Interaction between hydrogen sulfide-induced sulfhydration and tyrosine nitration in the K_\text{ATP} channel complex

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Kang M, Hashimoto A, Gade A, Akbarali HI. Interaction between hydrogen sulfide-induced sulfhydration and tyrosine nitration in the K_\text{ATP} channel complex. Am J Physiol Gastrointest Liver Physiol 308: G532–G539, 2015. First published December 31, 2014; doi:10.1152/ajpgi.00281.2014.—Hydrogen sulfide (H_2S) is an endogenous gaseous mediator affecting many physiological and pathophysiological conditions. Enhanced expression of H_2S and reactive nitrogen/oxygen species (RNS/ROS) during inflammation alters cellular excitability via modulation of ion channel function. Sulfhydration of cysteine residues and tyrosine nitration are the posttranslational modifications induced by H_2S and RNS, respectively. The objective of this study was to define the interaction between tyrosine nitration and cysteine sulfhydration within the ATP-sensitive K\textsuperscript{+} (K_\text{ATP}) channel complex, a significant target in experimental colitis. A modified biotin switch assay was performed to determine sulfhydration of the K_\text{ATP} channel subunits, Kir6.1, sulphonylurea 2B (SUR2B), and nitrotyrosine measured by immunoblot. NaHS (a donor of H_2S) significantly enhanced sulfhydration of SUR2B but not Kir6.1 subunit. 3-Morpholinosydnonimine (SIN-1) (a donor of peroxynitrite) induced nitration of Kir6.1 subunit but not SUR2B. Pretreatment with NaHS reduced the nitration of Kir6.1 by SIN-1 in Chinese hamster ovary cells cotransfected with the two subunits, as well as in enteric glia. Two specific mutations within SUR2B, C24S, and C1455S prevented sulfhydration by NaHS, and these mutations prevented NaHS-induced reduction in tyrosine nitration of Kir6.1. NaHS also reversed peroxynitrite-induced inhibition of smooth muscle contraction. These studies suggest that posttranslational modifications of the two subunits of the K_\text{ATP} channel interact to alter channel function. The studies described herein demonstrate a unique mechanism by which sulfhydration of one subunit modifies tyrosine nitration of another subunit within the same channel complex. This interaction provides a mechanistic insight on the protective effects of H_2S in inflammation.

Hydrogen sulfide (H_2S) is an endogenous gas involved in several biological functions, including neuromodulation, smooth muscle relaxation, inflammation, insulin release, and metabolic demand. H_2S is predominantly produced in mammalian tissues from L-cysteine by the enzymes cystathionine \( \beta \)-synthetase and cystathionine \( \gamma \)-lyase (16, 25). H_2S converts cysteine residues to thiocysteines (i.e., modifies the -SH group to -SSH; \( S \)-sulfhydration) in many proteins, including actin, tubulin, and GAPDH, and the K_\text{ATP} channel (17).

K_\text{ATP} channels play an important role in cellular function observed in a variety of tissues, including smooth muscles, pancreatic \( \beta \)-cells, myocardium, and neurons (18, 29). K_\text{ATP} channel in the smooth muscle colon is a heterooctamer consisting of two main subunits, a pore-forming subunit (Kir6.1) and a sulfonylurea receptor subunit (SUR2B) (8). H_2S \( S \)-sulfhydrates the sulfonylurea subunit, SUR2B, of the K_\text{ATP} channel in colonic smooth muscle. The \( S \)-sulfhydration of the SUR2B by H_2S provides an allosteric modulation of the channel, resulting in enhanced potency for the K_\text{ATP} channel opener, levomomalakilim (6).

Since the discovery of endogenous H_2S, many studies have explored the role of this gas in animal models. In an experimental model of colonic inflammation, enhanced H_2S synthesis is thought to result in faster resolution of the inflammation (22, 23). Furthermore, inhibition of K_\text{ATP} channel in colitis rats also induces significant mortality (24), indicating that H_2S and K_\text{ATP} channels are important components in ulcerative colitis. The mechanism by which K_\text{ATP} channel is affected may be through sulfhydration of specific cysteine residues (17). However, the molecular mechanisms involved in such modulation are not fully elucidated. Previously, we showed in mouse models of colonic inflammation that oxidative stress is also associated with increased tyrosine nitration in smooth muscle (1). The increased expression of tyrosine-nitrated calcium channels in inflamed colon prevents the binding and regulation of the calcium channel by \( c \)-Src kinase (11, 21).

In the present study, we explore the interaction of \( S \)-sulfhydration and tyrosine nitration within the K_\text{ATP} channel complex. Here, we demonstrate that sulfhydration of the SUR2B subunit prevents nitration of the pore-forming Kir6.1 subunit of the channel. This type of interaction provides mechanistic insight into the protective effects of H_2S in colonic inflammation.

MATERIALS AND METHODS

All animal protocols conformed to the guidelines and approval by the Virginia Commonwealth University Institutional Animal Care and Use Committee.

**Cell culture and transient transfection.** Chinese hamster ovary (CHO) cells were purchased from the American Type Cell Collection...
(ATCC), CHO cells were cultured in Dulbecco’s modified Eagle’s medium/F12 medium supplemented with 0.2 mg/ml geneticin and 10% fetal bovine serum (Invitrogen). Cells were grown in six-well plates overnight and transfected with Kir6.1, SUR2B, enhanced green fluorescent protein, a combination of plasmid DNAs, or with Cav1.2b and β2-subunit by Genejuicer (Stratagene), as described by the manufacturer. Untransfected CHO cells were maintained in the same media. After ~24–48 h of incubation, protein samples were isolated and were quantified using a protein assay kit (Pierce). Cells were maintained under standard cell culture conditions at 37°C and 5% CO2 in a humid environment.

**Immunohistochemistry.** Immunohistochemical staining was performed by in-cell Western blot analysis with specific antibodies to each protein. For quantitation of protein sulfhydration, samples were run on blots alongside total lysates. Anti-SUR2B and anti-Kir6.1 were used at 1:1,000 dilutions.

**In-cell Western blot assay.** Concentration-response measurements were performed by in-cell Western (ICW) blot assay. Enteric glial cells (EGCs) were obtained from the ATCC (CRL 2690) and cultured and maintained in Dulbecco’s modified Eagle’s medium/F12 medium supplemented with 0.2 mg/ml geneticin and 10% fetal bovine serum (Invitrogen). EGCs are derived from rat jejenum myenteric plexus. EGCs were stimulated by adding ONOO− (Cayman Chemical) twice at 5-min intervals. Protein samples were prepared as previously described (9) and were examined for nitration using anti-nitrotyrosine (NY) (cat. no. 487923-50UG RRID:AB_641022, Santa Cruz Biotechnology) or anti-SUR2B antibody (cat. no. sc-5793 RRID:AB_2219773, Santa Cruz Biotechnology). Standard protocols for Western blots were performed using site-specific antibodies. Protein samples were run on 5% and 10% SDS-PAGE and transferred on nitrocellulose membranes (Bio-Rad). Membranes were blocked with 3% nonfat milk blocking buffer with 0.1% Tween 20. Specific antibodies were used for blotting at concentrations of 1:200 to 1:1,000. The membranes were then incubated with secondary antibodies at concentrations of 1:1,000 to 1:5,000 and visualized using the Odyssey Infrared Imaging System (Licor Biosciences).

**Whole cell KATP currents.** Isometric tension recording. After a seal was obtained, cells were voltage clamped, and K+ currents were generated by progressive 10-mV depolarizing steps (500-ms duration, 5-s intervals) from a holding potential of −60 mV. KATP currents were measured in a high K+ (300 mM) in dimethyl sulfoxide CO2 in a humid environment.

**Immunofluorescence.** Immunofluorescence staining was carried out as described (6). In brief, cells treated with or without 1 mM NaHS (Cayman Chemical) were homogenized in 250 mM Hepes-NaOH (pH 7.4), 1 mM EDTA, 2.5% SDS, and 0.1 mM neocuproine (HEN buffer) supplemented with 100 µM deferoxamine. Protein samples (250 µg) were added to blocking buffer (HEN buffer adjusted to 1% SDS (HENS buffer). Biotin-N-[6-(biotinamido)hexyl]-3- pyridyldithio)propionamide (4 µM) in dimethyl sulfoxide was added to the suspension. After 3 h of incubation, biotinylated proteins were precipitated by streptavidin-agarose beads, which were then washed with HENS buffer. The biotinylated proteins were eluted by SDS-PAGE sample buffer and subjected to Western blot analysis with specific antibodies to each protein. For quantitation of protein sulfhydration, samples were run on blots alongside total lysates. Anti-SUR2B and anti-Kir6.1 were used at 1:1,000 dilutions.

**Site-directed mutagenesis.** The mutants were constructed using the QuikChange Site-Directed Mutagenesis System (Stratagene) according to the manufacturer’s procedures. Murine Kir6.1-cDNA (Open Biosystems) inserted into pcDNA3 (Invitrogen) was used as the template. Primers were designed by Vector NTI program (Invitrogen), containing the desired mutation in the middle region (Table 1). Sequences that are underlined indicate site mutated and change in the amino acid [cysteine (C) to serine (S)]. The presence of the desired mutation was confirmed by DNA sequencing of the relevant DNA region.

**Modified biotin switch assay.** The biotin switch assay was carried out as described (6). Table 1. **Primer sequences containing the desired mutations**

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>C6S</td>
<td>GAAATGAGCCTTCTTCTTTTGAGAACACATCTCC</td>
<td>GAGAGGAGATGGTTCCAGAAGAAGAGGCTTACCTCC</td>
</tr>
<tr>
<td>C2AS</td>
<td>GTGTCGCAAAACCTGCCTTTGAGAAGACTTACCG</td>
<td>GAGAGGAGATGGTTCCAGAAGAAGAGGCTTACCTCC</td>
</tr>
<tr>
<td>C263S</td>
<td>GGGGGGTCAGAAATTGTCCTTCGAAAAAGGACTTACCG</td>
<td>CTTTACATGCGCTCTTTGGAGGAGCTAATTGCTGACCCCC</td>
</tr>
<tr>
<td>C706S</td>
<td>GTACAGCAAGTTGCTCTTGAGAACAAATTCATTTCCTCC</td>
<td>GAGAGAAGAGATGGTTCCAGAAGAAGAGGCTTACCTCC</td>
</tr>
<tr>
<td>C785S</td>
<td>GCTGTCGAGGACTGAGCTGAGGAGAGCTTACCG</td>
<td>GAAGATGCTGCGAGAGAGGAGGAGGTGAGAGAGGAGGC</td>
</tr>
<tr>
<td>C818S</td>
<td>GTCGAGGAGGACTGAGCTGAGGAGAGCTTACCG</td>
<td>GAGAGGAGATGGTTCCAGAAGAAGAGGCTTACCTCC</td>
</tr>
<tr>
<td>C1410S</td>
<td>CTCGAGAGGACTGAGCTGAGGAGAGCTTACCG</td>
<td>CAGAGGAGGAGGCTTACCTCCAGTGACCCCC</td>
</tr>
<tr>
<td>C1455S</td>
<td>CAGAGGAGGACTGAGCTGAGGAGAGCTTACCG</td>
<td>AAAAGGCGCTGCGAGAGAGGAGGAGGTGAGAGAGGAGGC</td>
</tr>
</tbody>
</table>

Underlined sequences indicate site mutated and change in the amino acid, with cysteine (C) to serine (S).

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performed as described previously (21). Approximately 1.5-cm strips of mouse ileum were suspended in the longitudinal direction in an organ bath containing calcium-free Krebs solution (118 mM NaCl, 4.6 mM KCl, 1.3 mM NaH₂PO₄, 1.2 mM MgSO₄, 25 mM NaHCO₃, and 11 mM glucose), bubbled continuously with carbogen (95% O₂ and 5% CO₂) at 37°C under a resting tension of 1 g, and equilibrated for a period of 1 h. Isometric contractions were recorded by a force transducer (model no. GR-FT03, Radnoti) connected to a personal computer using Acqknowledge 382 software program (BIOPAC Systems). After equilibration in Krebs solution, tissues were incubated for 45 min in calcium-free high-potassium solution (80 mM) in which equimolar NaCl was replaced by KCl and changed every 15 min. Tissues were preincubated for 20 min either in the presence or absence of NaHS (100 μM). Tissues were washed with Krebs solution after the 20-min preincubation, and cumulative concentration-dependent contraction responses to CaCl₂ (1 μM to 3 mM) were performed. The results were expressed as percentages of the maximal response for CaCl₂ alone, and the curves were statistically compared.

Statistical analysis. All experiments were performed and repeated at least three times. Results are presented as the means ± SE for the number of cells (n). SigmaPlot 12.5 software was used for detection of differences in paired or unpaired t-tests as appropriate, and EC₅₀ values were calculated using four-parameter logistic nonlinear regression model in SigmaPlot. A P value of <0.05 was considered significant.

RESULTS

Dithiothreitol reduces sulfhydration of SUR2B. Recently, we reported that, in cells cotransfected with Kir6.1 and SUR2B and in freshly isolated mouse colonic smooth muscle cells, NaH₂S induces sulfhydration of SUR2B as cysteine thiols (–SH) react with H₂S to form hydropersulfides (–SSH) (6). Figure 1 shows the effect of the reducing agent, dithiothreitol (DTT), on Kₐtp channel sulfhydration in CHO cells transfected with Kir6.1 and SUR2B. Cell lysates were isolated and treated with 1 mM NaHS, 1 mM DTT, or the combination and subjected to the modified biotin switch assay with antibody against SUR2B to detect Kₐtp channel sulfhydration. There was basal sulfhydration of SUR2B (0.20 ± 0.025), and this sulfhydration was significantly enhanced with NaHS treatment (0.35 ± 0.07) (Fig. 1A). There was no significant effect of DTT on the basal sulfhydration of SUR2B subunit. However, treatment with DTT significantly reversed H₂S-mediated sulfhydration (0.21 ± 0.05). H₂S did not induce sulfhydration of Kir6.1 subunit of the channels (6) (not shown). Thus SUR2B sulfhydration is a covalent modification involving sulfhydryl groups. The presence of functional Kₐtp channels upon cotransfection of Kir6.1 and SUR2B was confirmed by whole cell voltage clamp in CHO cells. Levomakalim-induced currents were measured at a holding potential of -60 mV in the presence of high K⁺ solution with low internal ATP to isolate the Kₐtp currents as described previously (6). Levomakalim-induced currents were significantly inhibited by the channel blocker, glibencamide (Fig. 1B).

Cysteine residues affect sulfhydration. To determine the specific cysteine residues within SUR2B that are sulfhydrated, a preliminary analysis of the possible cysteine residues was carried out using the program PSIPRED, and the mutants were
constructed using the QuickChange Site-Directed Mutagenesis System. The following cysteine residues were identified as most likely to be involved, based on the preliminary analysis: C6, C24, C263, C706, C818, C1410, and C1455. CHO cells were transfected with Kir6.1 and SUR2B or mutants of SUR2B and subjected to the modified biotin switch assay with antibody against SUR2B to detect $\Delta k_{ATP}$ channel sulfhydration following NaHS. Figure 2 shows that mutation of cysteine 24 (C24S) and 1455 (C1455S) reduced sulfhydration, C24S (0.12 ± 0.023) and C1455S (0.12 ± 0.035) compared with control (0.31 ± 0.033) (Fig. 2). The other mutants did not affect the extent of sulfhydration of the channel.

**Sulfhydration prevents tyrosine nitration.** An interaction between tyrosine nitration and H2S has been suggested in neuronal cells (26), in interstitial cells of Cajal, and in processes involving apoptosis/necrosis and poly(ADP-ribose) polymerase 1 activation (4, 27, 30). We examined the interaction of H2S-induced sulfhydration with tyrosine nitration in the $\Delta k_{ATP}$ channel complex. In CHO cells cotransfected with the two subunits, treatment with SIN-1 and ONOO$^-$ resulted in nitration of Kir6.1 but not SUR2B (Fig. 3, A and B). Immunoprecipitation with anti-Kir6.1 followed by immunoblot with anti-NY revealed that the extent of nitration of Kir6.1 was significantly reduced by pretreatment with NaHS (1 mM). NaHS treatment did not affect the basal level of nitration of Kir6.1. The reduction in nitration by NaHS was also evident when examined by immunohistochemistry. Cells cotransfected with Kir6.1 and SUR2B or the C1455S were treated with SIN-1 or NaHS or the combination. Tyrosine nitration was readily observed in cells treated with SIN-1 but markedly reduced in the presence of NaHS (Fig. 3C). To further confirm that SUR2B subunit is the potential target for sulfhydration, the C1455S mutant was cotransfected with Kir6.1 and examined for tyrosine nitration following sulfhydration. As shown in Fig. 3C, bottom, mutation of C1455 resulted in robust expression of tyrosine nitration, indicative of the requirement of sulfhydration of C1455 for the reduction in tyrosine nitration. The effect of NaHS was further evaluated in EGCs by ICPW blot assay. Tyrosine nitration was evaluated over a 1,000-fold concentration range. We also examined the level of GFAP, marker for EGCs, to establish the validity of cells in each well. Significant increase in total cellular tyrosine nitration in EGCs was observed at the concentration of 1 and 3 mM ONOO$^-$ stimulation (1,054 ± 101-fold and 516 ± 49-fold, respectively, n = 4) (Fig. 3D). Pretreatment with NaHS reduced the level of nitration in the EGCs to 181 ± 19-fold (1 mM ONOO$^-$) and 84 ± 23-fold (3 mM ONOO$^-$), respectively (n = 4, ***p < 0.001). The expression of GFAP remained constant across the concentration range. These data suggest that NaHS may have the potential to inhibit tyrosine nitration formation.

*Mutation of cysteine residues prevents nitration.* The above data suggest that Kir6.1 is the potential target for tyrosine nitration, whereas cysteine residues in SUR2B subunit are sulfhydrated, and that sulfhydration prevents tyrosine nitration. To test this, the cysteine residues C24 and C1455 that were identified as potential sulfhydration sites were mutated to serine residues. In CHO cells cotransfected with SUR2B, wild-type, C263S (control), C24S, and C1455S with Kir6.1 were examined for prevention of nitration by NaHS. Figure 4, A and B, shows that SIN-1-induced nitration of Kir6.1 is reduced by NaHS in wild-type and in C263S SUR2B mutant but not in C24S or C1455S mutants. None of the mutants of SUR2B were nitrated by SIN-1 (Fig. 4C). We next examined whether tyrosine nitration of Kir6.1 could be reversed by NaHS. Enhanced tyrosine nitration of Kir6.1 was detected with SIN-1 treatment (2.024 ± 0.58), but, interestingly, NaHS did not reduce postnitration of Kir6.1 (2.11 ± 0.26) (Fig. 4D). These data suggest that sulfhydration of SUR2B is required for reducing nitration.

**Sulfhydration prevents Ca$^{2+}$ channel nitration.** Previously, we have shown that L-type calcium channels in colonic smooth muscle cells are tyrosine nitrated during colonic inflammation, resulting in reduced currents and inhibition of contractions (21). Cav1.2 channels in rat cardiomyocytes have been suggested to be sulfhydrated (31). We therefore examined whether tyrosine nitration of Cav1.2b is reduced by NaHS. Figure 5 shows that pretreatment with NaHS reduced the nitration of Cav1.2b.

**H$_2$S prevents ONOO$^-$-induced inhibition of CaCl$_2$-induced contraction.** To further evaluate the physiological role of H$_2$S on tyrosine nitration, CaCl$_2$-induced contractions of the isolated mouse ileum were measured in the presence and absence of ONOO$^-$. Mouse ileum tissues were incubated in a depolarizing calcium-free, high-potassium (80 mM) physiological saline solution. A cumulative concentration response to CaCl$_2$ was established in control tissues. Preincubation with ONOO$^-$ (150 µM) for 10 min significantly reduced the calcium-induced contraction and shifted the dose-response curve to the right (Fig. 6). Tissues preincubated with NaHS (100 µM) for 15 min followed by ONOO$^-$ resulted in a significant recovery of CaCl$_2$-induced contractions. The EC$_{50}$ values for CTL, H$_2$S + ONOO$^-$, and ONOO$^-$ were 143.3 ± 15.5 µM, 197.3 ± 23.5 µM, and 270.2 ± 23.7 µM, respectively. These data demon-
strate that the inhibition of the calcium influx under depolarizing conditions by ONOO−/H11002 is reduced by NaHS.

**DISCUSSION**

H2S is emerging as a highly significant endogenous gaseous mediator and potential target for pharmacological manipulation (5, 15). It is known to have several intracellular targets, of which KATP channels represent a significant target for H2S, particularly in colonic inflammation. Previously, we have shown that the KATP channel opener levcromakalim and NaHS-induced currents are enhanced in a 4,6-trinitrobenzene sulfonic acid model of colonic inflammation. An enhancement in the S-sulfhydration of the KATP channel subunit SUR2B is also seen after colonic inflammation, which may allow for the allosteric modulation of the channel. In the present study, we examined the potential sites within the KATP channel complex that are sulfhydrated and determined the interaction with nitration of tyrosine residues. We have found that 1) NaHS significantly enhanced sulfhydration of SUR2B but not Kir6.1 subunit; 2) SIN-1 (a donor of ONOO−) induced nitration of Kir6.1 subunit but not SUR2B; 3) two specific mutations within SUR2B, C245S and C1455S, prevented sulfhydration by NaHS, and these mutations prevented NaHS-induced reduction in tyrosine nitration of Kir6.1; 4) NaHS also reversed ONOO−-induced inhibition of smooth muscle contraction.

Ion channel activity can be significantly altered by the intracellular redox state maintained by reactive oxygen species (ROS) and reactive nitrogen species (RNS). Physiological generation of these compounds is highly regulated; however, an imbalance occurs in pathophysiological conditions, such as in colonic inflammation. Generated ROS/RNS can directly induce posttranslational modifications at specific residues and alter the ion channel function. These include redox modification of cysteine residues by ROS, S-nitrosylation by nitric oxide, and/or nitration of tyrosine residues by ONOO−. Our studies demonstrate that, within a single protein complex involving two subunits, there is significant specificity, as sulfhydration occurred in the SUR2B but not Kir6.1, whereas nitration occurred in Kir6.1 but not SUR2B. Since the discovery of endogenous H2S, many studies have explored the roles of this gas in animals and humans and have shown H2S to be involved in diverse physiological and pathophysiological pro-

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**Fig. 3. Tyrosine nitration is attenuated by H2S.** A and B: representative immunoblot analyses of tyrosine nitration proteins in CHO-transfected cells. Cells were lysed in immunoprecipitation assay buffer (RIPA), and Kir6.1 (A) or SUR2B (B) was immunoprecipitated (IP) using anti-Kir6.1 or anti-SUR2B and immunoblotted using anti-nitrotyrosine antibody (NY). The bar graph represents the ratio of nitration of Kir6.1 (A) and SUR2B (B) against total Kir and SUR2B, respectively (means ± SE from 3 independent experiments). *Significant difference between 3-morpholinosydnonimine (SIN-1)-induced nitration of Kir6.1 and in the presence of NaHS. C: representative immunostaining for tyrosine nitration in CHO-transfected cells. CHO cells were transfected with Kir6.1 and SUR2B or C1455S and treated with 100 µM SIN-1 for 20 min, 1 mM NaHS for 10 min, or a combination of both. Cells were visualized by fluorescence confocal microscopy. Scale bar = 20 µm. D: in-cell Western of enteric glial cells treated with the indicated doses of peroxynitrite (ONOO−) for 15 min in the absence or presence of NaHS (1 mM, 15 min) and processed for imaging (bottom), as described in MATERIALS AND METHODS. The image shows whole cell fluorescence from actual wells of a 96-well plate, highlighting the increase in tyrosine nitration signal. The signal is background subtracted and normalized to control. Bar graph (top) shows the fold induction of tyrosine nitration measured as intensity over the control (absence of ONOO−). Values represent the means ± SE. ***Significant difference between ONOO−-treated vs. NaHS + ONOO−-treated cells. GFAP, glial fibrillary acidic protein.
cesses. However, our understanding of the pharmacology and the pathophysiological roles of H$_2$S in the gastrointestinal (GI) tract is still quite rudimentary. There is growing evidence that, during colonic inflammation, including ulcerative colitis, there is an increased production of H$_2$S in the GI tract (23) that may have protective effects during colitis. In the present study, we demonstrate that sulfhydration of one subunit (SUR2B) of the KATP channel prevents the tyrosine nitration in the other subunit (Kir6.1). We also show that H$_2$S sulfhydrates SUR2B at C24 and C1455 residues and causes the reversal of tyrosine nitration of Kir6.1 in the KATP channel complex. This suggests that sulfhydration of cysteine residues of SUR2B on either side of the membrane is required to alter the tyrosine nitration of Kir6.1, as C24 is localized on the extracellular side, whereas C1455 is localized toward the intracellular side. The specific mechanism by which these cysteine residues affect modulation of the K$_{ATP}$ currents requires further work, but it is noteworthy that K$_{ATP}$ channel activity is markedly enhanced in a model of experimental colitis (8). In addition to the allosteric effects on channel function, the present studies show that sulfhydration also appears to reduce tyrosine nitration of the pore-forming subunit Kir6.1. Because postnitration of Kir6.1 is not reversed by NaHS after tyrosine nitration. Kir6.1 and wild-type SUR2B were transiently expressed and treated with SIN-1 (100 μM, 20 min) followed by NaHS (150 μM, 15 min). The cell lysates were immunoprecipitated using Kir6.1 antibody and then immunoblotted for tyrosine nitration. The graph represents normalized data for tyrosine nitration and is presented as means ± SE from 3 independent experiments. *Significant difference between wild-type and SIN-1.

Fig. 4. Tyrosine nitration of Kir6.1 and the effect of cysteine mutations of SUR2B. A and B: representative immunostaining for tyrosine nitration in CHO-transfected cells. Wild-type or mutants of SUR2B were transiently expressed, immunoprecipitated using Kir6.1 antibody, and then immunoblotted for tyrosine nitration of Kir6.1 using anti-NY antibody. The bar graph represents normalized data for tyrosine nitration and is presented as means ± SE from 3 independent experiments. *Significant differences between CTL vs. SIN-1 and SIN-1 vs. NaHS + SIN-1. Cells were treated with NaHS (150 μM, 15 min), SIN-1 (100 μM, 20 min), or both. B: representative Western blots of immunoprecipitated Kir6.1 and immunoblotted with anti-NY. C: representative immunoblot analyses of tyrosine nitration of SUR2B proteins in CHO-transfected cells. Wild-type or mutants of SUR2B were transiently expressed, treated with SIN-1 (100 μM, 20 min), immunoprecipitated using anti-SUR2B, and immunoblotted using antibody for tyrosine nitration proteins (NY). D: representative SIN-1-induced tyrosine nitration of Kir6.1 is not reversed by NaHS after tyrosine nitration. Kir6.1 and wild-type SUR2B were transiently expressed and treated with SIN-1 (100 μM, 20 min) followed by NaHS (150 μM, 15 min). The cell lysates were immunoprecipitated using Kir6.1 antibody and then immunoblotted for tyrosine nitration. The bar graph represents normalized data for tyrosine nitration and is presented as means ± SE from 3 independent experiments. *Significant difference between wild-type and SIN-1.
Previous work has shown that nitration of the tyrosine residues within the COOH terminus of Cav1.2 prevents Src-kinase-mediated tyrosine phosphorylation, leading to decreased calcium currents (9–12, 20, 21). Cav1.2 channels can also be sulfhydrated (31). The inhibition by tyrosine nitration of calcium-induced contractions that occurs as a result of calcium influx under depolarizing conditions is reduced by NaHS. Thus, similar to the findings for the K\textsubscript{ATP}/H11001 channel, sulfhydration may also reduce tyrosine nitration in Cav1.2. However, further works needs to done to identify the cysteine residues involved in the sulfhydration of the Cav1.2. Occurrence of this interaction in the K\textsubscript{ATP} channel complex in transfected CHO cells and EGCs and in Cav1.2 in transfected CHO cells demonstrates the ubiquitous nature of this phenomenon. This unique interaction within a protein complex may provide a mechanistic basis by which H\textsubscript{2}S affords a protective role in colonic dysmotility during colitis. Enhanced production of endogenous H\textsubscript{4}S and that via resident luminal bacteria may compete with the RNS produced during colonic inflammation. ONOO\textsuperscript{-} is enhanced during colonic inflammation as a result of the uncoupling of nitric oxide synthase (NOS) and enhanced expression of inducible NOS (3).

Ion channels play a central role in the regulation of cell excitability; therefore, altered regulation of ion channels as a consequence of protein modification is of significance, not only for colonic inflammation, but also in other disease states and may help provide novel approaches in channel-targeted therapeutics. Our findings that sulfhydration of the K\textsubscript{ATP} channel prevents the detrimental effects of tyrosine nitration within a

![Fig. 5. Sulfhydration prevents Ca\textsuperscript{2+} channel nitration. Bar graph represents nitration of calcium channel in CHO-transfected cells. Ca\textsuperscript{2+} channel (Cav1.2b) was transiently expressed and treated with NaHS (150 \mu M, 15 min), SIN-1 (100 \mu M, 20 min), or NaHS followed by SIN-1. Protein was immunoprecipitated using Cav1.2b antibody and then immunoblotted for tyrosine nitration. The bar graph represents normalized data for tyrosine nitration and is presented as means ± SE from 3 independent experiments. *Significant differences between NaHS and SIN-1.](image1)

![Fig. 6. Effect of ONOO\textsuperscript{-} or H\textsubscript{2}S + ONOO\textsuperscript{-} on the cumulative CaCl\textsubscript{2}-induced concentration-dependent contractions in isolated mouse ileum. Maximal contractions of control group (CTL) were considered as 100%. The CaCl\textsubscript{2} contractions were elicited in tissues depolarized by high-potassium (80 mM) physiological saline solution. A: representative graph showing the cumulative CaCl\textsubscript{2} concentration-response curves by ONOO\textsuperscript{-} and H\textsubscript{2}S + ONOO\textsuperscript{-} in mouse ileum. B: representative tracing of CaCl\textsubscript{2}-induced concentration-dependent contractions in isolated ileum from mouse. Points represent means ± SE. Data were analyzed by repeated-measures ANOVA followed by Bonferroni’s posttest. *P ≤ 0.05 compared with ONOO\textsuperscript{-}. #P ≤ 0.05, ##P ≤ 0.01 vs. ONOO\textsuperscript{-} compared with CTL, n = 3–4.](image2)

![Fig. 7. A schematic of the proposed protective effects of H\textsubscript{2}S in colonic inflammation. Exogenous H\textsubscript{2}S enhances sulfhydration of 2 cysteine residues (24 and 1455) within the SUR2B subunit of ATP-sensitive K\textsuperscript{+} (K\textsubscript{ATP}) channels, which prevents tyrosine nitration within the Kir6.1 subunit of the K\textsubscript{ATP} channel complex.](image3)
single ion channel complex provide a novel basis to examine the antioxidant properties of \( H_2S \). (Fig. 7).

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**GRANTS**

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**

Author contributions: M.K. and H.I.A. conception and design of research; M.K., A.H., and A.R.G. performed experiments; M.K. and H.I.A. analyzed data; M.K., A.H., A.R.G., and H.I.A. interpreted results of experiments; M.K. and A.R.G. prepared figures; M.K. and H.I.A. edited and revised manuscript; M.K., A.H., and H.I.A. drafted manuscript.

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