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Hydrogen sulfide-induced vasodilation mediated by endothelial TRPV4 channels

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Naik JS, Osmond JM, Walker BR, Kanagy NL. Hydrogen sulfide-induced vasodilation mediated by endothelial TRPV4 channels. *Am J Physiol Heart Circ Physiol* 311: H1437–H1444, 2016. First published October 7, 2016; doi:10.1152/ajpheart.00465.2016.—Hydrogen sulfide (H_2S) is a recently described gaseous vasodilator produced within the vasculature by the enzymes cystathionine γ-lyase and 3-mercaptopyruvate sulfurtransferase. Previous data demonstrate that endothelial cells (EC) are the source of endogenous H_2S production and are required for H_2S-induced dilation. However, the signal transduction pathway activated by H_2S within EC has not been elucidated. TRPV4 and large-conductance Ca^{2+}-activated K channels (BK channels) are expressed in EC. H_2S-induced dilation is inhibited by luminal administration of iberiotoxin and disruption of the endothelium. Calcium influx through TRPV4 may activate these endothelial BK channels (eBK). We hypothesized that H_2S-mediated vasodilation involves activation of TRPV4 within the endothelium. In pressurized, phenylephrine-constricted mesenteric arteries, H_2S elicited a dose-dependent vasodilation blocked by inhibition of TRPV4 channels (GSK2193874A, 300 nM). H_2S (1 μM) increased TRPV4-dependent (1.8-fold) localized calcium events in EC of pressurized arteries loaded with fluo-4 and Oregon Green. In pressurized EC tubes, H_2S (1 μM) and the TRPV4 activator, GSK101679A (30 nM), increased calcium events 1.8- and 1.5-fold, respectively. H_2S-induced an iberiotoxin-sensitive outward current measured using whole cell patch-clamp techniques in freshly dispersed EC. H_2S increased K^+ currents from 10 to 30 pA/pF at +150 mV. Treatment with Na_2S increased the level of sulfhydration of TRPV4 channels in aortic ECs. These results demonstrate that H_2S-mediated vasodilation involves activation of TRPV4-dependent Ca^{2+} influx and BK channel activation within EC. Activation of TRPV4 channels appears to cause calcium events that result in the opening of eBK channels, endothelial hyperpolarization, and subsequent vasodilation.

hydrogen sulfide; vasodilation; endothelium; TRPV4; BK channel

NEW & NOTEWORTHY

This is the first study to demonstrate that H_2S-induced vasodilation involves TRPV4 channels and strengthens our previous observation that endothelial large-conductance Ca^{2+}-activated K^+ channels (BK) participate in vasodilation in small mesenteric arteries.

HYDROGEN SULFIDE (H_2S) is a recently described gaseous vasodilator produced within the vasculature by the enzymes cystathionine γ-lyase (CSE) and 3-mercaptopyruvate sulfurtransferase. Several studies suggest diminished H_2S production associated with the development of hypertension in humans (16, 46, 51) and animal models (54). In addition, genetic deletion of CSE in mice results in the development of hypertension with age. Arterial pressure of CSE−/− mice was 20 mmHg higher at 12 wk of age compared with age-matched controls, suggesting that impairment of the CSE/H_2S system contributes to elevated blood pressure (54).

Our laboratory has demonstrated in mesenteric arteries that endothelial cells (EC) are the source of endogenous vascular H_2S production and are required for exogenous H_2S-mediated dilation (14, 16). Indeed, disruption of the endothelium abolished dilation in response to exogenous H_2S (14). Recent evidence suggests H_2S-dependent dilation in some arteries requires activation of large-conductance Ca^{2+}-activated K^+ channels (BK channels, 14–16, 26). However, the signal transduction pathway activated by H_2S within EC is not defined.

Current dogma suggests ECs express few BK channels and/or that endothelial BK (eBK) do not participate in vascular control. However, increasing evidence substantiates that native endothelial cells express functional eBK (14, 50). In vascular smooth muscle (VSM), BK channels are activated by rapid, localized Ca^{2+}-release events through ryanodine channels, termed Ca^{2+} sparks (17). In addition, the endothelium produces a number of dilator substances including heme oxygenase (HO)-derived carbon monoxide (24) and the cytochrome 2C eicosanoid, 11,12-EET (4), that target the BK channel directly or indirectly through Ca^{2+} spark activation. Indeed, the mechanisms of BK channel activation and their role in vasodilation are well established in vascular smooth muscle (VSM). However, it is unclear if eBK channels are regulated in a similar manner to BK channels in VSM.

Transient receptor potential cation channel V4 (TRPV4) conduct Ca^{2+} into the cytosol in response to a number of stimuli (1, 3, 34, 43, 56) and are expressed in ECs (29) and VSM cells (7). Activation of EC TRPV4 channels produces rapid, spatially localized, transient calcium events, termed Ca^{2+} sparklets (44, 45). Endothelial small- and intermediate-conductance Ca^{2+}-sensitive K^+ channels are activated by calcium entry through TRPV4 channels by a mechanism analogous to the Ca^{2+} spark in VSM (2, 42). However, it is unknown if eBK channels are regulated by localized calcium-release events through endothelial TRPV4 channels. Thus we hypothesized that H_2S-mediated vasodilation involves activation of TRPV4 and BK channels within the endothelium.

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MATERIALS AND METHODS

Male Sprague-Dawley rats (Harlan Laboratories, 250–275 g) were used for all experiments. Rats were euthanized with a lethal concentration of pentobarbital sodium (200 mg/kg ip), and mesenteric arteries were collected for all experiments. The Institutional Animal Care and Use Committee of the University of New Mexico School of Medicine reviewed and approved all animal protocols. All protocols conformed to National Institutes of Health guidelines for animal use.

Isolated mesenteric artery preparation. Fourth- or fifth-order mesenteric artery segments (<100 μm) were isolated in HEPES buffer [containing (in mM) 130 NaCl, 4 KCl, 1.2 $MgSO_4$, 4 $NaHCO_3$, 1.8 $CaCl_2$, 10 HEPES, 1.18 KH_2PO_4, 6 glucose], cannulated, and then pressurized to 65–75 mmHg in a vessel chamber (Living System Instrumentation). Endothelium-intact arteries were equilibrated in physiological salt solution [PSS; containing (in mM) 129.8 NaCl, 5.4 KCl, 0.83 $MgSO_4$, 0.43 NaH_2PO_4, 19 $NaHCO_3$, 1.8 $CaCl_2$, and 5.5 glucose], which was heated to 37°C, gassed with 21% 0_2-6% CO_2-balance N_2, and superfused at a rate of 5 ml/min. To examine the heterogeneity of the response to H_2S along the vascular tree, experiments were also performed using large (>400 μm) mesenteric arteries.

Vasodilation studies. Arterial inner diameter was recorded in cannulated, pressurized (75 mmHg) arteries using edge-detection software (IonOptix). Arteries were equilibrated at 37°C in warmed, oxygenated PSS for 30 min prior to the start of the experiment. Mesenteric arteries from naive rats exhibit little myogenic tone (16). Thus, following equilibration, arteries were preconstricted to ~50% resting diameter using phenylephrine (PE), and vasodilation was measured during cumulative addition of the H_2S donor NaHS or the TRPV4 agonist (GSK1016790A). Arteries were incubated in Ca^{2+}-free PSS (in mM: 129.8 NaCl, 5.4 KCl, 0.83 $MgSO_4$, 0.43 NaH_2PO_4, 19 $NaHCO_3$, 3.7 tetrasodium EGTA, and 5.5 glucose) to determine passive diameter at the end of the experiment. In a subset of experiments, artery segments were pretreated with the TRPV4 channel antagonist, GSK2193874 (300 nM, GlaxoSmithKline), luminal iberiotoxin (100 nM, IbTx), or *N*-nitro-L-arginine (100 μM, L-NNA) or underwent endothelial disruption by rubbing the lumen of the artery with a strand of moose mane inserted into the free distal end of a vessel as previously described (18). In these arteries, dislodged endothelial cells were flushed from the artery before the distal end was cannulated and secured with silk sutures.

Endothelial cell Ca^{2+} measurements. Isolated, cannulated, and pressurized mesenteric arteries (65 mmHg) were loaded intraluminally with high-K_d Ca^{2+} indicator fluo-4 AM (5 μM), low-K_d Ca^{2+} indicator Oregon Green 488 BAPTA-1 AM (5 μM), and 0.25% pluronic F-127 in HEPES buffer for 15 min at room temperature. After equilibration at 33°C, endothelial cell Ca^{2+} events were assessed by exciting the fluorophores with a solid-state 488-laser and emissions > 500 nm were collected using an Olympus IX71 microscope with a ×40 oil-immersion objective and a spinning-disk confocal scanning unit (Andor Technology). Five hundred images were collected at a frame rate of 50–60 Hz. Ca^{2+} events were quantified using the ImageJ plugin LC Pro, with a 15-pixel diameter region of interest (ROI) and a $P < 0.05$ level of significance.

Preparation of endothelial cell tubes. Mesenteric arteries (~200 μm) were placed in HEPES buffer containing 10 μM sodium nitroprusside and 0.1% bovine serum albumin (BSA) and cleared of adipose tissue. Arteries were flushed of blood and exposed to a HEPES-buffered digestion solution containing 0.1% BSA, 0.62 mg/ml papain, 1.5 mg/ml collagenase II, and 1 mg/ml dithiothreitol for 40 min at 37°C. Arteries were individually triturated to remove adventitia and smooth muscle layers. An individual endothelial cell tube was then transferred to a vessel chamber (Living Systems Instrumentation) and cannulated, pressurized to 2 mmHg, and loaded with fluo-4 AM, Oregon Green 488 BAPTA-1 AM, and 0.25% Pluronic F-127 in HEPES buffer for 15 min at room temperature (RT). Endothelial cell calcium events under basal conditions and in response to NaHS (1–10 μM) or GSK1016790A (10 nM) were measured as described above.

Whole cell K^+ currents. Mesenteric arteries were cut into 2-mm segments and exposed to digestion solution containing 0.2 mg/ml dithiothreitol, 5 U/ml elastase, 7 U/ml displace, and 12 U/ml papain in HEPES buffer at 37°C for 45 min. Arteries were removed from the digestion solution and placed in 1 ml of HEPES buffer containing 2 mg/ml BSA. Single endothelial cells were released by gentle trituration with a small-bore Pasteur pipette. A few drops of the resulting cell suspension were seeded on a glass coverslip mounted on an inverted fluorescence microscope (Olympus IX71) for 30 min prior to superfusion. Endothelial cells were superfused under constant flow (2 ml/min) at room temperature (22–23°C) in an extracellular solution (in mM: 150 Na^+-glutamate, 5.0 K^+-glutamate, 2 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, 5 glucose, buffered to pH 7.4 with NaOH). Whole cell current data were generated using an Axopatch 200B amplifier (Axon Instruments). Biophysical criteria (seal resistance > 1 GΩ, series resistance < 5× pipette resistance) were checked following membrane rupture and monitored throughout the course of the experiment. Cells were held at −60 mV and dialyzed for 5 min with an intracellular solution (in mmol/l: 135 K^+-glutamate, 5 Mg_2ATP, 10 HEPES, 5 EGTA tetrasodium salt adjusted to pH 7.2 with KOH). $CaCl_2$ was added to yield a free-Ca^{2+} concentration of 1 μmol/l, as calculated using WinMAXC chelator software. Voltage ramps were performed from −100 mV to +150 mV over 200 ms. Perforated patch experiments were performed with pipettes back-filled with intracellular solution containing 300 μM amphotericin B.

Sulfhydration of TRPV4. Sulfhydration of TRPV4 was measured using methods modified from Sen et. al. (40). Briefly, rat aortic endothelial cells (passage 5) were treated with vehicle or 10 μM Na_2S for 30 min at 37°C. Cells were then lysed, homogenized, and TRPV4 was immunoprecipitated using the Dynabeads Co-Immunoprecipitation kit (Invitrogen). Each sample (n = 4/group) was divided into three tubes with one tube from each sample reserved for the determination of total TRPV4 protein using standard Western blot. The remaining two samples from each rat were treated with Alexa Fluor 680-conjugated C2 maleimide (2 μM) for 2 h at 4°C, and then one was treated with and the other without DTT (1 mM) for 1 h at 4°C. Samples underwent gel electrophoresis, were transferred to PVDF membranes, and scanned with the Li-COR Odyssey system. Images were quantified using ImageJ. The level of fluorescence in each sample was normalized to the total TRPV4 protein level in each sample.

Statistical analysis. Data are presented as means ± SE and were analyzed using a one-way ANOVA, two-way ANOVA, or Student's *t*-test, as appropriate (Graphpad Prism). $P < 0.05$ was considered statistically significant for all analyses.

Reagents. GSK2193874A was generously provided by GlaxoSmithKline. Oregon Green 488 BAPTA-1 AM, fluo-4 AM, and pluronic F-127 were purchased from Life Technologies (Grand Island, NY). Collagenase II and papain were purchased from Worthington Biochemical (Lakewood, NJ). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

RESULTS

Effect of TRPV4 inhibition on exogenous H_2S dilation. Endothelial small- (SK) and intermediate-conductance (IK) Ca^{2+}-sensitive K^+ channels are activated by calcium entry through TRPV4 channels by a mechanism analogous to the Ca^{2+} spark in VSM (2, 42). The role of TRPV4 Ca^{2+} entry in H_2S-mediated vasodilation was assessed in pressurized mesenteric arteries (Fig. 1). Administration of the H_2S donor NaHS to the superfusate elicited a concentration-dependent vasodilation (Fig. 1, 1×10^{-9} to 1×10^{-6} M), which was

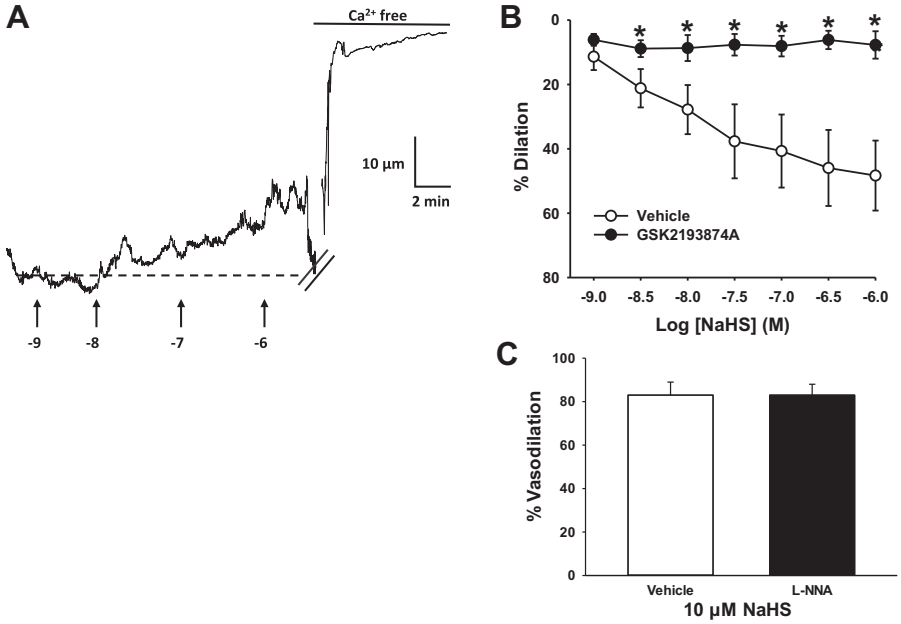

Fig. 1. H_2S-induced vasodilation in mesenteric arteries. *A*: representative trace of the dilation in response to NaHS administration in a phenylephrine preconstricted artery. *B*: exogenous H_2S (NaHS) produced a concentration-dependent vasodilation in pressurized mesenteric arteries that was blocked by luminal administration of the TRPV4 inhibitor GSK2193874A (300 nM), $n = 5$/group. *C*: NaHS-induced dilation is unaltered by nitric oxide synthase inhibition (L-NNA, 100 μM). *$P < 0.05$, different from Veh.

abolished in arteries pretreated with the TRPV4 inhibitor GSK2193874A. Interestingly, a bolus of 1 μM H_2S dilated 100 μm or less inner diameter mesenteric arteries by 90% ± 13. However, arteries > 400 μm did not respond to H_2S administration (3% ± 1) but dilated in response to treatment with 1 μM acetylcholine (96% ± 1).

Effect of nitric oxide synthase inhibition on H_2S dilation. To examine the role of NO in H_2S-mediated vasodilation, the response to a H_2S donor was examined in the presence and absence of nitric oxide synthase inhibition (Fig. 1*C*, 100 μM L-NNA). Administration of the H_2S donor NaHS (10 μM) to the superfusate under control conditions produced a dilation which was unaltered in arteries pretreated with L-NNA.

Role of eBK in TRPV4-dependent vasodilation. To assess if direct activation of TRPV4 channels produced an eBK-dependent vasodilation, a single concentration of TRPV4 agonist GSK101679A (10 nM) was administered in the superfusate to pressurized, PE-constricted mesenteric arteries (Fig. 2). To ensure GSK1016790A produced a TRPV4-sensitive dilation, we showed that the TRPV4 antagonist blocks GSK1016790A dilation. Moreover, disruption of the endothelium or pretreatment with luminal IbTx inhibited the vasodilatory response to GSK1016790A.

Analysis of TRPV4-dependent Ca^{2+} events. The spatiotemporal characteristics of H_2S-stimulated TRPV4-dependent Ca^{2+}-events were assessed using isolated, pressurized mesenteric arteries (Fig. 3, *A–D*). H_2S increased total endothelial Ca^{2+} events, which were prevented in arteries pretreated with GSK219387A. Basal Ca^{2+} events were not affected by inhibiting TRPV4 channels (Fig. 3*A*). Endothelial Ca^{2+} events that were H_2S-induced and TRPV4-sensitive displayed an average duration of 0.10 ± 0.01 s (Fig. 3*B*), had a spatial spread of 13.5 ± 0.48 μm^2 (Fig. 3*C*), and an average amplitude of 1.14 ± 0.005 F/F_0 (Fig. 3*D*).

Ca^{2+} dynamics in isolated endothelial cell tubes. TRPV4 channels are expressed on VSM and endothelial cells (7). To confirm that GSK1016790A acts directly on the endothelium to activate TRPV4-dependent Ca^{2+} events, endothelial cell tubes were prepared and loaded with fluorescent Ca^{2+} indicators. The addition of 10 nM GSK1016790A to the superfusate increased the frequency of localized Ca^{2+} events (Fig. 4).

BK currents in mesenteric endothelial cells. Conventional whole cell patch-clamp recordings were performed in enzymatically dissociated mesenteric endothelial cells (Fig. 5). Pretreating cells with the SK and IK channel inhibitors (apamin and TRAM-34, respectively) abolished K^+ currents in a majority of endothelial cells. However, a subpopulation of cells displayed residual K^+ current that was sensitive to the BK channel inhibitor, IbTx. Using the perforated patch configuration in the presence of SK/IK channel inhibition, cells that exhibited a residual current responded to TRPV4 activation with increased outward current (Fig. 6). In contrast, GSK1016790A had no effect in cells that lacked a significant

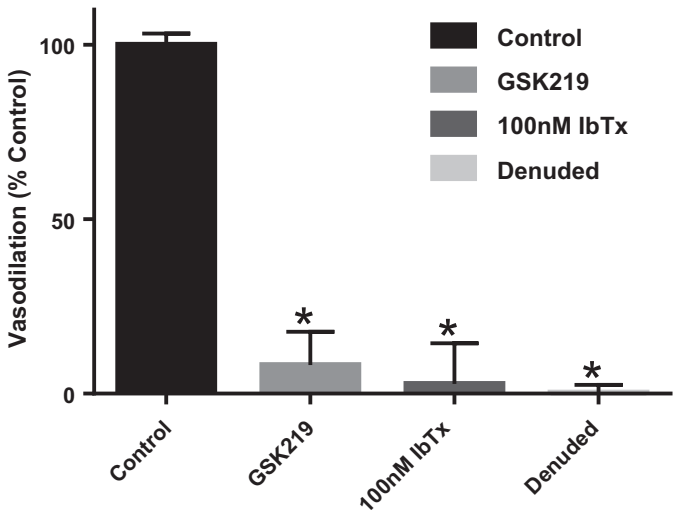

Fig. 2. TRPV4-dependent vasodilation (GSK1016790A; 10 nM) was inhibited by disruption of the endothelium (denuded) or pretreating arteries with the TRPV4 antagonist GSK2193874A (300 nM) or luminal iberiotoxin (Ibtx, 100 nM). $n = 5$/group. *$P < 0.05$, different from control.

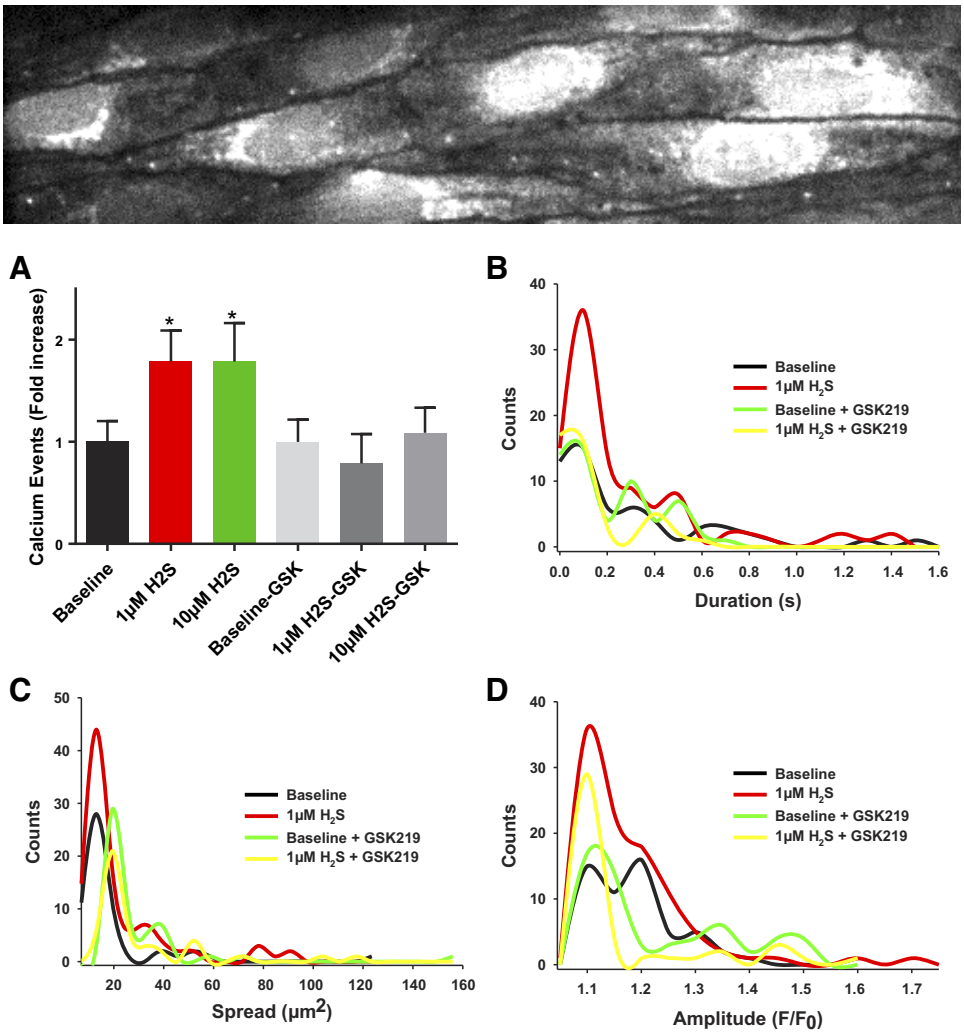

Fig. 3. Endothelium of pressurized mesenteric arteries was loaded with the fluorescent Ca^{2+} indicators, fluo-4 AM (5 μM) and Oregon green BAPTA-1 AM (5 μM). *A*: H_2S increased Ca^{2+}-event frequency that was prevented by pretreatment with the TRPV4 antagonist GSK2193874A (300 nM); n = 7/group. *B–D*: histogram analysis of 1 μM H_2S-elicited Ca^{2+}-events (n = 418 events): duration (s; *B*), spatial spread (μm^2; *C*), and amplitude (F/F_0; *D*). *$P < 0.05$, different from baseline.

baseline current when SI/IK channels were inhibited. Two cells that had a residual current in the presence of Tram-34 and apamin exhibited a decrease in current in response to GSK1016790A and were excluded from the analysis.

TRPV4 sulfhydration in aortic endothelial cells. The maleimide sulfhydration assay was performed in rat aortic endothelial cells treated with Na_2S or vehicle. Fluorescently labeled maleimide binds to sulfhydryl groups. Treatment with DTT reduces only sulfhydrated cysteines, resulting in a decrease of the fluorescent signal in proteins with sulfhydrated residues. TRPV4 exhibited basal persulfide modification that was significantly increased in response to Na_2S treatment (Fig. 7).

DISCUSSION

H_2S is an important, endogenously generated gaseous signaling molecule (for review see 52, 53). However, the mechanisms mediating the biological activity of H_2S have not been clearly established. This study provides support for the novel hypothesis that TRPV4 channels in the endothelium of small mesenteric arteries are required for H_2S-mediated vasodilation (Fig. 1). Activation of TRPV4 channels with H_2S or GSK1016790A elicits an IbTx- and endothelium-dependent vasodilation (Fig. 2) and increased the number of rapid, localized Ca^{2+} events in endothelial cells (Figs. 3 and 4). In the presence of SK/IK channel inhibition, a subpopulation of native mesenteric artery endothelial cells exhibit eBK currents (Fig. 5) and TRPV4-mediated outward currents (Fig. 6). Last, H_2S treatment increases persulfide levels of endothelial TRPV4 channels (Fig. 7). Taken together, these results suggest that H_2S-mediated vasodilation involves eBK channel activation that is dependent on Ca^{2+}-influx through endothelial TRPV4 channels.

In VSM, TRPV4 have been implicated in epoxyeicosatrienoic acid-induced vasodilation in mammary and cerebral arteries through ryanodine receptor-dependent activation of smooth muscle cell BK channels (8, 28). Alternatively, a significant number of studies have provided support for a role of TRPV family members in endothelium-dependent vasodilation in response to varied stimuli. Endothelium-dependent vasodilation in response to arachidonic acid (56), muscarinic receptor activation (42, 43), and the plant-derived phenylpropene, eugenol (34), has been shown to involve activation of TRPV4 channels on EC. In addition to TRPV4, TRPV1 (5) and TRPV3 (35) can also mediate endothelium-dependent vasodilation in different vascular beds. Interestingly, Mendoza et al. (29) showed TRPV4 mRNA and protein expression in ECs but

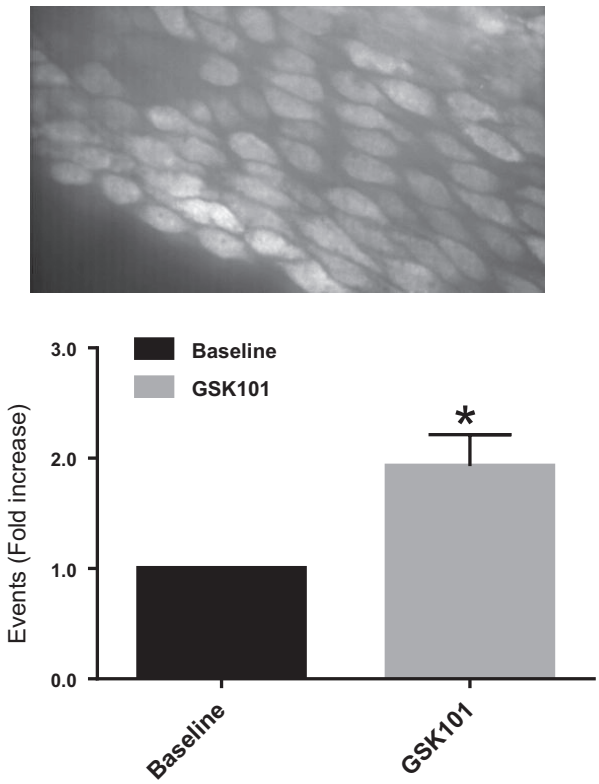

Fig. 4. Pressurized mesenteric artery endothelial cell tubes were loaded with the fluorescent Ca^{2+} indicators, fluo-4 AM (5 μM) and Oregon Green 488 BAPTA-1 AM (5 μM). GSK1016790A (10 nM)-induced calcium events were imaged using a spinning disk confocal microscope (40×) and detected using the ImageJ plugin LC_PRO; $n = 4$/group. *$P < 0.05$, different from baseline.

not VSM from mouse mesenteric arteries, supporting a role for this channel in endothelium-dependent responses in this bed. Previous work as well as the current study supports the postulate that endothelial proteins are the target of both exogenous

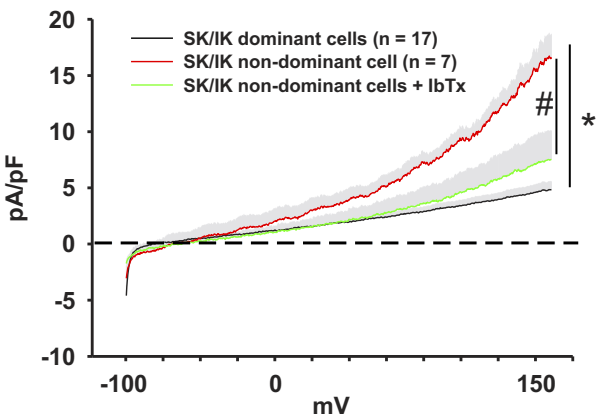

Fig. 5. Whole cell K^+ currents were measured in freshly isolated mesenteric endothelial cells using conventional whole cell patch clamp. Experiments were performed in the presence of SK/IK inhibition (apamin; 300 nM and Tram-34; 1 μM). Voltage ramps were performed from −100 to 150 mV. Cells that displayed residual K^+ current were treated with iberiotoxin (100 nM) and a second voltage ramp was performed. SE is shown in gray. *$P < 0.05$, different from SK/IK dominant cells. #$P < 0.05$, different from SK/IK nondominant cells + IbTx.

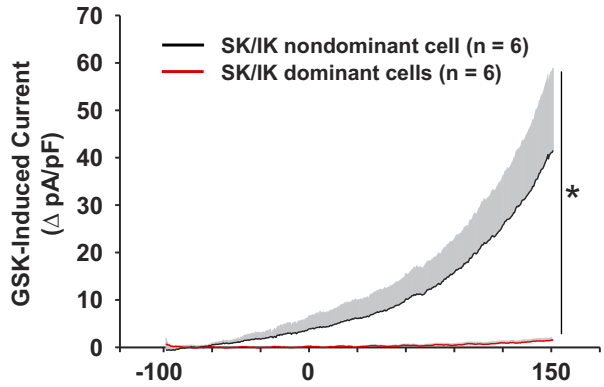

Fig. 6. Whole cell K^+ currents were measured in freshly isolated mesenteric endothelial cells using the perforated patch-clamp configuration. Experiments were performed in the presence of SK/IK inhibition (apamin; 300 nM and Tram-34; 1 μM). Voltage ramps were performed from −100 to 150 mV under baseline conditions and following treatment with GSK1016790A (3 nM). Subtraction currents are shown. SE is shown in gray. *$P < 0.05$, different from SK/IK dominant cells.

and endogenous H_2S (14–16). Moreover, H_2S hyperpolarizes endothelial cells from both wild-type and CSE−/− mice (31).

Our results provide further evidence of a role for endothelial TRPV4 channels in H_2S-induced vasodilation. Vasodilation in response to direct activation of TRPV4 with GSK1016790A

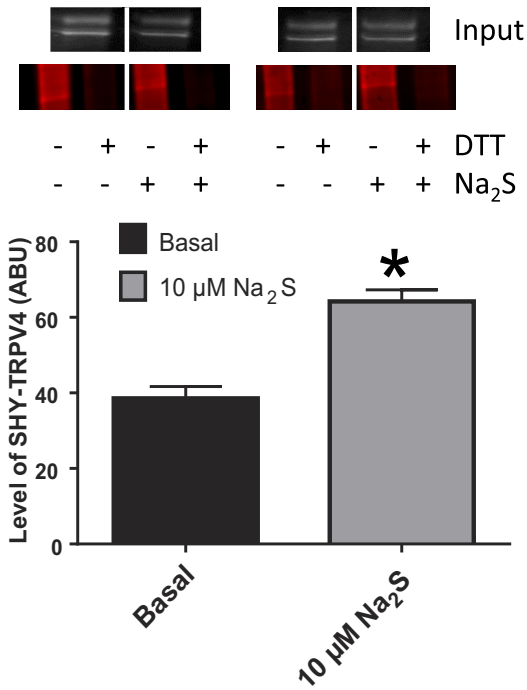

Fig. 7. Sulfhydration of TRPV4 was detected using the maleimide assay in cultured rat aortic endothelial cells ($n = 4$/group). Cells were treated with Vehicle or 10 μM Na_2S for 30 min at 37°C. TRPV4 was immunoprecipitated from each of the whole cell lysates with each dish treated as a single sample. Each sample was then divided into three aliquots, one for standard Western blotting to determine total TRPV4 in the input, one treated with DTT then probed with maleimide (+DTT), and one treated with vehicle prior to maleimide labeling (−DTT). Thus the input is the same for both +DTT and −DTT; and therefore the total protein for each pair of treatments is represented by a single lane. Maleimide fluorescence was normalized to total TRPV4 protein determined by standard Western blot. *$P < 0.05$, different from basal.

was blocked by disruption of the endothelium similar to our previous observations that exogenous H_2S-induced dilation is abolished in denuded mesenteric arteries (14). In the present study, H_2S-induced vasodilation was also blocked by pretreatment with TRPV4 antagonist GSK2193874, and H_2S elicited an increase in TRPV4-dependent Ca^{2+} events in endothelial cells of pressurized mesenteric arteries. Although there is considerable variation in the reported biophysical characteristics of TRPV4 Ca^{2+} events (2, 45), the spatial and temporal characteristics we observed are consistent with previous work (45). This H_2S-induced activation of endothelial TRPV4 may be mediated either directly through sulfhydration of the channel (30, 31), which is supported by the present study, or indirectly through activation of a second messenger, providing a Ca^{2+} source for activation of endothelial dilator pathways.

Previous work has provided evidence for a signaling complex formed between Ca^{2+} conducting TRPV4 channels and Ca^{2+}-sensitive SK and IK channels within endothelial cells (27, 42) that is analogous to the ryanodine-mediated Ca^{2+} sparks/BK channel coupling observed in cardiac and VSM. Indeed, TRPV4 and SK/IK channels cluster within caveolae of human microvascular endothelial cells supporting the postulate that TRPV4 acts as a Ca^{2+} source for K_{Ca} channels (10). Previous work from our laboratory demonstrated that myogenic tone is augmented in mesenteric arteries following exposure to intermittent hypoxia in an endothelium-dependent manner; however, this effect was not mimicked by inhibition of SK/IK channels (16), suggesting alternative K^+ channels operate within the endothelium of small mesenteric arteries. This led us to examine a role for eBK channels in this vascular bed. In addition to SK/IK channels, TRP family members have also been shown to provide a source of activator Ca^{2+} for BK channels in VSM. For example, TRPC1 colocalizes with BK channels and Ca^{2+} influx through TRPC1 channels activates VSM BK channels in second-order mesenteric arteries (21). In mouse coronary arteries, the TRPV1 agonist, capsaicin, elicited an IbTx-sensitive vasodilation (11). In the present study, vasodilation in small mesenteric arteries (<100 μm) in response to TRPV4 activation was blocked by either endothelial disruption or by luminal administration of IbTx, consistent with the postulate that Ca^{2+} influx through TRPV4 channels activates eBK.

High extracellular K^+ blocks H_2S-induced vasodilation suggesting activation of K^+ channels by H_2S (31). The first K^+ channel found to respond to H_2S was the K_{ATP} channel (23, 47). However, studies showing K_{ATP} channel activation by H_2S used high concentrations of H_2S donors that may not reflect physiological pathways. In contrast, we observed that inhibiting K_{ATP} channels has little effect on vasodilation to low concentrations of a H_2S donor (16). H_2S vasodilation has also been shown to involve stimulation of endothelial charybdotoxin/apamin-sensitive K^+ channels as well as smooth muscle K_{ATP} channels in superior mesenteric artery at much higher concentrations than used in the present study (6). However, the role for BK channels in H_2S-mediated vasodilation has been equivocal. Whereas a number of studies have shown that H_2S can activate the BK channel (14–16, 26, 41, 57), others have demonstrated BK channel inhibition in response to H_2S (25, 48, 49). Because the efficacy of iberiotoxin treatment in preventing H_2S dilation in small mesenteric arteries is greater with luminal than abluminal administration (14) and disruption of the endothelium prevents both H_2S and TRPV4-dependent vasodilation, functional BK channels appear to be present in endothelial cells of these arteries. Others have reported that H_2S-induced relaxation of rat aortic rings is not altered by iberiotoxin, suggesting BK channels are not involved in H_2S-mediated relaxation in conduit arteries (55). Indeed, in the present study, exogenous H_2S failed to dilate large (>400 μM) mesenteric arteries or ~200 μM gracilis arteries (unpublished observations), suggesting a lack of functional eBK channels in these conduit and larger parenchymal arteries might preclude responsiveness to H_2S.

There is significant controversy concerning the expression of BK channels in native endothelial cells. Although it is readily accepted that functional eBK channels are present in cultured endothelial cells (19, 20), some investigations do not observe active BK channels in native endothelial cells (9, 22), or only observe these channels in pathophysiological settings (13, 36, 37). However, others have identified eBK channels in native rat, rabbit, and porcine endothelial cells (14, 32, 38, 50). The majority of studies that did not identify BK channels in native endothelial cells were performed in cells collected from large-conduit vessels without culturing (39). However, Nelson and colleagues (12) did not detect functional BK channels in endothelial cells from rat parenchymal cerebral arteries. It is possible that this discrepancy is due to variations in channel expression between species, vascular beds or to phenotypic variation along the vascular tree. The possibility that BK channel expression differs along the vascular tree is supported by data in the present study. Cells harvested for patch-clamp experiments were from third (large)- to sixth (small)-order arterial branches of the superior mesenteric artery. Whole cell K^+ currents in the majority of cells were SK/IK channel mediated (TRAM-34/apamin-sensitive), whereas a subset of cells (~41%) exhibited a residual iberiotoxin-sensitive current. In addition, in the presence of TRAM-34/apamin, GSK1016790A elicited an outward current only in the subset of cells that exhibited an SK/IK-insensitive current. Our work supports the postulate that endothelial TRPV4 channels activate eBK resulting in an endothelium-dependent vasodilation.

A likely mechanism by which the physiological effects of H_2S are mediated is through the direct modification of proteins. H_2S can sulfhydrate cysteine residues on target proteins, resulting in the formation of hydropersulfide moieties (reviewed in 33). Indeed, H_2S has been shown to sulfhydrate a diverse population of proteins (30, 33), including ion channels in the vasculature. H_2S induced vasodilation linked to sulfhydration of Cys43 of the Kir6.1 subunit of the ATP-dependent K^+ channel on vascular smooth muscle cells (31). In the present study, treatment of rat aortic endothelial cells with Na_2S increased persulfide levels of TRPV4 channels. Fluorescent maleimide was used to detect TRPV4 sulfhydration which selectively labels sulfhydryl groups ($-$SH and $-$SSH, but not $-$SNO) (40). Treatment of samples with DTT reduces only persulfides; thus the decrease in fluorescent intensity is proportional to the level of sulfhydrated cysteines in the sample. Because maleimide binds to both thiols and persulfides, the observed increase in fluorescent band intensity after treatment with Na_2S could be due to H_2S reacting with *S*-nitrosylated cysteines to form maleimide-sensitive persulfides. Data are presented as the change in fluorescent intensity normalized to total TPV4 protein in the sample. Although this does not

directly show that sulfhydration of TRPV4 increases channel activity, it does suggest a possible mechanism for TRPV4 activation by H_2S. Site-directed mutagenesis studies would conclusively verify the role of sulfhydration in regulating TRPV4 channel activity.

Taken together, the results of these studies indicate that H_2S-mediated vasodilation requires activation of TRPV4-dependent Ca^{2+} influx and eBK channels within EC. Furthermore, TRPV4 channels generate spatially discrete Ca^{2+} events that appear to increase the opening of eBK channels leading to vasodilation and are sulfhydrated by elevations in H_2S.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

J.S.N., J.M.O., B.R.W., and N.L.K. conception and design of research; J.S.N. and J.M.O. performed experiments; J.S.N. and J.M.O. analyzed data; J.S.N., J.M.O., B.R.W., and N.L.K. interpreted results of experiments; J.S.N. and J.M.O. prepared figures; J.S.N. drafted manuscript; J.S.N., J.M.O., B.R.W., and N.L.K. edited and revised manuscript; J.S.N., J.M.O., B.R.W., and N.L.K. approved final version of manuscript.

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