H2S donor GYY in a model of MSU crystal–induced inflammation in mice. Our study showed that GYY treatment reduced levels of pro-inflammatory mediators. The moderate GYY effect obtained in vivo compared with the striking effects measured in vitro could be related to different H2S release kinetics and efficient H2S doses in the two experimental settings. Additionally, the limited effect of GYY on IL-1β activation we observed in MSU-induced peritonitis could reflect the limited caspase-1 contribution on IL-1β activation in vivo. Indeed, several experimental results (reviewed in Ref. 59) suggest that the in vivo part of IL-1β is activated via other enzymatic mechanisms that do not act via NLRP3/caspase-1 inflammasome.
We propose that H2S, endogenously produced in macrophages by CSE or locally produced by other cells at the site of inflammation, is one of the major negative regulators of NLRP3 inflammasome in MSU-induced inflammation. H2S has multiple targets in the pathway of NLRP3 inflammasome activation (XO activity, mitochondrial ROS production, ASC speck formation, and caspase-1 activity), thereby ensuring its inhibitory effect on IL-1β secretion (Fig. 6). Remarkably, even under extreme conditions (e.g. when IL-1β was strongly decreased via Nrf2 or HO-1 inhibition as shown in our study and in others (12, 39)), H2S was still inhibitory. Conclusively, our in vitro and in vivo results coherently substantiated the inhibitory effect of H2S on NLRP3 inflammasome activation. The fact that not only exogenous H2S but also endogenous H2S control NLRP3 inflammasome activation at multiple levels strongly suggest that this gas is of paramount significance in inflammation resolution.

Experimental procedures

Preparation of MSU crystals

Sterile, pyrogen-free MSU crystals were synthesized and determined to be endotoxin free (<0.01 endotoxin units/10 mg of crystal) by Limulus amebocyte lysate assay as previously described (40). Crystals were suspended in sterile phosphate-buffered saline (PBS) and dispersed by brief sonication.

Crystal-induced peritonitis

C57BL/6J mice (10–16 weeks old) were intraperitoneally injected with 1 mg of MSU in 0.5 ml of DMSO (final concentration 0.5%). To test H2S donors, mice were intraperitoneally injected with 50 mg/kg of GYY4137 (in 0.5 ml of DMSO 0.5% final concentration) 30 min before crystal administration. After 5 h, mice were euthanized by CO2 administration, and peritoneal exudate cells were subsequently harvested by performing lavage with 3 ml of PBS. Lavage fluids were centrifuged at 450 × g for 10 min and the supernatants were used for analysis of cytokines. Cells were subjected to flow cytometric analysis. This study was carried out in accordance with the guidelines set by the “Service de la consommation et des affaires vétérinaires du Canton de Vaud.” The protocol was approved by the state veterinarian.

Flow cytometric analysis

Peritoneal exudate cells were resuspended in fluorescence-activated cell sorting (FACS) buffer (5% fetal calf serum plus 5 mM EDTA in PBS) and incubated with conjugated monoclonal antibodies (mAb). The mAbs used were phycoerythrin-conjugated anti-Ly-6G (clone RB6–8C5), fluorescein isothiocyanate (FITC)–conjugated anti-CD11b (clone M1/70), and allophycocyanin-conjugated anti-F4/80 (clone BM8) (all from eBioscience). Peritoneal cells (1 × 10^7) were incubated with appropriate conjugated antibodies for 30 min at 4 °C in the dark. Stained cells were subsequently washed twice with FACS buffer and fixed in BD CellFIX solution (BD Biosciences). All data acquisition was performed on a FACS Calibur flow cytometer (BD Biosciences) using CellQuest software (BD Biosciences). Data analysis was performed using FlowJo software (Tree Star).

Preparation of BMDM

Bone marrow cells were isolated from the tibia and femurs of 8–12-week-old wt, CSE-deficient (60) or Nrf2-deficient mice (61) all in C57BL/6 background. For differentiation into BMDMs, the isolated cells were incubated for 7 days on Petri dishes in Dulbecco’s modified Eagle’s medium (Life Technologies) with 30% L929 conditioned medium (source of M-CSF), 10% FBS (PAA Laboratories GmbH, Austria), 1% HEPES (Life Technologies), and 1% penicillin/streptomycin (Life Technologies). After differentiation, cells were detached using cold PBS, and plated for stimulation experiments in Dulbecco’s modified Eagle’s medium with 10% FBS, 1% HEPES, and 1% penicillin/streptomycin.

Preparation of human primary macrophages and THP-1 cells

Human primary macrophages were obtained by culturing monocytes for 7 days in the presence of 50 ng/ml of human M-CSF (Milenyi Biotec). Monocytes were obtained from peripheral blood mononuclear cells kindly provided by Dr. Thierry Roger. THP-1 cells were cultured in RPMI1640 medium with 10% FBS, 1% HEPES, 1% penicillin/streptomycin (Invitrogen).

Cell stimulation

BMDM, THP-1 cells, or human macrophages were plated in 96-well dishes at a density of 1 × 10^5 cells/well. BMDM and human macrophages were primed overnight with 100 ng/ml of Pam3CSK4 (Invitrogen) and 100 ng/ml of ultrapure LPS (Invitrogen), respectively. THP-1 cells were primed overnight with 100 ng/ml of phorbol myristate acetate (Invivogen). Then, primed cells were stimulated with nigericin (10 μM, AppliChem GmbH, Germany) or MSU crystals (500 μg/ml) for the indicated time and treated or not with the H2S donors, STS or GYY (Sigma). Pan (pantothenate)-caspase inhibitor Z-YVAD-fmk (20 μM, Enzo Life Sciences), the hemoxigenase inhibitor Zn(II) deuteroporphyrin IX 2,4-bis-ethyleneglycol (2.5 μM, Enzo Life Sciences), or 10 μM of the Nrf2 activator TBHQ (10 μM, Sigma) were used.
**ELISA**

Mouse and human IL-1β and IL-6 ELISA kits (eBioscience, Inc., San Diego, CA) were used to measure the corresponding cytokine levels in the supernatants according to the manufacturer’s instructions.

**Measurement of LDH and MTT**

LDH is a stable cytosolic enzyme released from cells after cell damage. LDH in the supernatants was measured using CytoTox-ONE™ Homogeneous Membrane Integrity Assay (Promega) according to the manufacturer’s instructions. LDH release (%), therefore the percentage of cytotoxicity, was calculated by using the following formula: LDH release (%) = [(value in samples) – (background)] /[(value in Triton X-100-treated samples) – (background)] × 100. To measure viability, MTT reagent (1 mg/ml in sterile PBS) was directly added to wells and incubated for 3 h at 37 °C protected from light. MTT solution was removed and ethanol (95%) was added to dissolve formazan crystals. The solution was transferred in a V bottom plate and spun down. Supernatant was transferred to a flat bottom transparent plate to measure absorbance at 570 nm using as reference 630 nm. The percentage of cell viability was calculated using the following formula: viability (%) = (OD sample – OD 0% viability)/(OD 100% viability – OD 0% viability).

**Measurement of mitochondrial ROS levels**

Mitochondrial ROS in cells was measured by MitoSOX (Invitrogen) fluorescence as described previously (17).

**XO activity**

Cell lysates were tested for XO activity using the pterin assay. Briefly, cell lysates from 10⁶ cells or XO purified from butter milk (Life Technologies, A22182) were incubated with 50 μM pterin and the conversion to isoxanthopterin was measured by fluorescence as described in Ref. 17.

**Immunoblot**

Cells were lysed in a buffer containing 20 mM HEPES, pH 7.5, 0.5% Nonidet P-40, 50 mM KCl, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA and protease inhibitors. Cell culture supernatants were precipitated by methanol/chloroform as previously described. Samples were then immunoblotted using the following antibodies: anti-mouse caspase-1, anti-NLRP3, anti-ASC. Samples were then precipitated by methanol/chloroform as previously described. The pellets were frozen immediately after obtaining, were used as negative control. The other assay, based on a specific bioluminescent method, was performed on cell supernatants to detect caspase-1 (Caspase-Glo 1 Inflammasome Assay, Promega) and caspase-8 (Caspase-Glo 8 Assay, Promega) activities following the manufacturer’s instructions using Ac-YVAD-CHO as a specific caspase-1 inhibitor. Caspase-9 and -3 activities were measured in cell lysates (Caspase-Glo 9 and 3 Assay, Promega) of cells stimulated 36 h with 500 nm staurosporine (Sigma).

**Determination of ASC oligomerization**

Cell lysates were centrifuged at 330 × g for 10 min at 4 °C, and the pellets were washed, resuspended in PBS, and then cross-linked by incubation with disuccinimidyl suberate for 30 min. Pellets and soluble lysates were simultaneously analyzed by Western blot using anti-ASC antibody (MBL).

**Caspase-1, -8, -9, and -3 activity**

For caspase-1 activity, two different assays were performed. The first was a cell-free assay performed as described in Ref. 62. Briefly, cells were lysed in buffer W (20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl₂, 1 mM sodium EDTA, 1 mM sodium EGTA, and 0.1 mM PMSF) and lysates were incubated with or without H₂S donors or Z-YVAD-fmk under agitation at 37 °C for 45 min. A cell lysate sample, frozen immediately after incubation, was used as negative control. The other assay, based on a specific bioluminescent method, was performed on cell supernatants to detect caspase-1 (Caspase-Glo 1 Inflammasome Assay, Promega) and caspase-8 (Caspase-Glo 8 Assay, Promega) activities following the manufacturer’s instructions using Ac-YVAD-CHO as a specific caspase-1 inhibitor. Caspase-9 and -3 activities were measured in cell lysates (Caspase-Glo 9 and 3 Assay, Promega) of cells stimulated 36 h with 500 nm staurosporine (Sigma).

**H₂S inhibits NLRP3 inflammasome**

Briefly, cells were lysed in TRIzol (Invitrogen), and RNA was extracted using the DirectZol RNA extraction kit (ZymoResearch) and reverse transcribed to complementary DNA using Superscript II (Invitrogen). Relative expression levels of RNA transcripts were determined using gene-specific primers, SYBR Green, and the LightCycler 480 system (Roche). Gene-specific primers were TATA-binding protein (Tbp), 5’-CCGTTGAATTCTTGCTGTAAAC-3’ and 5’-TCCAGAATCTGAAATACCAACGC-3’; Gapdh, 5’-CTCATGACCACTGCTATGC-3’, 3’-CACATTTGGGATGAGAAC-5’; Il-1β, 5’-CTGACGAA- GTGACGCCATCTGTGAG-3’ and 5’-ACATCGACAGCCCTACCAAGTCCA-3’; and Nqo-1, 5’-TGCTAGAGATGAC- TCDDGAAGG-3’ and 5’-AGGTGGAGGAGGTACTGAATC-3’. Relative expression levels of genes of interest were calculated using a 2^ΔΔCT method with Tbp and Gapdh as reference genes.

**AIM2 inflammasome assay**

Primed immortalized BMDM (iBMDM) (63) and THP1 were transfected with p(dA-dT) (Invitrogen) using 2 μl/ml of Lipofectamine 2000 (Thermo Fisher Scientific, Invitrogen), and treated or not for 6 h with 25 mM STS (for THP1) or 100 μM NaHS (for iBMDM). Supernatants were analyzed by ELISA or IL-1β and Caspase-1 immunoblot.

**Statistical analysis**

All data are expressed as mean ± S.D. of triplicates from one representative experiment of at least two independent experiments. For two-group comparisons, Student’s t test was used. For multiple comparisons, one-way analysis of variance was used. All data were statistically analyzed using GraphPad PRISM software version 6.01 (GraphPad, La Jolla, CA). Differences with a probability value of <0.05 were considered significant (*, p < 0.05; **, p < 0.01; †††, p < 0.001; ††††, p < 0.0001).

**Author contributions**—M. C. performed the majority of the experiments and wrote the paper. J. L. performed some experiments and reviewed the manuscript. D. E. performed the in vivo experiment. S. N. set-up some assays and reviewed the manuscript. I. L. gave the CSE-deficient mice and reviewed the manuscript. A. S. reviewed the manuscript. F. M. contributed to experimental design and in the writing of the manuscript. N. B. conceived, coordinated the study, and wrote the manuscript. All authors reviewed the results and approved the final version of the manuscript.
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