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Hydrogen Sulfide-Induced Dual Vascular Effect Involves Arachidonic Acid Cascade in Rat Mesenteric Arterial Bed

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ABSTRACT

Hydrogen sulfide (H_2S), a novel gaseous transmitter, is considered a physiological regulator of vascular homeostasis. Recent evidence suggests H_2S as an endothelium-hyperpolarizing factor (EDHF) candidate. To address this issue, we evaluated the vascular effect of sodium hydrogen sulfide (NaHS), an H_2S donor, on the rat mesenteric arterial bed. NaHS concentration-response curve was performed on preconstricted mesenteric arterial bed. To assess the contribution of EDHF, we performed a pharmacologic dissection using indomethacin, N^G-nitro-L-arginine methyl ester (L-NAME), or apamin and charybdotoxin as cyclooxygenase, nitric-oxide synthase, and calcium-dependent potassium channel inhibitors, respectively. In another set of experiments, we used 4-(4-octadecylphenyl)-4-oxobutenoic acid, baicalein, or proadifen as phospholipase A_2 (PLA_2), lipoxygenase, and cytochrome P450 inhibitors, respectively. Finally, an immunofluorescence study was performed to support the involvement of PLA_2 in mesenteric artery challenged by NaHS. NaHS promoted a dual vascular effect (i.e., vasoconstriction and vasodilation). L-NAME or baicalein administration affected neither NaHS-mediated vasodilation nor vasoconstriction, whereas apamin and charybdotoxin significantly inhibited NaHS-induced relaxation. Pretreatment with PLA_2 inhibitor abolished both the contracting and the relaxant effect, whereas P450 cytochrome blocker significantly reduced NaHS-mediated relaxation. The immunofluorescence study showed that NaHS caused a migration of cytosolic PLA_2 close to the nucleus, which implicates activation of this enzyme. Our data indicate that H_2S could activate PLA_2, which in turn releases arachidonic acid leading, initially, to vasoconstriction followed by vasodilation mediated by cytochrome P450-derived metabolites. Because EDHF has been presumed to be a cytochrome P450 derivative of the arachidonic acid, our results suggest that H_2S acts through EHDF release.

Introduction

Hydrogen sulfide (H_2S) is a well known pungent gas that has been widely studied for its toxic effect (Beauchamp et al., 1984). It has recently been recognized as a vascular gaseous mediator involved in cardiovascular homeostasis (Lowicka and Bełtowski, 2007; Yang et al., 2008; Wagner, 2009). H_2S is produced endogenously from L-cysteine by cystathionine β-synthase and/or cystathionine-γ-lyase (CSE). The expression of both enzymes has been detected in many human and other mammalian cells in a tissue-specific manner (Hosoki et al., 1997). Cystathionine β-synthase is located mostly in the central nervous system, and its activity is 30-fold greater than CSE (Abe and Kimura, 1996). In contrast, CSE is predominant in vascular tissues, such as aorta, mesenteric artery, and portal vein (Hosoki et al., 1997; Levonen et al., 2000). CSE has been found to be localized to the endothelial layer of blood vessel (Yang et al., 2008).

H_2S is a vasodilator factor in various types of tissues (e.g., rat aorta and mesenteric arteries). It induces vasorelaxation partially mediated by directly opening ATP-dependent (K_{ATP}) (Zhao et al., 2001; Cheng et al., 2004) or voltage-dependent potassium channels (Schleifenbaum et al., 2010) as demonstrated recently. The vascular effect of H_2S seems to be endothelium-dependent because removal of the endothelium attenuated relaxation of the rat aorta and the mesenteric bed (Zhao and Wang, 2002; Cheng et al., 2004). The key role of the endothelium in the H_2S pathway has clearly been demonstrated by Yang et al. (2008). Indeed, they have

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ABBREVIATIONS: H_2S, hydrogen sulfide; NaHS, sodium hydrogen sulfide; PLA_2, phospholipase A_2; $cPLA_2$, cytosolic, phospholipase A_2; CSE, cystathionine γ-lyase; COX, cyclooxygenase; INDO, indomethacin; NO, nitric oxide; NOS, nitric-oxide synthase; OBAA, 4-(4-octadecylphenyl)-4-oxobutenoic acid; ChTX, charybdotoxin; MTX, methoxamine; ACh, acetylcholine; PRD, proadifen; L-NAME, N^G-nitro-L-arginine methyl ester; LOX, lipoxygenase; EDHF, endothelium-derived hyperpolarizing factor; $K_{Ca^{+2}}$, calcium-dependent potassium channels; DAPI, 4,6-diamidino-2-phenylindole.

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shown that CSE localized on endothelial cells represents the major physiological source of H_2S in the vascular system. It is interesting that stimulation of endothelial cells by acetylcholine determines a marked increase in H_2S level that can be blocked by an anticholinergic drug. In addition, acetylcholine-induced endothelium-dependent relaxation of resistance mesenteric arteries was significantly diminished in CSE-deficient mice (Yang et al., 2008).

The endothelial control of the vascular tone involves the release of soluble factors, such as nitric oxide (NO) and prostacyclin, as well as factors that cause hyperpolarization of the underlying smooth muscle cells. These latter factors are designated endothelium-derived hyperpolarizing factors (EDHF). It has recently been suggested that either H_2S itself is an EDHF or that H_2S can induce the release of EDHF from the endothelium (Wang, 2009). Because the nature of EDHF(s) is still unknown, it is mainly defined through some key features determined through specific bioassays. It is now widely accepted that EDHF mediates the hyperpolarization of vascular smooth muscle cells by activating calcium-dependent potassium channels ($K_{Ca^{+2}}$). In particular, the effect of EDHF is mainly mediated by small-conductance $K_{Ca^{+2}}$ channels and aided by intermediate-conductance $K_{Ca^{+2}}$ channels, which can be blocked by the coapplication of apamin and charybdotoxin (Busse et al., 2002; Gluais et al., 2005). The involvement of $K_{Ca^{+2}}$ in H_2S induced relaxation in rat aortic and mesenteric arteries, it has also been demonstrated (Zhao et al., 2001; Cheng et al., 2004).

Isolated and perfused mesentery bioassay is exquisitely sensitive to EDHF. Indeed, as the caliber of the artery diminishes, the effect of EDHF on endothelium-dependent dilation increases (Shimokawa et al., 1996). We have used this bioassay to investigate the involvement of EDHF in the H_2S pathway because the mechanisms of vascular action of H_2S are still unclear.

Materials and Methods

Tissue Preparation. Male Wistar rats (200–220 g; Charles River Italica, Calco, Italy) were used for ex vivo experiments. The experimental procedures performed in this study followed the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health, publication 86-23, revised 1985) as well as the specific guidelines of the Italian (N.116/1992) and European Council law (N.86/609/CEE). Animals were kept under temperature 23 ± 2°C, humidity range of 40 to 70%, and 12-h light/dark cycles. Food and water were fed ad libitum.

Mesenteric bed preparation was performed according to Warner (1990). In brief, rats were anesthetized with urethane solution (15% w/v; 10 ml/kg). The superior mesenteric artery was cannulated to perfuse the whole vascular bed with Krebs' buffer containing heparin (10 IU/ml; Sigma-Aldrich, Milan, Italy) for 5 min at 2 ml/min. The mesenteric bed was separated from the intestine by cutting along the closed intestinal border and connected to a pressure transducer (Bentley 800 Trantec; Ugo Basile, Comerio, Italy). It was perfused with Krebs' buffer (2 ml/min) composed of 115.3 mM NaCl, 4.9 mM KCl, 1.46 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25.0 mM $NaHCO_3$, and 11.1 mM glucose (Carlo Erba Reagents, Milan, Italy), warmed at 37°C, and oxygenated (95% O_2, 5% CO_2). To inhibit the prostanoid component, all of the experiments were performed with Krebs' solution medicated with indomethacin (INDO; 10 μM; Sigma-Aldrich). Changes in perfusion pressure were measured by a recorder (Unirecord 7050; Ugo Basile). After approximately 20 min of equilibration, methoxamine (MTX, 100 μM; Sigma-Aldrich), an adrenergic α_1-agonist, was added to the Krebs' solution, and the endothelium integrity was evaluated by a bolus injection of acetylcholine (ACh; 1 and 10 pM; Sigma-Aldrich).

Experimental Design. A concentration-response curve to sodium hydrogen sulfide (NaHS; 10 μM–1 mM; Sigma-Aldrich), an H_2S donor, was constructed. NaHS was infused on MTX (100 μM) stable tone at 50 μl/min for 15 min to reach the final concentration of perfusion from 10 μM to 1 mM, as described elsewhere (Cheng et al., 2004). To visualize NO synthase (NOS) and cyclooxygenase (COX)-independent relaxation (i.e., EDHF), L-NAME (100 μM; Sigma-Aldrich) as a NOS inhibitor was also added to the Krebs' solution containing INDO. NaHS then was infused as described above on a MTX stable tone. Likewise, to also investigate the contributions of the endogenous H_2S pathway, we performed a concentration-response curve by using L-cysteine (10 μM–1 mM; Sigma-Aldrich) infused at 50 μl/min for 30 min (Cheng et al., 2004) on the stable tone of MTX.

To investigate on H_2S intracellular signaling, we designed experiments by solely using NaHS; for this purpose, several inhibitors were used: charybdotoxin (ChTX, 100 nM; Sigma-Aldrich) plus apamin (5 μM; Sigma-Aldrich) as $K_{Ca^{+2}}$ blockers, 4-(4-octadecylphenyl)-4-oxobutenoic acid (OBAA, 10 μM; Tocris Bioscience, Bristol, UK), a powerful PLA_2 inhibitor of both the secretory ($sPLA_2$) and cytosolic ($cPLA_2$) form, and baicalein (10 μM; Tocris Bioscience) or proadifen (PRD, 10 μM; Sigma-Aldrich) as lipoxygenase (LOX) or cytochrome P450 inhibitors, respectively. To validate the efficacy of the inhibitors used, in our experimental conditions, a bolus injection of ACh (10 pM) was given 30 min before and after each treatment.

Immunofluorescence Studies. Mesenteric artery was isolated and incubated in Krebs' solution with NaHS (1 mM) for 5, 10, or 20 min. In another set of experiments, tissues were pretreated with OBAA (10 μM) for 30 min and then challenged with NaHS (1 mM) for 5 min. Tissues were fixed in paraformaldehyde [4% (v/v)] for 15 min, permeabilized with prechilled methanol [100% (v/v)] at −20°C for 10 min, and then incubated with rabbit anti-$cPLA_2$ (Santa Cruz Biotechnology, Milan, Italy). Anti-rabbit Texas Red (Santa Cruz Biotechnology) was used as a secondary antibody and incubated for 1 to 2 h at room temperature. Isotype control served as negative control. The slides were examined with a fluorescence microscope (Carl Zeiss GmbH, Jena, Germany) and Axioplan Imaging Programme (AxioCam Programme; Carl Zeiss GmbH).

Data Analysis. Data were expressed as mean ± S.E.M. ($n = 5$) for each treatment. Statistical analysis was performed using Student's t test or one-way analysis of variance followed by Bonferroni's post-test, as needed. P values less than 0.05 were considered significant. The decrease in perfusion pressure, i.e., vasodilation, was calculated as area under the curve (millimeters of mercury × minutes), whereas the increase in perfusion pressure, i.e., contraction, was calculated in millimeters of mercury compared with MTX-induced constricted tone.

Results

NaHS Induced a Dual Vascular Effect on the Mesenteric Arterial Bed. NaHS is a salt that releases H_2S in solution (Zhao et al., 2001). The infusion of NaHS (10 μM–1 mM) promoted a dual vascular effect (Fig. 1A). In fact, an increase in perfusion pressure, i.e., constriction (Fig. 1B), was followed by a decrease in perfusion pressure, i.e., vasodilation (Fig. 1C). The dual effect observed was concentration-dependent ($P < 0.05$; Fig. 1, B and C). The contractile effect was evident at the concentration of 10 μM. At the dose of 100 μM, the constriction was followed by a vasodilation (Fig. 1, B and C). At higher concentration (e.g., 1 mM), the vasoconstriction did not occur, whereas vasodilation was dominant (Fig. 1C). An equal volume of Krebs' solution was infused to exclude any unspecific effect related to the method.

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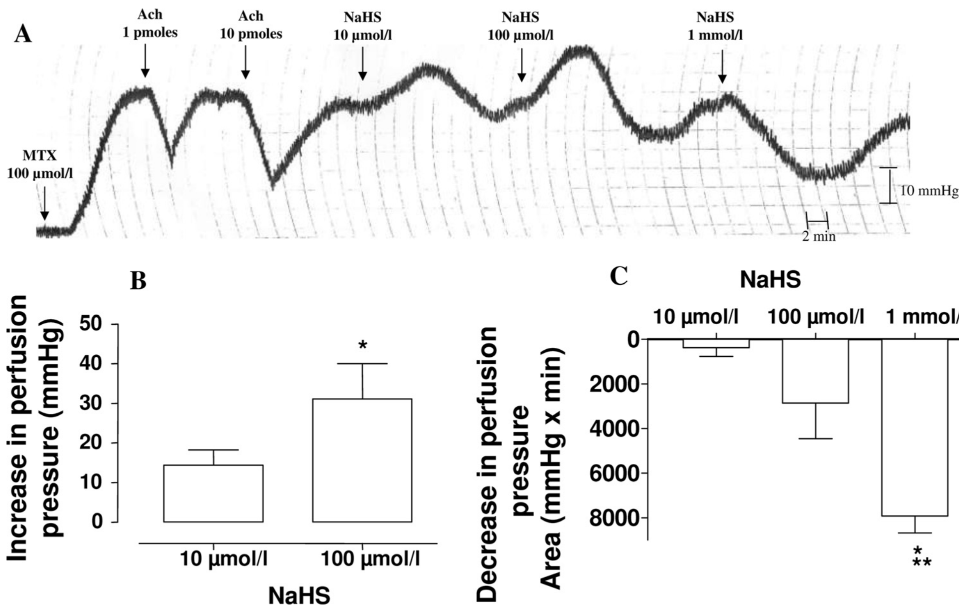

Fig. 1. A, tracer of NaHS infusion (10 µM–1 mM) on preconstricted mesenteric arterial bed. B, NaHS induced an increase in perfusion pressure (contraction) at 10 and 100 µM in a concentration-dependent manner (*, $P < 0.05$). C, NaHS induced a decrease in perfusion pressure (vasodilation) in a concentration-dependent manner (*, $P < 0.05$ versus 100 µM; **, $P < 0.001$ versus 10 µM). All of the experiments were performed in the presence of endothelium and INDO (10 µM). The increase in perfusion pressure, i.e., contraction, was calculated in millimeters of mercury compared with the MTX-induced constricted tone. The decrease in perfusion pressure, i.e., vasodilation, was calculated as area under the curve (mm Hg × min). Data represent mean ± S.E.M.; $n = 5$.

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NaHS-Induced Constriction Is COX- and NOS-Independent. Because all experiments were performed in the presence of INDO, COX products were not involved in NaHS-induced vasoconstriction. The NO involvement was also ruled out given that the addition of L-NAME (100 µM) did not affect NaHS-induced increase in perfusion pressure, i.e., constriction (Fig. 2). To evaluate whether other arachidonic acid metabolites were involved in NaHS constriction effect, we used baicalein (10 µM) or OBAA (10 µM) as LOX and PLA_2 inhibitors, respectively. The PLA_2, but not the LOX, inhibition abrogated the increase in perfusion pressure induced by NaHS (Fig. 2, $P < 0.01$). $K_{Ca^{+2}}$ inhibitors or PRD did not influence the contractile effect (data not shown).

NaHS-Induced Relaxation Is COX- and NOS-Independent. L-Cysteine (10 µM–1 mM), the endogenous source of H_2S, caused a decrease in perfusion pressure (i.e., vasodilation in presence of INDO plus L-NAME implicating the involvement of EDHF) (Fig. 3).

Likewise, under our experimental conditions, NaHS-induced vasodilation persisted in the presence of INDO plus L-NAME (Fig. 4A). To carry out the intracellular mechanism of H_2S, we used NaHS as its exogenous source. The combination of apamin (5 µM) and ChTX (100 nM), as $K_{Ca^{+2}}$ inhibitors, significantly reduced NaHS-induced vasodilation (Fig. 4 A, $P < 0.01$). It is noteworthy that PLA_2 inhibitor OBAA (10 µM) or the cytochrome P450 inhibitor PRD (10 µM) significantly reduced NaHS-induced relaxation (Fig. 4B; $P < 0.05$). Baicalein (a LOX inhibitor) did not inhibit the NaHS-induced vasodilation (data not shown). ACh-induced vasodilation was abrogated by OBAA or PRD pretreatment (data not shown).

NaHS-Induced $cPLA_2$ Migration Closed to the Nucleus. The migration of the $cPLA_2$ from the cytoplasm to the nuclear membrane implicates the activation of the enzyme and in turn that of arachidonic acid synthesis (Freeman et al., 1998). As shown in Fig. 5, stimulation of the mesenteric artery with NaHS (1 mM) induced a higher localization of the $cPLA_2$ close to the nucleus, as shown by the Texas Red-positive staining closer to the DAPI-positive staining. Prolonged NaHS incubation resulted in a lower migration of $cPLA_2$ to the nucleus. Treatment with OBAA before NaHS challenge reduced the nuclear localization of $cPLA_2$ (Fig. 5). OBAA by itself did not affect $cPLA_2$ localization (data not shown). The isotype control (IgG) did not show any positive staining (data not shown).

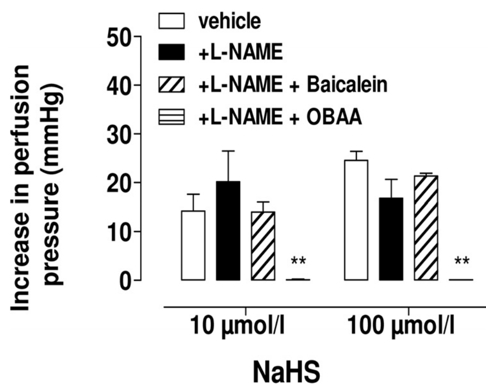

Fig. 2. L-NAME (100 µM) or baicalein (10 µM) did not affect NaHS-induced increase in perfusion pressure (contraction). The addition of OBAA (10 µM) abrogated NaHS-induced increase in perfusion pressure (**, $P < 0.01$ versus L-NAME). The increase in perfusion pressure, i.e., contraction, was calculated in millimeters of mercury compared with the MTX-induced constricted tone. All of the experiments were performed in the presence of endothelium and INDO (10 µM). Data represent mean ± S.E.M.; $n = 5$.

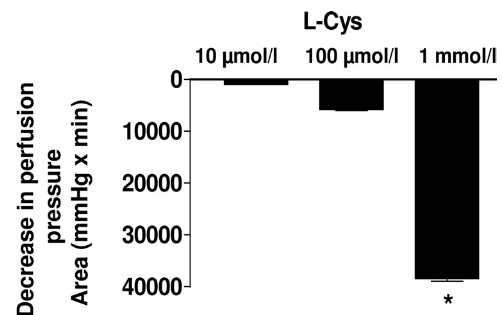

Fig. 3. L-Cysteine (10 and 100 µM and 1 mM) caused a concentration-dependent decrease in perfusion pressure on MTX (100 µM) stable tone in the presence of INDO plus L-NAME (*, $P < 0.05$ versus 10 and 100 µM). The decrease in perfusion pressure, i.e., vasodilation, was calculated as area under the curve (mm Hg × min). Data represent mean ± S.E.M.; $n = 5$.

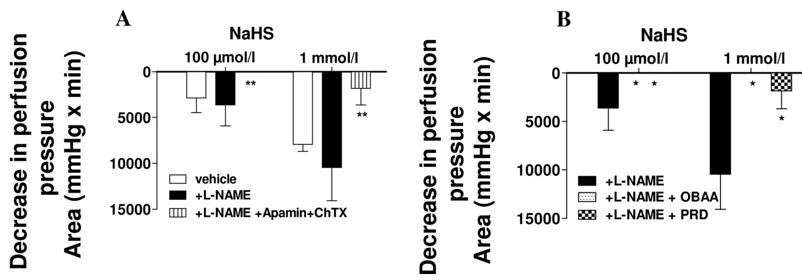

Fig. 4. A, L-NAME (100 μM) did not modify NaHS-induced decrease in perfusion pressure (vasodilation); apamin (5 μM) plus ChTX (100 nM) significantly reduced NaHS-induced decrease in perfusion pressure (**, $P < 0.01$ versus L-NAME). B, NaHS-induced decrease in perfusion pressure (vasodilation) was significantly (*, $P < 0.05$) reduced by OBAA (10 μM) as PLA_2 inhibitor or by PRD (10 μM) as cytochrome P450 inhibitor. All of the experiments were performed in the presence of endothelium and INDO (10 μM). The decrease in perfusion pressure, i.e., vasodilation, was calculated as area under the curve (mm Hg × min). Data represent mean ± S.E.M.; $n = 5$.

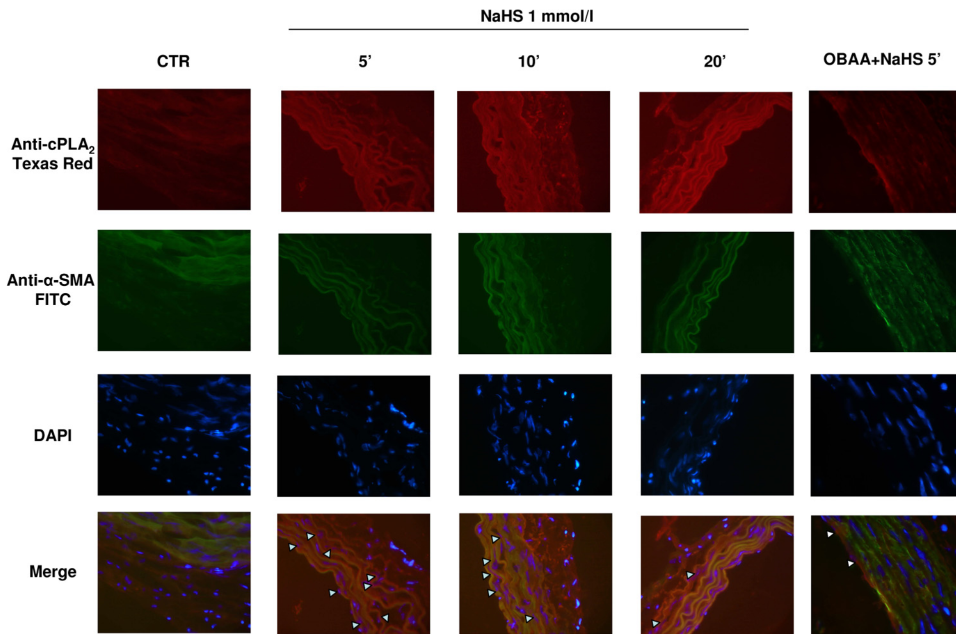

Fig. 5. Mesenteric artery was isolated and treated with NaHS (1 mM) for 5, 10, and 20 min. $cPLA_2$ was evaluated by immunofluorescence technique by use of anti-$cPLA_2$ Texas Red. Anti–α-smooth muscle cell actin (SMA) and DAPI were used to differentiate the smooth muscle cell and the nucleus, respectively. The stimulation of the mesenteric artery with NaHS induced a localization of the $cPLA_2$ closed to the nucleus, highlighted by the DAPI staining. The pretreatment of the mesenteric artery with OBAA (10 μM) reduced the nuclear localization of $cPLA_2$ compared with NaHS alone. The panels are representative of three different experiments.

Discussion

Infusion of NaHS, an H_2S donor, in the isolated and perfused mesentery artery caused a biphasic effect. At lower concentrations, NaHS caused vasoconstriction, whereas at higher concentrations, NaHS caused vasodilation. These vascular effects induced by NaHS on preconstricted mesenteric bed resulted from being NOS- and COX-independent. A similar result was also obtained by using L-cysteine as the endogenous source of H_2S. To study the intracellular mechanism of H_2S-induced vascular effect, we used a pharmacological modulation.

The NaHS-vasoconstricting effect seems to involve the arachidonic acid. In fact, it was strongly inhibited by OBAA, a PLA_2 inhibitor, but was not affected by LOX blockade. Moreover, neither cytochrome P450 metabolites nor $K_{Ca^{+2}}$ channels were involved in NaHS-induced contracting effect. Our data are consistent with literature showing that arachidonic acid causes constriction by releasing calcium from intracellular stores, stimulating calcium entry, or involving the Rho-kinase pathway (Gong et al., 1992; Vacher et al., 1992; van der Zee et al., 1995).

NaHS-induced vasodilation in mesenteric bed, as stated before, resulted as NOS- and COX-independent but it was $K_{Ca^{+2}}$ channel-dependent as revealed by apamin plus ChTX treatment. This latter result suggests that there is an EDHF-like activity dependent upon H_2S stimulus. Indeed, it is well known that EDHF-induced relaxation is NOS- and COX-independent and is blocked by $K_{Ca^{+2}}$ inhibitors (Busse et al., 2002; Gluais et al., 2005). To ascertain the involvement of EDHF in NaHS-induced vasodilation, we also used a LOX inhibitor. The LOX inhibitor was ineffective; therefore, we can also exclude LOX metabolite involvement in NaHS-induced vasodilation. To obtain more convincing data concerning the similar pattern of vascular activity between EDHF(s) and H_2S, we further investigated the arachidonic acid cascade. It is noteworthy that the inhibition of PLA_2 or cytochrome P450 abrogated the NaHS-induced relaxation in mesenteric plexus. These findings are in agreement with the hypothesis that EDHF could be a cytochrome P450 derivative of the arachidonic acid cascade (Hecker et al., 1994; Adeagbo, 1997; Campbell and Falck, 2007) and fits well with a major role for H_2S as EDHF(s) as proposed by others (Yang et al., 2008; Wang, 2009).

In mesenteric microcirculation, arachidonic acid is the main source of NaHS-induced vasoactive effects. A similar effect has been shown in perfused methoxamine-preconstricted kidney, another example of microcirculation where infusion of arachidonic acid promotes a transient contraction followed by relaxation (Kamata et al., 2006).

Arachidonic acid acts as a second messenger, and it is released from membrane phospholipids in response to a wide variety of extracellular stimuli from PLA_2 (Hirabayashi and Shimizu, 2000). Moreover, $cPLA_2$, but not secretory PLA_2, seems to play a major role in the vascular bed (Murakami et al., 1993; Duan et al., 2008). To exert its function, $cPLA_2$ is translocated into the nuclear envelope and endoplasmic re-

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ticulum by a calcium-dependent mechanism (Nalefski et al., 1994; Freeman et al., 1998; Millanvoye-Van Brussel et al., 1999; Duan et al., 2008). To gain insight into the involvement of H_2S in $cPLA_2$ activation, we performed an immunofluorescence study using the mesenteric artery. NaHS challenge promoted a rapid migration of $cPLA_2$ close to the nucleus, and this effect was reduced by OBAA pretreatment. Because H_2S-induced vasorelaxation relies on extracellular calcium entry (Zhao and Wang, 2002) and $cPLA_2$ activation is calcium-dependent, it is feasible that H_2S through calcium entry triggers $cPLA_2$ activation. Our results are in line with the literature showing that H_2S elicits a dual effect either in rat or human vessels. Several other different mechanisms have been proposed to explain this effect (Kubo et al., 2007a,b; Lim et al., 2008; Webb et al., 2008; Liu and Bian, 2010; Schleifenbaum et al., 2010). In addition, it is important to point out that H_2S vascular effects are dependent upon the vascular district, the endothelium (physiological or pathological conditions), the NaHS concentration, and the method of precontraction. All of these variables that influence the experimental output justify the controversy emerged in this field. For instance, the cross-talk between NO and H_2S as well as their chemical interference has been widely studied (Whiteman et al., 2006; Kubo et al., 2007a). In addition, the vascular responses induced by NaHS in rat aorta (contraction or vasodilation) have been shown recently to be highly dependent on the presence of HCO_3^-. H_2S stimulates anion exchangers to transport HCO_3^- in exchange of O_2^- to inactivate NO (Liu et al., 2010). However, in our experimental conditions, we can rule out the involvement of NO in H_2S-induced contraction as well as vasodilation. Indeed, L-NAME did not modify the NaHS-induced vascular activity in mesenteric plexus. Furthermore, local O_2 concentration changes seem to modulate the vasoactive effect of H_2S in large capacitance vessels such as rat aorta (Koenitzer et al., 2007; Kiss et al., 2008). However, we cannot exclude the contribution of O_2 tension in the H_2S vascular effect, even if in our experimental condition the NaHS vascular response was completely abrogated by PLA_2 inhibition. Another additional mechanism implicated in the vascular effect of H_2S involves cAMP. In fact, it was found that H_2S increases cAMP production in brain cells and in aorta tissue but inhibits the adenyl cyclase/cAMP pathway in cardiac myocytes (Kimura, 2000; Lim et al., 2008 Yong et al., 2008). The effects of H_2S again seem to be dependent upon the tissue considered.

In this scenario, our study adds insight into understanding the vascular effects induced by H_2S in the mesenteric plexus. Here, we show that, in the microcirculation, the effects of both NaHS constrictor and vasodilator are dependent upon arachidonic acid release. In the presence of exogenous H_2S, $cPLA_2$ translocates to the nucleus. This translocation triggers both the constriction and the vasodilation in a PLA_2-dependent manner as suggested by the modulatory effect operated by PLA_2 inhibitors. The vasodilation, but not the constriction, requires activation of both cytochrome P450 metabolism activation as well as $K_{Ca^{+2}}$ channels. The effect of NaHS on the regulation of vascular tone in the microcirculation involves the $cPLA_2$/arachidonic acid pathway. The involvement of PLA_2 in the inflammation induced by H_2S has also been proposed recently (di Villa Bianca et al., 2010).

In conclusion, this study shows that exogenous H_2S causes a dose-dependent biphasic effect in rat microcirculation. Both effects are dependent on arachidonic acid generated by $cPLA_2$ and not by COX or LOX metabolites. Cytochrome P450 metabolites of arachidonic acid are involved in the vasodilatory effect generated by higher concentrations of exogenous H_2S, suggesting that H_2S promotes the release of EHDF in the mesenteric circulation.

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Authorship Contributions

Participated in research design: d'Emmanuele di Villa Bianca and Ra. Sorrentino.

Conducted experiments: d'Emmanuele di Villa Bianca, Ro. Sorrentino, Coletta, Mitidieri, Rossi, and Vellecco.

Performed data analysis: d'Emmanuele di Villa Bianca, Coletta, Mitidieri, and Ra. Sorrentino.

Wrote or contributed to the writing of the manuscript: d'Emmanuele di Villa Bianca, Pinto, Cirino, and Ra. Sorrentino.

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