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Hydrogen sulphide triggers VEGF-induced intracellular Ca²⁺ signals in human endothelial cells but not in their immature progenitors

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Abstract: Hydrogen sulphide (H₂S) is a newly discovered gasotransmitter that regulates multiple steps in VEGF-induced angiogenesis. An increase in intracellular Ca²⁺ concentration ([Ca²⁺]_i) is central to endothelial proliferation and may be triggered by both VEGF and H₂S. Albeit VEGFR-2 might serve as H₂S receptor, the mechanistic relationship between VEGF- and H₂S-induced Ca²⁺ signals in endothelial cells is unclear. The present study aimed at assessing whether and how NaHS, a widely employed H₂S donors, stimulates pro-angiogenic Ca²⁺ signals in Ea.hy926 cells, a suitable surrogate for mature endothelial cells, and human endothelial progenitor cells (EPCs). We found that NaHS induced a dose-dependent increase in [Ca²⁺]_i in Ea.hy926 cells. NaHS-induced Ca²⁺ signals in Ea.hy926 cells did not require extracellular Ca²⁺ entry, while they were inhibited upon pharmacological blockade of the phospholipase C/inositol-1,4,5-trisphosphate (InsP₃) signalling pathway. Moreover, the Ca²⁺ response to NaHS was prevented by genistein, but not by SU5416, which selectively inhibits VEGFR-2. However, VEGF-induced Ca²⁺ signals were suppressed by DL-propargylglycine (PAG), which blocks the H₂S-producing enzyme, cystathionine γ -lyase. Consistent with these data, VEGF-induced proliferation and migration were inhibited by PAG in Ea.hy926 cells, albeit NaHS alone did not influence these processes. Conversely, NaHS elevated [Ca²⁺]_i only in a modest fraction of circulating EPCs, whereas neither VEGF-induced Ca²⁺ oscillations nor VEGF-dependent proliferation were affected by PAG. Therefore, H₂S-evoked elevation in [Ca²⁺]_i is essential to trigger the pro-angiogenic Ca²⁺ response to VEGF in mature endothelial cells, but not in their immature progenitors.



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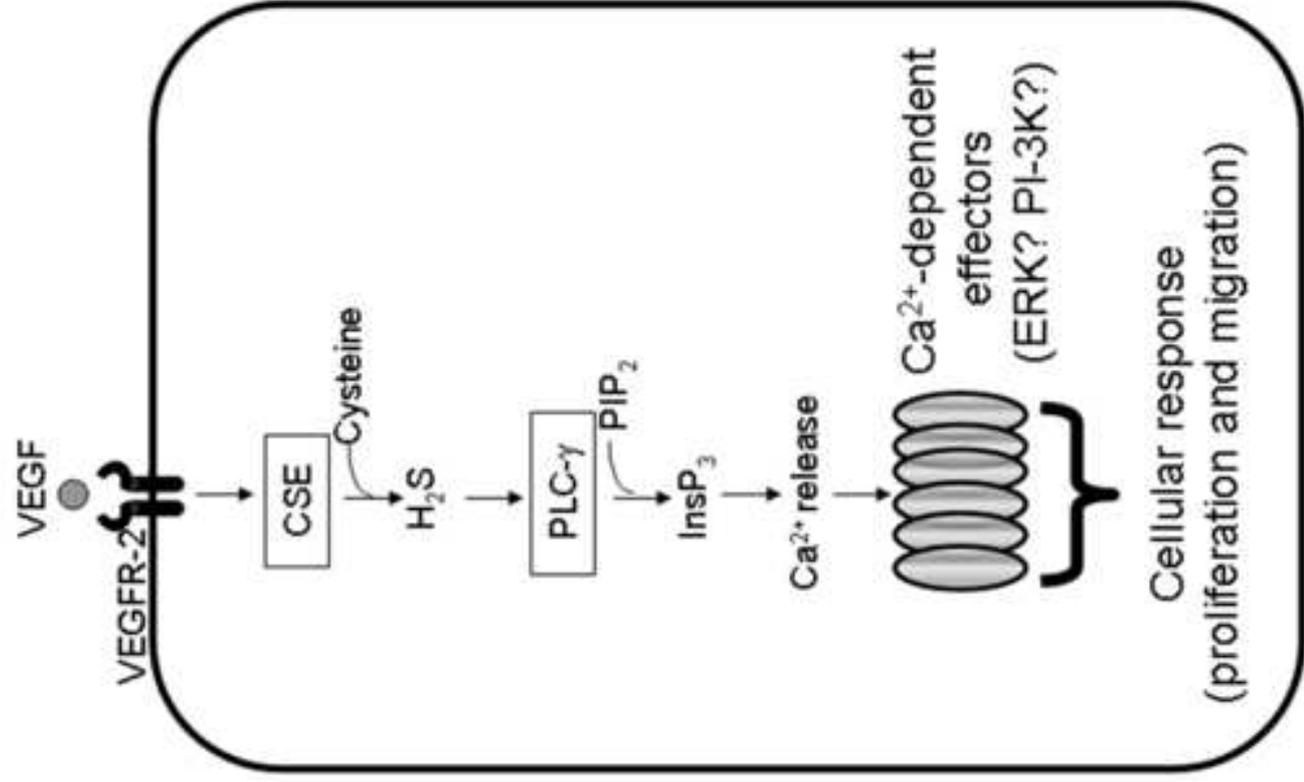
Dear Prof. Verkhratsky,

We did appreciate referees' comments on our manuscript entitled “Hydrogen sulphide triggers VEGF-induced intracellular Ca²⁺ signals in human endothelial cells but not in their immature progenitors”. We reviewed and amended the text according to their suggestions. We added more references and shortened the Discussion, as suggested by Referee #2, albeit we had to address some additional issues, as required by Referee #1. We provided a point-to-point reply to the Reviewers indicating all the changes made to the text and the corresponding Page and Line. We do hope that you will now find the manuscript suitable for publication on Cell Calcium.

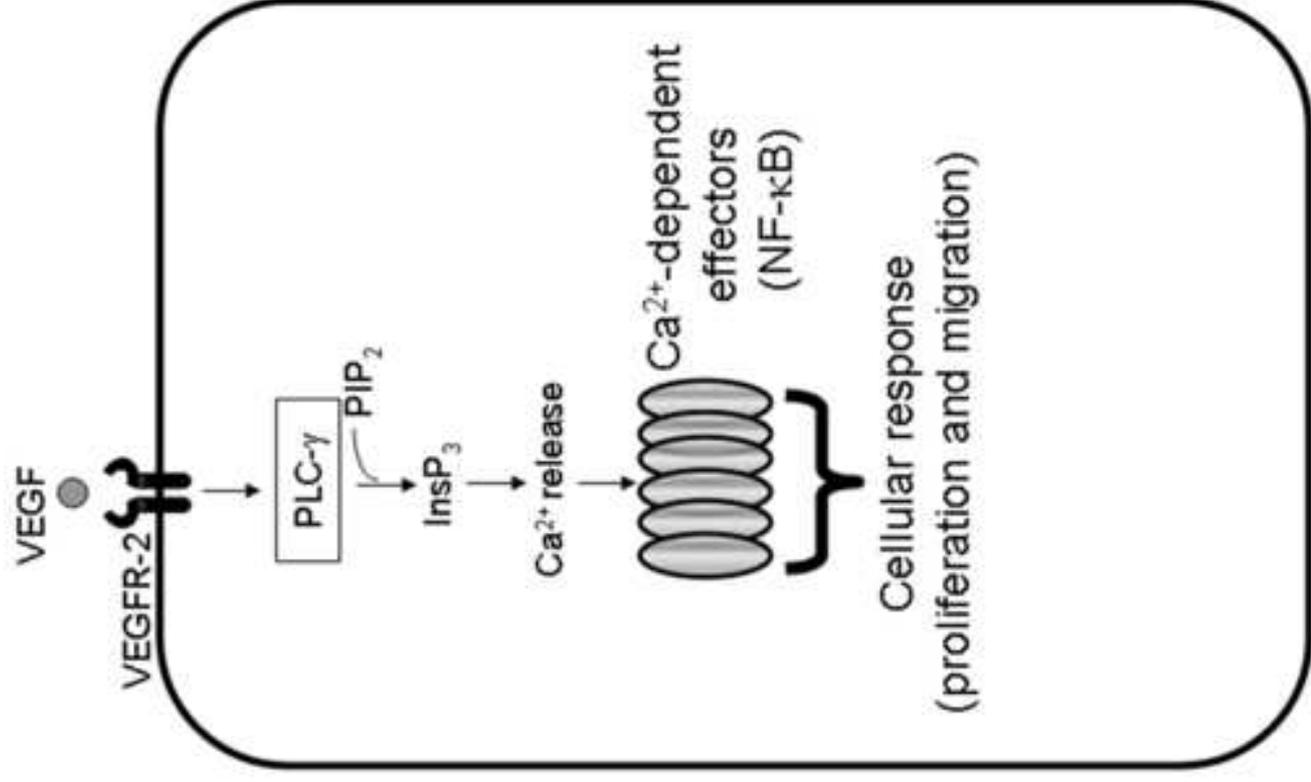
With my kindest regards,

Francesco Moccia

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HUVECS



EPCS

***Highlights (for review)**

Hydrogen sulphide triggers VEGF-induced intracellular Ca²⁺ signals in human endothelial cells but not in their immature progenitors

Highlights

We studied the pro-angiogenic Ca²⁺ signals induced by H₂S in mature and immature endothelial cells.

H₂S induces InsP₃-dependent Ca²⁺ release in Ea.hy926 cells, a surrogate of mature endothelial cells.

H₂S does not elicit any detectable Ca²⁺ signal in endothelial progenitor cells (EPCs).

The blockade of H₂S synthesis prevents the Ca²⁺ response to VEGF in Ea.hy926 cells, but not in EPCs.

The blockade of H₂S abrogates VEGF-induced angiogenesis in Ea.hy926 cells, but not in EPCs.

H₂S-induced Ca²⁺ signals in human endothelial cells

Hydrogen sulphide triggers VEGF-induced intracellular Ca²⁺ signals in human endothelial cells but not in their immature progenitors

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Running title: H₂S-induced Ca²⁺ signals in human endothelial cells

H₂S-induced Ca²⁺ signals in human endothelial cells

Abstract

Hydrogen sulphide (H₂S) is a newly discovered gasotransmitter that regulates multiple steps in VEGF-induced angiogenesis. An increase in intracellular Ca²⁺ concentration ([Ca²⁺]_i) is central to endothelial proliferation and may be triggered by both VEGF and H₂S. Albeit VEGFR-2 might serve as H₂S receptor, the mechanistic relationship between VEGF- and H₂S-induced Ca²⁺ signals in endothelial cells is unclear. The present study aimed at assessing whether and how NaHS, a widely employed H₂S donors, stimulates pro-angiogenic Ca²⁺ signals in Ea.hy926 cells, a suitable surrogate for mature endothelial cells, and human endothelial progenitor cells (EPCs). We found that NaHS induced a dose-dependent increase in [Ca²⁺]_i in Ea.hy926 cells. NaHS-induced Ca²⁺ signals in Ea.hy926 cells did not require extracellular Ca²⁺ entry, while they were inhibited upon pharmacological blockade of the phospholipase C/inositol-1,4,5-trisphosphate (InsP₃) signalling pathway. Moreover, the Ca²⁺ response to NaHS was prevented by genistein, but not by SU5416, which selectively inhibits VEGFR-2. However, VEGF-induced Ca²⁺ signals were suppressed by DL-propargylglycine (PAG), which blocks the H₂S-producing enzyme, cystathionine γ -lyase. Consistent with these data, VEGF-induced proliferation and migration were inhibited by PAG in Ea.hy926 cells, albeit NaHS alone did not influence these processes. Conversely, NaHS elevated [Ca²⁺]_i only in a modest fraction of circulating EPCs, whereas neither VEGF-induced Ca²⁺ oscillations nor VEGF-dependent proliferation were affected by PAG. Therefore, H₂S-evoked elevation in [Ca²⁺]_i is essential to trigger the pro-angiogenic Ca²⁺ response to VEGF in mature endothelial cells, but not in their immature progenitors.

Keywords: HUVECs, endothelial progenitor cells, hydrogen sulphide, Ca²⁺ release, DL-propargylglycine, proliferation

1.1 Introduction

Hydrogen sulphide (H₂S) is the latest entry in the growing family of gasotransmitters, already including nitric oxide (NO) and carbon monoxide (CO), that can be released by vascular endothelium to regulate cardiovascular functions [1, 2]. Endothelial H₂S is synthesized from either L-cysteine or cystathionine by the pyridoxal-5'-phosphate-dependent enzyme cystathionine γ -lyase (CSE), which is absent in adjoining vascular smooth muscle cells [3]. It remains to be elucidated whether endothelial cells impinge on the intracellular pool of sulfur that liberates H₂S in rodent neurons and astrocytes in the presence of physiologic levels of endogenous reducing substances, such as glutathione and cysteine [4]. Endothelial-derived H₂S has been shown to regulate a myriad of processes within the cardiovascular system [1-3]. For instance, H₂S affords cardioprotection against the ischemia/reperfusion injury by engaging both protein kinase C (PKC) and the phosphatidylinositol 3-kinase (PI3-K)/Akt signaling pathway [5-7]. Moreover, H₂S induces vasorelaxation in small resistance arteries by activating ATP-dependent K⁺ channels or intermediate- and small-conductance K_{Ca} (IK_{Ca} and SK_{Ca}) channels to hyperpolarize smooth muscle fibres, thereby reducing the vascular tone [8, 9]. Similar to NO and CO, H₂S exerts an auto/paracrine action on the innermost layer of blood vessels, by regulating a wide range of endothelial functions. These include protection from apoptosis [10] and oxidative stress [11], defense against hypoxia-induced injury [12], delay of cell senescence [13], and inhibition of leukocyte adhesion and rolling [14, 15]. A recent series of studies has further demonstrated that sodium hydrosulfide (NaHS), a widely employed H₂S donor, stimulates endothelial cell proliferation and tube formation both *in vitro* and *in vivo* [16-19]. NaHS-liberated H₂S induces human umbilical vein ECs (HUVECs) to replicate and assemble into a three-dimensional capillary-like network when plated on Matrigel [20]. Consistently, the intraperitoneal injection of NaHS restores regional tissue blood flow in a rodent model of hind limb ischemia [17]. H₂S stimulates HUVEC proliferation by enhancing the phosphorylation of Akt, extracellular signal-related kinase (ERK) and p38 [20]. The pro-angiogenic role of H₂S is corroborated by the evidence that VEGF signalling is suppressed by the genetic/pharmacological ablation of CSE and that H₂S is generated in response to VEGF stimulation [20, 21]. In addition, exogenous H₂S administration may increase both the hypoxic tissue levels of VEGF [22] and the extent of VEGFR-2 activation in endothelial cells, thereby sustaining blood vessel regrowth after an ischemic insult [17]. VEGFR-2 is the most important receptor sustaining VEGF-induced mitogenesis, motility and tubulogenesis [23, 24]. Apart from VEGF binding, VEGFR-2 phosphorylation in HUVECs is induced by H₂S-induced breaking of the disulfide bond

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between Cys1045 and Cys1024, which leads to the recruitment of the PI3-K/Akt signalling pathway [18]. Therefore, VEGFR-2 might serve as the long-sought H₂S receptor, albeit these data require further experimental challenge.

An increase in intracellular Ca²⁺ concentration ($[Ca^{2+}]_i$) is a key event in the complex molecular network driving endothelial cell proliferation, migration and tubulogenesis [25-29]. For instance, both inositol-1,4,5-trisphosphate (InsP₃)-dependent Ca²⁺ release and store-operated Ca²⁺ entry (SOCE) are recruited by VEGF to stimulate HUVEC proliferation and migration in *in vitro* angiogenic settings [27, 30, 31]. Recent work from our group has unveiled that NaHS elevates $[Ca^{2+}]_i$ in rat aortic endothelium by activating the forward mode (3 Na⁺ out : 1 Ca²⁺ in) of the Na⁺/Ca²⁺ exchanger (NCX), while it inhibits inositol-1,4,5-trisphosphate (InsP₃)-dependent Ca²⁺ release and SOCE [32]. Similarly, H₂S elicits Ca²⁺ entry in human breast carcinoma (B-TECs) upon the recruitment of a non-selective cation channel that mediates both cell migration and the concomitant Ca²⁺ response to VEGF [21]. VEGF-induced Ca²⁺ signals and B-TEC motility are abrogated when CSE is inhibited with DL-propargylglycine (PAG) [21]. It is, however, still unknown whether H₂S targets VEGFR-2 to generate intracellular Ca²⁺ waves [4].

The present investigation aimed at assessing whether and how H₂S evokes an increase in $[Ca^{2+}]_i$ in two widely employed cellular models to study human endothelial signaling: human umbilical vein-derived EA.hy926 cells, which are an established model to study *in vitro* angiogenesis in mature endothelium [30], and human endothelial progenitor cells (EPCs), which are committed to acquire a mature endothelial phenotype once released into peripheral circulation [24]. We demonstrated that H₂S elicits robust intracellular Ca²⁺ signals in Ea.hy926 cells, but not in EPCs. The Ca²⁺ response to H₂S in fully differentiated endothelial cells depends on Ca²⁺ mobilization from InsP₃-sensitive stores and independent on extracellular Ca²⁺ inflow. However, the pharmacological blockade of VEGFR-2 does not suppress H₂S-induced elevation in $[Ca^{2+}]_i$. Conversely, CSE inhibition prevents VEGF-induced intracellular Ca²⁺ movements and angiogenesis in Ea.hy926 cells, whereas it is ineffective in EPCs. Therefore, H₂S is central to the onset of the pro-angiogenic Ca²⁺ signals promoted by VEGF in mature endothelial cells.

2. Materials and Methods

2.1 Ea.hy926 cell culture

The HUVEC-derived cell line Ea.hy926 was kindly provided by Dr. Livia Visai (Department of Molecular Medicine, University of Pavia) at passages >45. Cells were grown in DMEM containing 15% FCS, 50 units/ml penicillin, 2 mM/ml L-glutamine, 50 µg/ml streptomycin and were maintained at 37 °C in 5% CO₂ atmosphere.

2.2 Isolation and cultivation of endothelial progenitor cells

Blood samples (40 mL) were obtained from healthy human volunteers aged from 22 to 28 years old. The Institutional Review Board at “Istituto di Ricovero e Cura a Carattere Scientifico Policlinico San Matteo Foundation” in Pavia approved all protocols. Informed consent was obtained and all procedures were performed according to the Declaration of Helsinki. Among the different subtypes of EPCs described in literature, we focussed on the so-called endothelial colony forming cells (ECFCs), which truly belong to the endothelial phenotype, organize into bidimensional capillary-like networks when plated on Matrigel, and form patent vessels in vivo [23, 29]. To isolate ECFCs, mononuclear cells (MNCs) were separated from peripheral blood (PB) by density gradient centrifugation on lymphocyte separation medium for 30 min at 400g and washed twice in EBM-2 with 2% FCS. A median of 36 x 10⁶ MNCs (range 18-66) were plated on collagen-coated culture dishes (BD Biosciences) in the presence of the endothelial cell growth medium EGM-2 MV Bullet Kit (Lonza) containing endothelial basal medium (EBM-2), 5% foetal bovine serum, recombinant human (rh) EGF, rhVEGF, rhFGF-B, rhIGF-1, ascorbic acid and heparin, and maintained at 37°C in 5% CO₂ and humidified atmosphere. Discard of non-adherent cells was performed after 2 days; thereafter medium was changed three times a week. The outgrowth of endothelial colonies from adherent MNCs was characterized by the formation of a cluster of cobblestone-appearing cells [33, 34]. That ECFC-derived colonies belonged to endothelial lineage was confirmed as described in [33] and [34].

2.3 Solutions

Physiological salt solution (PSS) had the following composition (in mM): 150 NaCl, 6 KCl, 1.5 CaCl₂, 1 MgCl₂, 10 Glucose, 10 Hepes. In Ca²⁺-free solution (0Ca²⁺), Ca²⁺ was substituted with 2 mM NaCl, and 0.5 mM EGTA was added. Solutions were titrated to pH 7.4 with NaOH. The osmolality of

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the extracellular solution, as measured with an osmometer (Wescor 5500, Logan, UT), was 300-310 mmol/kg.

2.4 [Ca²⁺]_i measurements

The cells were loaded with 4 μM fura-2 acetoxymethyl ester (fura-2/AM; 1 mM stock in dimethyl sulfoxide) in PSS for 1 hour min at room temperature. After washing in PSS, the coverslip was fixed to the bottom of a Petri dish and the cells observed by an upright epifluorescence Axiolab microscope (Carl Zeiss, Oberkochen, Germany), usually equipped with a Zeiss ×40 Achroplan objective (water-immersion, 2.0 mm working distance, 0.9 numerical aperture). The cells were excited alternately at 340 and 380 nm, and the emitted light was detected at 510 nm. A first neutral density filter (1 or 0.3 optical density) reduced the overall intensity of the excitation light and a second neutral density filter (optical density=0.3) was coupled to the 380 nm filter to approach the intensity of the 340 nm light. A round diaphragm was used to increase the contrast. The excitation filters were mounted on a filter wheel (Lambda 10, Sutter Instrument, Novato, CA, USA). Custom software, working in the LINUX environment, was used to drive the camera (Extended-ISIS Camera, Photonic Science, Millham, UK) and the filter wheel, and to measure and plot on-line the fluorescence from 10-15 rectangular “regions of interest” (ROI) enclosing 10-15 single cells. Each ROI was identified by a number. Since cell borders were not clearly identifiable, a ROI may not include the whole EPC or may include part of an adjacent EPC. Adjacent ROIs never superimposed. [Ca²⁺]_i was monitored by measuring, for each ROI, the ratio of the mean fluorescence emitted at 510 nm when exciting alternatively at 340 and 380 nm (shortly termed "ratio"). An increase in [Ca²⁺]_i causes an increase in the ratio [35, 36]. Ratio measurements were performed and plotted on-line every 3 s. The experiments were performed at room temperature (22°C). All the data have been collected from EPCs isolated from the peripheral blood of at least three healthy volunteers (EPCs) or three different coverslips of Ea.hy926 cells. Statistical comparisons were made by using One way Anova. P<0.05 was considered significant.

2.5 Proliferation assay on Ea.hy926 cells

Ea.hy926 cells were plated in DMEM 15% FCS in a 96-well culture plate (0.05*10⁴ cells/well) for 24 hours, and then starved in DMEM 0% FCS for 48 hours. Cells were then incubated with treatments dissolved in DMEM 0% for 72 hours and proliferation rate was assessed with the colorimetric kit CellTiter 96 AQueous One Solution (Promega, USA) and absorbance was revealed at

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490 nm in a microplate reader (Bio-Rad) after 3 hours of incubation. Cells treated with PAG (5 mM) were pre-incubated for 30 minutes before adding the other treatments. Experiments were performed in triplicate.

2.6 *In vitro* wound healing assay

Cells were seeded in a 24-well culture plate (4.5*10⁴ cells/well) for 48 hours to reach the confluence, and then starved in DMEM 0% FCS for 6 hours. Motility assay was performed generating a wound in confluent cellular monolayers scraping with a P10-pipette tip. Floating cells were removed by wash in PBS solution, and monolayers were treated with test conditions (in duplicate). Cells treated with PAG 5mM were pre-incubated for 30 minutes before adding the other treatments. Experiments were performed using a Nikon Eclipse Ti (Nikon Corporation, Tokio, Japan) inverted microscope equipped with an incubator to keep cells at 37°C and 5% CO₂. Images were acquired at 2 hour time intervals using a Nikon Plan 4X/0.10 objective and a CCD camera. MetaMorph software (Molecular Devices, Sunnyvale, CA, USA) was used to acquire images. All treatments were dissolved in DMEM 0% and experiments were performed in triplicate.

2.7 *EPC Proliferation assay*

1x10⁵ ECFC-derived cells (1st passage) were plated in 30 mm collagen treated dishes in EGM-2 MV medium with or without 5 mM PAG [34, 37]. Cultures were incubated at 37°C (in 5% CO₂ and humidified atmosphere) and cell growth assessed every day until confluence was reached in the control cultures (0 mM PAG). At this point, cells were recovered by trypsinization from all dishes and the cell number assessed by counting in a haemocytometer. The percentage of growth inhibition by the drug was calculated by dividing the total number of cells obtained in presence of 5 mM PAG by the number of cells in control experiments and multiplying the ratio by 100.

2.8 *Chemicals*

Fura-2/AM was purchased from Molecular Probes (Molecular Probes Europe BV, Leiden, The Netherlands). *N*-(4-[3,5-Bis(trifluoromethyl)-1H-pyrazol-1-yl]phenyl)-4-methyl-1,2,3-thiadiazole-5-carboxamide (BTP-2) was provided by Calbiochem (La Jolla, CA, USA). All other chemicals were obtained from Sigma. H₂S was administered in the form of sodium hydrosulfide (NaHS), the most commonly employed H₂S donor. When dissolved in neutral solution, NaHS dissociates to Na⁺ and HS⁻, which then reacts with H⁺ to yield H₂S. Free H₂S gas accounts for approximately 30-33% of a molar

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concentration of NaHS at 20°C, while the Na⁺ content and the pH of the solution are not appreciably altered [1].

3. Results

3.1 NaHS elicits intracellular Ca²⁺ signals in Ea.hy926 cells

The Ca²⁺ response to NaHS was first assessed in Ea.hy926 cells: this is a widely employed hybrid cell line established by fusing HUVEC with the A549/8 human lung carcinoma cell line and possesses the angiogenic features of differentiated endothelial cell. Ea.hy926 cells did not display any detectable increase in [Ca²⁺]_i when NaHS concentration ([NaHS]) was maintained below a threshold level of 1 μM (not shown). The percentage of responding cells augmented at [NaHS] ≥ 1 μM and displayed a bell-shaped dose-response relationship with a peak at 30 μM (Fig. 1A). The amplitude of the Ca²⁺ signal manifested a rather complex behaviour, whereas the Ca²⁺ peak increased when [NaHS] was raised from 1 μM up to 10-30 μM, decreased for further elevations to 50-100 μM, and finally augmented again when the donor was administered at 200 μM (Fig. 1B). Figure 1C depicts representative traces of the Ca²⁺ signals induced in Ea.hy926 cells by NaHS at 1, 30, and 100 μM. Thirty μM NaHS caused an immediate, but slow elevation in [Ca²⁺]_i which was followed by a slow phase of recovery to the baseline. However, a complete decay of the Ca²⁺ signal was barely achieved even after agonist washout; this feature has also been observed in primary cultures of endothelial cells dissociated from saphenous vein (SVECs) [38] and in B-TECs [21] and prevented us from probing the effect of consecutive applications of NaHS. Overall, these results show that Ea.hy926 cells are a useful tool to investigate the signal transduction machinery shaping the Ca²⁺ response to H₂S in mature endothelial cells. All the subsequent experiments were carried out by using NaHS at 30 μM, which is the dose activating the larger number of Ea.hy926 cells (Fig. 1A).

3.2 The Ca²⁺ response to NaHS is mediated by inositol-1,4,5-trisphosphate-dependent Ca²⁺ release

In order to assess the role played by extracellular Ca²⁺ entry and intracellular Ca²⁺ release in generating the Ca²⁺ response to NaHS, external Ca²⁺ was removed from the bath (0Ca²⁺). Under such conditions, the H₂S donor caused an increase in [Ca²⁺]_i (Fig. 2A), whose amplitude (Fig. 2C) and time-to-peak (TTP) (Fig. 2D) were not different from those recorded in the presence of Ca²⁺. This result strongly suggests that Ca²⁺ influx plays a minor role, if any, in shaping NaHS-induced Ca²⁺ signals. This issue was further elucidated by assessing the contribution of SOCE. Store-dependent Ca²⁺ inflow mediated by the physical interaction between Stim1 and Orai1 is the major Ca²⁺-permeable pathway

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activated in HUVEC after physiological depletion of intracellular Ca²⁺ reserves [31]. However, when Ea.hy926 cells were pre-treated with BTP-2 (20 μM; 20 min), a widely employed SOCE inhibitor [28, 29], the Ca²⁺ response to NaHS was not significantly altered as respect to control cells (Fig. 2B-2D). Collectively, these results indicate that NaHS-induced Ca²⁺ signaling requires intracellular Ca²⁺ mobilization in Ea.hy926 cells. The endoplasmic reticulum contains the main Ca²⁺ pool in mature endothelial cells and discharges Ca²⁺ in response to endogenous InsP₃ synthesized after phospholipase C (PLC) activation [27]. The contribution of the PLC/InsP₃ signalling pathway in the signal transduction cascade leading to NaHS-induced [Ca²⁺]_i elevations was ascertained by first pre-incubating the cells with (1-(6-((17β-3-methoxyestr-1,3,5(10)-trien-17-yl)amino)-hexyl)-1H-pyrrole-2,5-dione) (U73122; 10 μM), a popular PLC antagonist [35, 36]. This manoeuvre reversibly suppressed the Ca²⁺ response to NaHS in 120 out of 120 cells (100%) (Fig. 3A). Likewise, 2-nitro-4-carboxyphenyl-N,N-diphenylcarbamate (NCDC; 50 μM), another structurally unrelated PLC inhibitor [39], hindered NaHS-evoked increase in [Ca²⁺]_i in 36 out of 40 cells (90%) (Fig. 3B). However, the intracellular Ca²⁺ activity did not resume upon NCDC removal from the bath (Fig. 3B). Next, the InsP₃-sensitive Ca²⁺ store was depleted by exposing the cells to the selective SERCA inhibitor, cyclopiazonic acid (CPA) (10 μM), in 0Ca²⁺ to prevent SOCE [35, 39]. Under such conditions, ATP (100 μM), that mobilizes Ca²⁺ from InsP₃Rs in HUVEC [31], did not cause any detectable increase in [Ca²⁺]_i in 57 out of 57 cells (100%). Similarly, the subsequent administration of NaHS failed to augment intracellular Ca²⁺ levels (Fig. 3C). Consistently, 2-aminoethoxydiphenyl borate (2-APB; 50 μM), a widely employed InsP₃R blocker, abrogated the Ca²⁺ response to NaHS in 121 out of 121 cells (100%) (Fig. 3D). Taken together, these data show that the NaHS activates PLC to liberate intraluminally stored Ca²⁺ in Ea.hy926 cells.

3.3 Interaction between H₂S and VEGF in the modulation of intracellular Ca²⁺ homeostasis in Ea.hy926 cells

Recent evidence has indicated VEGFR-2 as the long-sought receptor for H₂S-mediated signalling in endothelial cells [18]. Therefore, we challenged the hypothesis that H₂S-evoked [Ca²⁺]_i elevations in Ea.hy926 cells are driven by VEGFR-2 by first pre-incubating the cells with genistein (10 μM, 10 min), a protein tyrosine kinase inhibitor [39]. Figure 4A shows that this treatment reversibly abolished the NaHS-induced intracellular Ca²⁺ wave in 82 out of 89 cells (92.1%). However, 1,3-Dihydro-3-[(3,5-dimethyl-1H-pyrrol-2-yl)methylene]-2H-indol-2-one (SU5416; 10 μM, 30 min), a

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specific antagonist of VEGFR-2 [18], did not affect NaHS-evoked intracellular Ca²⁺ signals in Ea.hy926 cells (Fig. 4B). More specifically, neither the fraction of responding cells (70% vs. 65% in not treated cells) nor the amplitude of the elevation in [Ca²⁺]_i (0.153±0.008 vs. 0.149±0.007 in not treated cells, n=65 and 72, respectively) were significantly affected by SU5416. In order to confirm that SU5416 was able to interfere with intracellular Ca²⁺ signalling in Ea.hy926 cells, we first assessed the Ca²⁺ response to VEGF (10 ng/ml). As shown in Fig. 4C (black tracing), VEGF augmented intracellular Ca²⁺ levels in 49 out of 78 (62.8%) control cells. Conversely, the pre-treatment with SU5416 (10 μM, 30 min) irreversibly abolished VEGF-induced increase in [Ca²⁺]_i in 27 out of 28 cells (96.4%) (grey tracing Fig. 4C). Nevertheless, the pharmacological blockade of H₂S synthesis with PAG (5 mM, 30 min), a specific CSE inhibitor [21, 32], prevented the Ca²⁺ response to VEGF (10 ng/ml) in 69 out of 74 cells (93.2%) (Fig. 4D). The Ca²⁺ response to the growth factor immediately resumed upon washing PAG out of the bath (Fig. 4D). Therefore, H₂S does not target VEGFR-2 to produce an increase in [Ca²⁺]_i in Ea.hy926 cells, but VEGFR-2 recruits CSE to ignite Ca²⁺ signals in these cells.

3.4 Endogenous H₂S is required for Ea.hy926 cells proliferation and migration

In order to evaluate whether and how H₂S-mediated, VEGF-induced Ca²⁺ signals impact on Ea.hy926 cells-dependent angiogenesis, we performed proliferation and *in vitro* migration experiments. The selective inhibition of CSE with PAG (5 mM, 72 hours) induced a significant reduction in Ea.hy926 proliferation induced by VEGF (10 ng/ml), while NaHS 30 μM did not affect cell growth (Fig. 5A). Figure 5B reports the percentage of migration in wound healing experiments after 8 hours of treatment. As expected, we observed a pro-migratory response induced by VEGF (10 ng/ml), but this effect was prevented by pre-incubating the cells with PAG (5 mM, 30 min), thereby suggesting that the endogenous production of H₂S is involved in the pro-angiogenic response to VEGF. Conversely, exogenous administration of H₂S (NaHS, 30 μM) was not able to affect Ea.hy926 cell migration. Overall, these data strongly suggest that, although H₂S alone is not able to stimulate Ea.hy926 cell proliferation and migration, VEGF utilizes this gasotransmitter to generate pro-angiogenic Ca²⁺ signals.

3.5 NaHS does not reproducibly generate intracellular Ca²⁺ signals in endothelial progenitor cells

Finally, we analyzed the Ca²⁺ response to NaHS in circulating EPCs, which are extremely useful to investigate the basic features of Ca²⁺ signalling in endothelial progenitors [23, 28, 29, 40, 41]. A recent series of papers from our group has indeed demonstrated that Ca²⁺ signaling plays a key role

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in driving EPC proliferation and migration [33, 34, 36, 40]. However, we found that NaHS induces an increase in [Ca²⁺]_i in a modest percentage of cells when administrated at doses ranging from 1 μM to 50 μM (Fig. 6A). The fraction of responding cells slightly, but significantly, increases when [NaHS] is raised up to 250 μM. No Ca²⁺ signal was observed when EPCs are challenged with higher [NaHS] (Fig. 6A). Interestingly, the average amplitude of NaHS-induced Ca²⁺ signals remained constant whatever the concentration of the donor we employed (Fig. 6B). Figure 6C illustrates the typical elevation in [Ca²⁺]_i induced by NaHS in EPCs: a rapid Ca²⁺ spike followed by a plateau phase of intermediate magnitude onto which rapid Ca²⁺ spikes could be overlapped. Intracellular Ca²⁺ levels were restored when NaHS was washed out from the bath. The low fraction of cells sensitive to NaHS did not permit us to reliably investigate the mechanisms underlying Ca²⁺ signalling in EPCs. Nevertheless, we sought to assess whether H₂S underpins the pro-angiogenic Ca²⁺ oscillations elicited by VEGF in these cells [35, 36]. Unlike Ea.hy926 cells, PAG (5 mM, 30 min) did not significantly impair either the percentage of EPCs displaying Ca²⁺ oscillations in response to VEGF (10 ng/ml) (Fig. 6D and Fig. 6E) or the rate of VEGF-induced proliferation (Fig. 6C). Overall, these results strongly suggest that H₂S is not involved in the generation of pro-angiogenic Ca²⁺ signals in human endothelial progenitors.

4. Discussion

Hydrogen sulphide has recently been described as a key signalling gasotransmitter in vascular endothelium [2, 3]. H₂S may promote endothelial proliferation and migration, thus stimulating angiogenesis and restoring local blood perfusion in several rodent models of hindlimb ischemia [17, 22]. A growing number of studies established a tight and finely tuned interplay between H₂S and VEGF. The growth factor may activate endothelial cells by inducing H₂S synthesis and, *viceversa*, H₂S may elevate VEGF levels and stimulate VEGFR-2 to trigger its downstream phosphorylative cascades. Ca²⁺ signalling serves a central role in angiogenesis and stands amid the network of molecular mechanisms engaged by H₂S in mature endothelial cells [4, 23, 26, 41]. Therefore, we undertook the present investigation: 1) to compare the Ca²⁺ response between Ea.hy926 cells, a widely employed mature endothelial cell line and endothelial committed progenitors; 2) to assess whether VEGFR-2 contributes to shape the Ca²⁺ response to H₂S and, *viceversa*, whether H₂S triggers VEGF-induced Ca²⁺ signals; and 3) to ascertain whether the pharmacological abrogation of H₂S synthesis interferes with the pro-angiogenic activity of VEGF in both endothelial cell types.

While a previous report from our group showed that H₂S-evoked Ca²⁺ signals could be involved in VEGF-elicited motility in tumour endothelium [21], herein we dissected the Ca²⁺ response to this gasotransmitter in the HUVEC-derived endothelial cell line EA.hy926. The following pieces of evidence indicate that H₂S-evoked Ca²⁺ signals are patterned by intracellular Ca²⁺ release through InsP₃Rs. First, NaHS evoked similar Ca²⁺ signals both in the presence and in the absence of extracellular Ca²⁺. Likewise, H₂S-induced increase in [Ca²⁺]_i was not affected when SOCE, the most important pathway for Ca²⁺ entry in HUVECs [31], was inhibited with BTP-2 [23, 28]. Therefore, H₂S-elicited [Ca²⁺]_i elevation primarily requires intracellular Ca²⁺ mobilization. Second, depleting the InsP₃-sensitive Ca²⁺ pool with CPA prevented the subsequent Ca²⁺ response to NaHS. Third, the pharmacological blockade of the PLC/InsP₃ signalling pathway with either U73122/NCDC or 2-APB suppressed NaHS-induced Ca²⁺ waves. Therefore, H₂S stimulates PLC, perhaps via S-sulphydrilation [4], to synthesize InsP₃ and mobilize intraluminally stored Ca²⁺ in Ea.hy926 cells. Ryanodine receptors are seemingly not expressed in these cells [42], so that their contribution to the H₂S-induced Ca²⁺ signals is unlikely. This mechanism is strikingly different from that depicted in B-TECs, HMVECs and rat aortic endothelial cells, which heavily depend on extracellular Ca²⁺ entry to produce a Ca²⁺ response to NaHS [4, 21, 32]. Conversely, H₂S utilizes InsP₃Rs and ryanodine receptors to shape intracellular

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Ca²⁺ signals in SVECs [38], thereby suggesting that the molecular mechanisms recruited by this gasotransmitter to modulate endothelial Ca²⁺ homeostasis depend on both species and vessel type, which may dictate the pattern of expression of the components of the Ca²⁺ machinery [27]. Our data are consistent with those recently described in HeLa cells, where the slow H₂S-release donor morpholin-4-ium-4-methoxyphenyl-(morpholino)-phosphinodithioate (GYY4137) increases [Ca²⁺]_i by up-regulating InsP₃R1 and InsP₃R2 [43]: however, our results do not fit with parallel findings in mouse airway smooth muscle cells (ASMCs) [44]. Herein, exogenous H₂S reduces InsP₃Rs to repress InsP₃-dependent Ca²⁺ release and the consequent muscle relaxation [44]. The Authors of the study concluded that a reducing ER environment leads the luminal protein ERp44 to bind to and inhibit InsP₃Rs. However, this process requires a strong drop in ER Ca²⁺ levels [45]. Interestingly, the inhibitory effect of H₂S in ASMCs has been observed upon a first InsP₃-dependent ER depletion in the absence of the gasotransmitter; this initial response might have caused a local reduction in intraluminal Ca²⁺ nearby InsP₃Rs, thereby rendering them more sensitive to the reducing conditions associated to H₂S stimulation. Conversely, both in our present study and others [38] the InsP₃-dependent Ca²⁺ pool was not depleted before presenting the cells with H₂S which might explain the differences with the study on ASMCs [44].

We further attempted to decipher the signaling pathway upstream PLC recruitment by H₂S. A recent study showed that H₂S caused VEGFR-2 autophosphorylation in Tyr1175, which is required to stimulate PI3-K and Akt in HUVECs [18]. First, we demonstrated that genistein reversibly prevented the onset of the Ca²⁺ response to Ea.hy926 cells, thus indicating the involvement of PLC γ in H₂S-induced Ca²⁺ signals [39]. However, SU5416, a popular VEGFR-2 inhibitor that prevents PI3-K and Akt activation in HUVECs [18], did not interfere with the Ca²⁺ response to NaHS. These observations suggest that H₂S targets VEGFR-2 to promote the phosphorylation of PI3-K and Akt, but not the recruitment of PLC γ . An alternative explanation is that PLC γ is enlisted by Src family kinases [46], which are non-receptor tyrosine kinases involved in H₂S signaling [47]. A different scenario implies that H₂S activates a distinct PLC isoform, such as the β subtype, and that genistein-induced inhibition of InsP₃-dependent Ca²⁺ mobilization rather reflects the blockade of InsP₃R phosphorylation by the non-receptor tyrosin kinase, Fyn [48]. Although H₂S stimulates intracellular Ca²⁺ release without activating VEGFR-2, VEGF recruits CSE-dependent H₂S production to increase [Ca²⁺]_i in Ea.hy926 cells: consistently, the pharmacological blockade of the enzyme prevented the Ca²⁺ response to NaHS. This result concurs with our previous observations that VEGF-induced elevation in [Ca²⁺]_i is inhibited

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by pre-incubating B-TECs with PAG [21]. In addition, this finding sheds more light on the molecular mechanisms whereby CSE silencing prevents the pro-angiogenic effects of VEGF [19, 20]. Indeed, PAG inhibited both Ea.hy926 cell proliferation and migration, indicating that H₂S-mediated intracellular Ca²⁺ signals control VEGF-dependent angiogenesis. Future studies will have to unveil whether the intracellular events triggered by VEGF in endothelial cells require the concomitant H₂S-dependent increase in [Ca²⁺]_i or serve as two parallel, yet not redundant, signaling pathways. It should, however, be pointed out that H₂S *per se* failed to affect the biological activity of Ea.hy926 cells. This result is consistent with previous findings by Wang's group, who demonstrated that the administration of NaHS alone influence Ea.hy926 tube formation only in hypoxic, but not in normoxic conditions [49]. Therefore, albeit both intracellular Ca²⁺ waves and ERK and PI-3K/Akt may be engaged by H₂S, they are not sufficient to activate the pro-angiogenic program in these cells, which require the participation of additional mechanisms recruited by VEGF.

Once characterized the basic underpinnings of the Ca²⁺ response to NaHS in a well established model of mature endothelial cells, we turned to their more immature progenitors. Endothelial progenitor cells are released from either bone marrow or the arterial wall to replace senescent or dysfunctional endothelial cells, thereby restoring blood flow upon occlusion of feeding arteries in the heart, in peripheral limbs and in the brain [23, 24, 40, 41]. A series of studies from our group has demonstrated that the concerted interplay between InsP₃-dependent Ca²⁺ mobilization and SOCE underlies the pro-angiogenic effect of VEGF in circulating human EPCs [33-36]. A recent study has unveiled that H₂S regulate human bone marrow-derived mesenchymal stem cells (BMMSCs) self-renewal and osteogenic differentiation by inducing Ca²⁺ entry through TRPV3 and TRPV6 [50]. Unlike Ea.hy926 cells and BMMSCs, we found that NaHS induces a detectable Ca²⁺ signal only in a modest fraction of circulating EPCs. While the percentage of responding cells slightly increases by augmenting [NaHS] from 5 μM up to 250 μM, the amplitude of the Ca²⁺ peak is unchanged over this range of stimulus intensities. No elevation in [Ca²⁺]_i could be evoked by further raising [NaHS]. Therefore, increasing [NaHS] does not lead to an enhancement in the magnitude of the Ca²⁺ response, but to the recruitment of more cells. This rather unusual dose-response relationship is peculiar to human EPCs, since the amplitude of the Ca²⁺ response increases with the extent of NaHS stimulation in B-TECs [21], human microvascular endothelial cells (HMVECs) [21], SVECs [38], and Ea.hy926 cells (present study). Assuming that the Ca²⁺ response in EPCs is shaped by the PLCγ/InsP₃ pathway described for Ea.hy926 cells, we speculate that higher doses of NaHS release more H₂S, thereby augmenting the

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probability to activate its underlying signalling cascade in a larger number of cells. However, the H₂S-sensitive PLC γ pool must be limited and already saturated at low NaHS concentrations. This feature would explain why the percentage of activated cells, but not the magnitude of the [Ca²⁺]_i elevation, increases up to stimulation with 250 μ M NaHS. The absence of any detectable Ca²⁺ signal at 500 μ M NaHS supports previous findings obtained on primary cultures of rat hippocampal astrocytes, where the Ca²⁺ response to NaHS was significantly reduced at doses higher than 320 μ M [51]. This same range of NaHS doses suppresses glutamatergic synaptic transmission in rat hippocampus [52]. Similarly, it is conceivable that 500 μ M is such a high [NaHS] concentration to be toxic for Ea.hy926, which might explain why it fails to induce an increase in [Ca²⁺]_i. An additional difference between endothelial committed progenitors and mature endothelial cells is the lower sensitivity of EPCs to the gasotransmitter. The proportion of cells displaying a robust Ca²⁺ activity in the presence of NaHS is dramatically higher in B-TECs [21], HMVECs [21], SVECs [38], and Ea.hy926 cells (present study). Consistent with these data, the pharmacological CSE abrogation with PAG affected neither VEGF-induced Ca²⁺ oscillations nor proliferation in human EPCs, suggesting that H₂S plays a little role, if any, in the pro-angiogenic response to the growth factor. However, we cannot rule out the impact of H₂S on alternative signalling pathways, such as K_{ATP} channels, PI-3K, and MAPK [3]. The full comprehension of the molecular mechanisms whereby H₂S engages PLC to trigger Ca²⁺ release through InsP₃Rs in Ea.hy926 cells will permit to understand why it fails to do so in EPCs.

In conclusion, this study contributes to elucidate the complex network of signaling pathways recruited by H₂S in endothelial cells. First, H₂S evokes a robust Ca²⁺ activity in Ea.hy926 cells, a human umbilical vein endothelial cell line which is widely employed to investigate *in vitro* angiogenesis, but not in more immature endothelial committed progenitors. Second, H₂S mobilizes the intracellular Ca²⁺ pool by likely activating PLC γ and InsP₃ release. Third, CSE contributes to trigger the pro-angiogenic Ca²⁺ response to VEGF in Ea.hy926 cells, but not in EPCs. It has long been known that VEGF-induced Ca²⁺ signals in human umbilical vein endothelium are ignited upon the activation of the PLC γ /InsP₃-dependent signalling axis [53]. Our results suggest that, in addition to direct phosphorylation by VEGFR-2, PLC γ might be recruited to the plasma membrane by the concomitant synthesis of H₂S. Indeed, under physiological conditions, H₂S could induce a sub-membranal Ca²⁺ elevation to facilitate the activation of this Ca²⁺-dependent enzyme. This scenario is subtly different from that envisaged by other authors and will deserve particular attention by future studies. Indeed, it

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has recently been proposed that VEGF causes H₂S synthesis by discharging intracellular Ca²⁺ to stimulate the Ca²⁺-sensitive CSE [19]. The results described elsewhere [21] and in the present study might lead to the re-evaluation of this hypothesis.

5. Figure legends

Figure 1. NaHS is a reliable activator of intracellular Ca²⁺ signals in Ea.hy926 cells. A, percentage of Ea.hy926 cells exhibiting an elevation when stimulated with increasing doses of NaHS. B, dose-response relationship of the Ca²⁺ response to NaHS in Ea.hy926 cells. Data are expressed as mean±SE. In both Panel A and Panel B, for each concentration, n ranged from 172 to 435 cells recorded from 3-5 independent coverslips. C, representative tracing of the increase in [Ca²⁺]_i elicited by three different doses of NaHS in Ea.hy926 cells. *P<0.05 as respect to 10, 30 and 200 μM; **P<0.05 as respect to 10, 30 and 200 μM.

Figure 2. Ca²⁺ entry does not trigger the Ca²⁺ response to NaHS in Ea.hy926 cells. A, removal of extracellular Ca²⁺ (0 Ca²⁺) does not prevent the onset of NaHS-induced Ca²⁺ signals in Ea.hy926 cells. NaHS has been applied at 30 μM. B, 30 min pre-incubation with 20 μM BTP-2, a powerful inhibitor of store-operated Ca²⁺ channels, did not interfere with the Ca²⁺ response to 20 μM BTP-2. C, mean±SE of the amplitude of NaHS-evoked increases in [Ca²⁺]_i in the absence of extracellular Ca²⁺ or in the presence of 20 μM BTP-2. D, mean±SE of the time to peak of NaHS-evoked [Ca²⁺]_i increases in the absence of extracellular Ca²⁺ or in the presence of 20 μM BTP-2.

Figure 3. Phospholipase C and inositol-1,4,5-trisphosphate receptors shape the elevation in [Ca²⁺]_i induced by NaHS in Ea.hy926 cells. The inhibition of PLC with either U73122 (10 μM) (A) or NCDC (50 μM) (B) prevented the Ca²⁺ response to NaHS (30 μM) in Ea.hy926 cells. C, the depletion of the InsP₃-sensitive Ca²⁺ pool with cyclopiazonic acid (20 μM) in 0Ca²⁺ abrogated NaHS-induced Ca²⁺ signals. NaHS has been applied at 30 μM. D, the inhibition of InsP₃Rs with 2-APB (50 μM) suppressed the increase in [Ca²⁺]_i elicited by NaHS (30 μM).

Figure 4. The relationship between H₂S and VEGF in modulating intracellular Ca²⁺ signals in Ea.hy926 cells. A, genistein (10 μM) prevented the onset of NaHS-induced Ca²⁺ signals in Ea.hy926 cells. NaHS has been applied at 30 μM. B, pre-incubation with SU5416 (10 μM, 30 min), a blocker of VEGFR-2 activity, did not affect the Ca²⁺ response to NaHS (30 μM). C, VEGF-evoked elevation in [Ca²⁺]_i was inhibited by SU5416 (10 μM, 30 min (grey tracing), while it occurred in control cells

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(black tracing). C, VEGF-induced Ca²⁺ signals were abrogated by pre-treating the cells with PAG (5 mM, 30 min), a selective CSE inhibitor.

Figure 5. Effects of exogenous and endogenous H₂S on Ea.hy926 cell proliferation and migration. A, Pre-incubation with the selective CSE inhibitor PAG (5 mM for 30 min), significantly reduced cell growth induced by VEGF (10 ng/ml, 72 hours). Absorbance at 490 nm is reported on Y axis. B, Histograms report the percentage of migration evaluated in wound healing experiments at 8 hours of treatment. Pre-incubation with PAG (5 mM, 30 min) reduced cells migration induced by VEGF 10 ng/ml (DMEM 0% was used as Ctl). Conversely, NaHS (30 μM) did not affect cell migration. All data are representative of three independent experiments. *P < 0.05; ** p < 0.01 compared to VEGF 10 ng/ml.

Figure 6. NaHS is not a powerful stimulator of Ca²⁺ signalling in human endothelial progenitor cells. A, fraction of EPCs displaying a detectable increase in [Ca²⁺]_i when challenged with increasing doses of NaHS. For each concentration, n ranged between 50 and 120 cells isolated from three different healthy donors. B, mean±SE of the amplitude of the elevations in [Ca²⁺]_i induced by NaHS in ECFCs. C, representative tracing of the Ca²⁺ signals elicited by 1 μM NaHS in human EPCs. D, mean±SE of the percentage of EPCs responding to VEGF (10 ng/ml) with Ca²⁺ oscillations in the absence and presence of PAG (5 mM). The numbers of cells computed for the statistical analysis were equal to 151 and 89, respectively, from three separate donors. E, sample traces of intracellular Ca²⁺ oscillations evoked by VEGF (10 ng/ml) in the presence (black tracing) and absence (grey tracing) of PAG (5 mM). *P<0.05. F, mean±SE of EPCs counted after three days in culture in the presence of EBM-2 (which served as control), EBM-2 + VEGF (10 ng/ml), and EBM-2 + VEGF (10 ng/ml) + PAG (5 mM). The results are representative of three different experiments conducted on cells harvested from three different donors.

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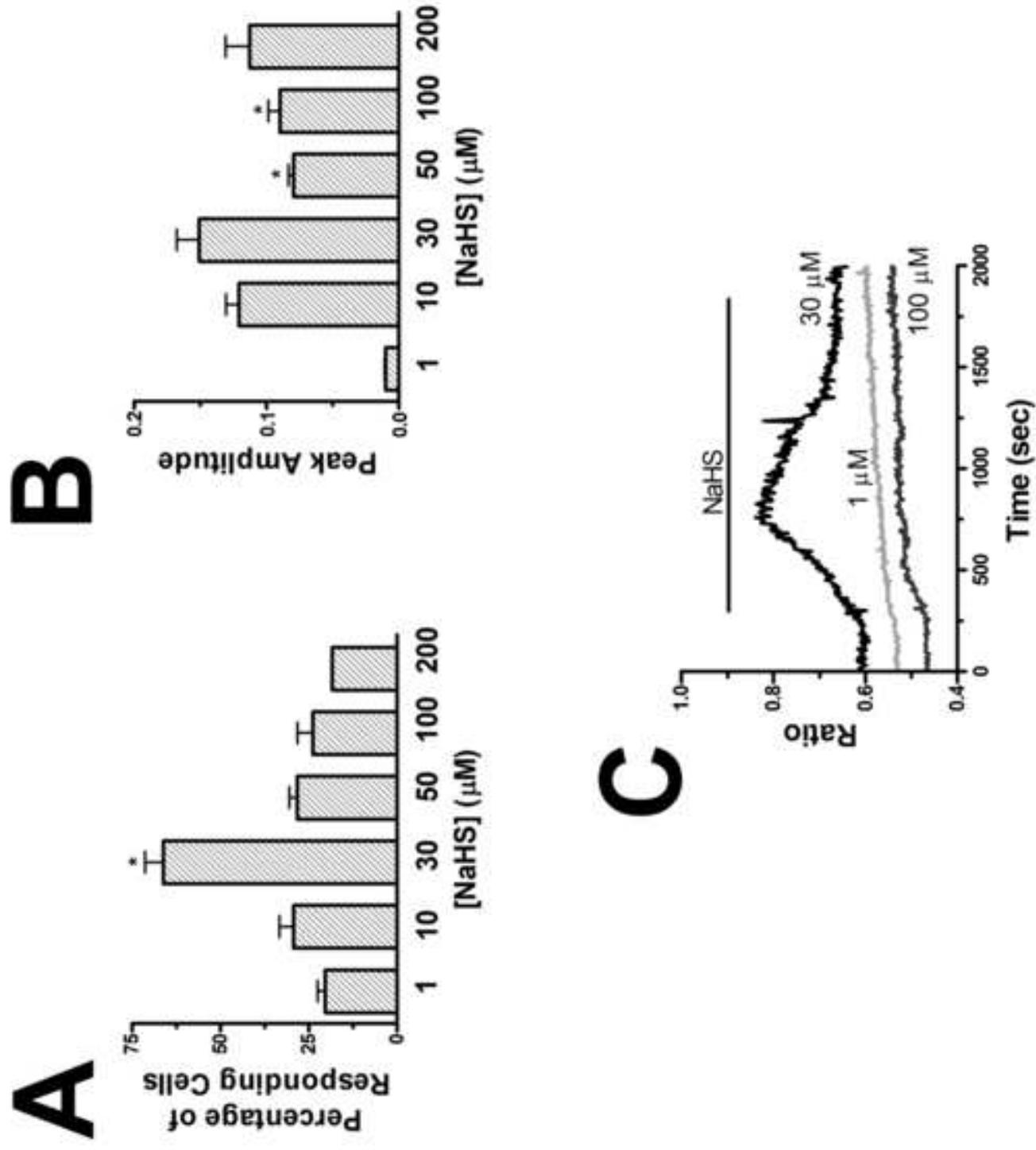
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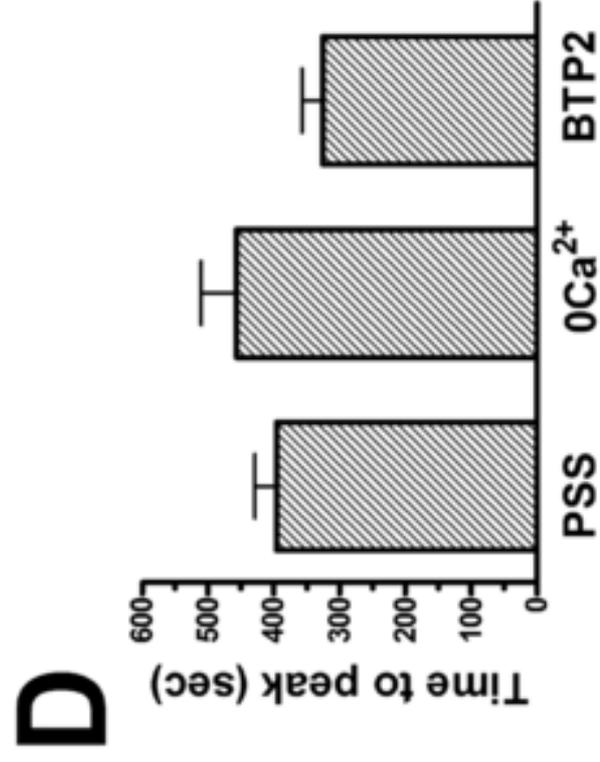
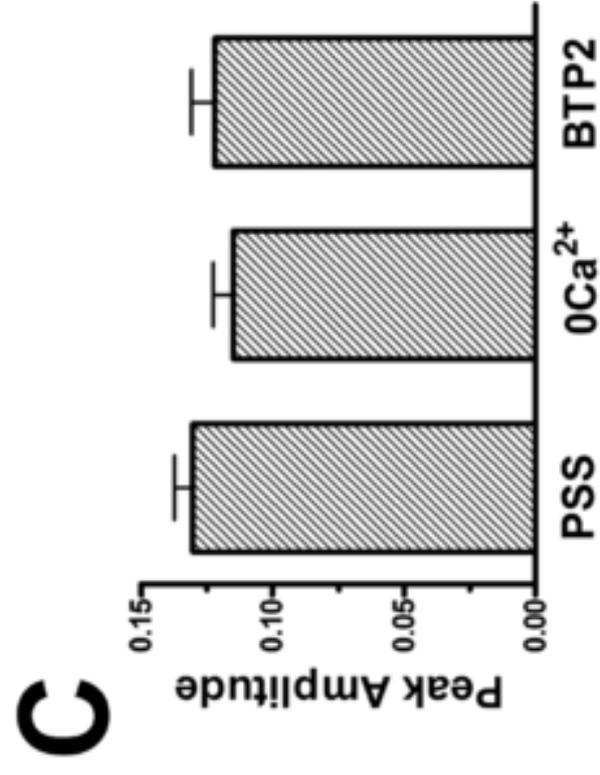
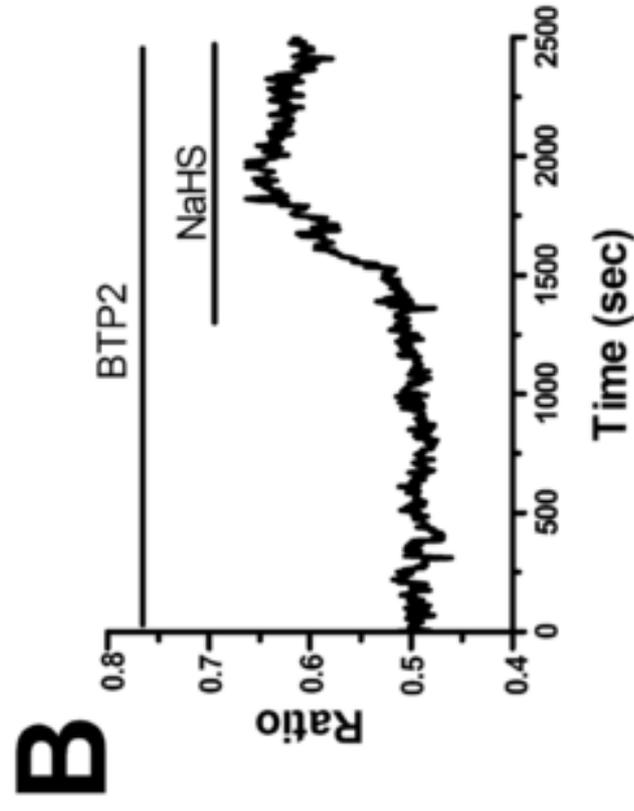
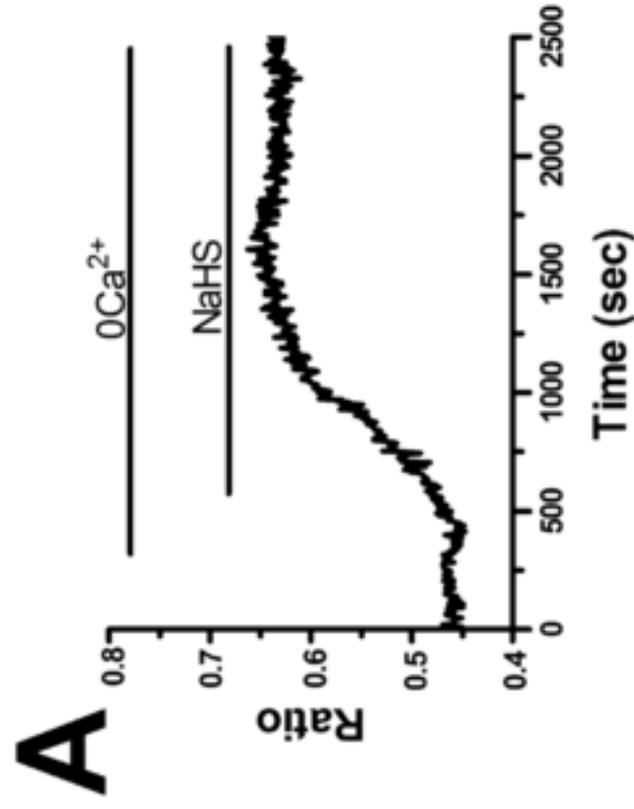
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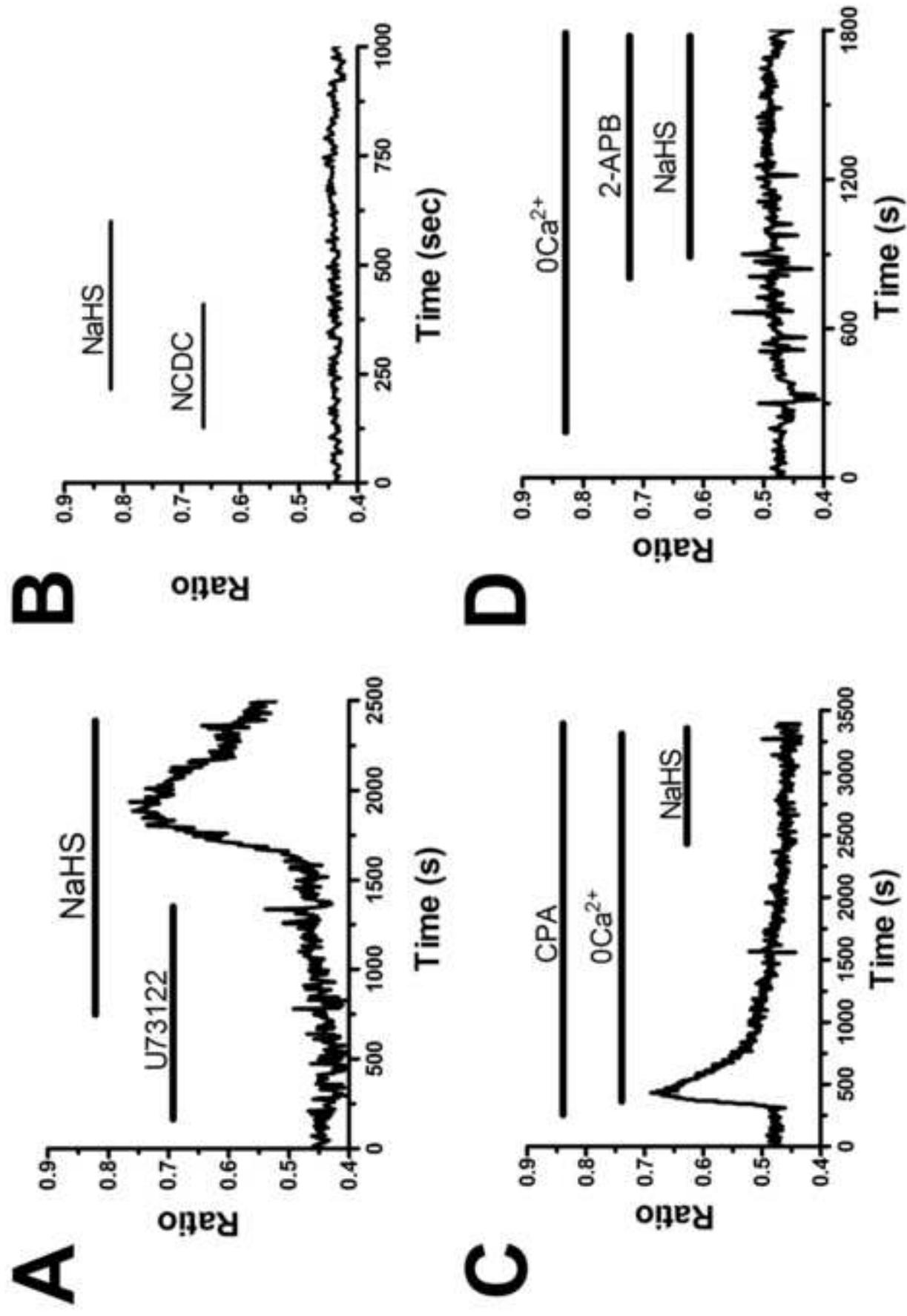
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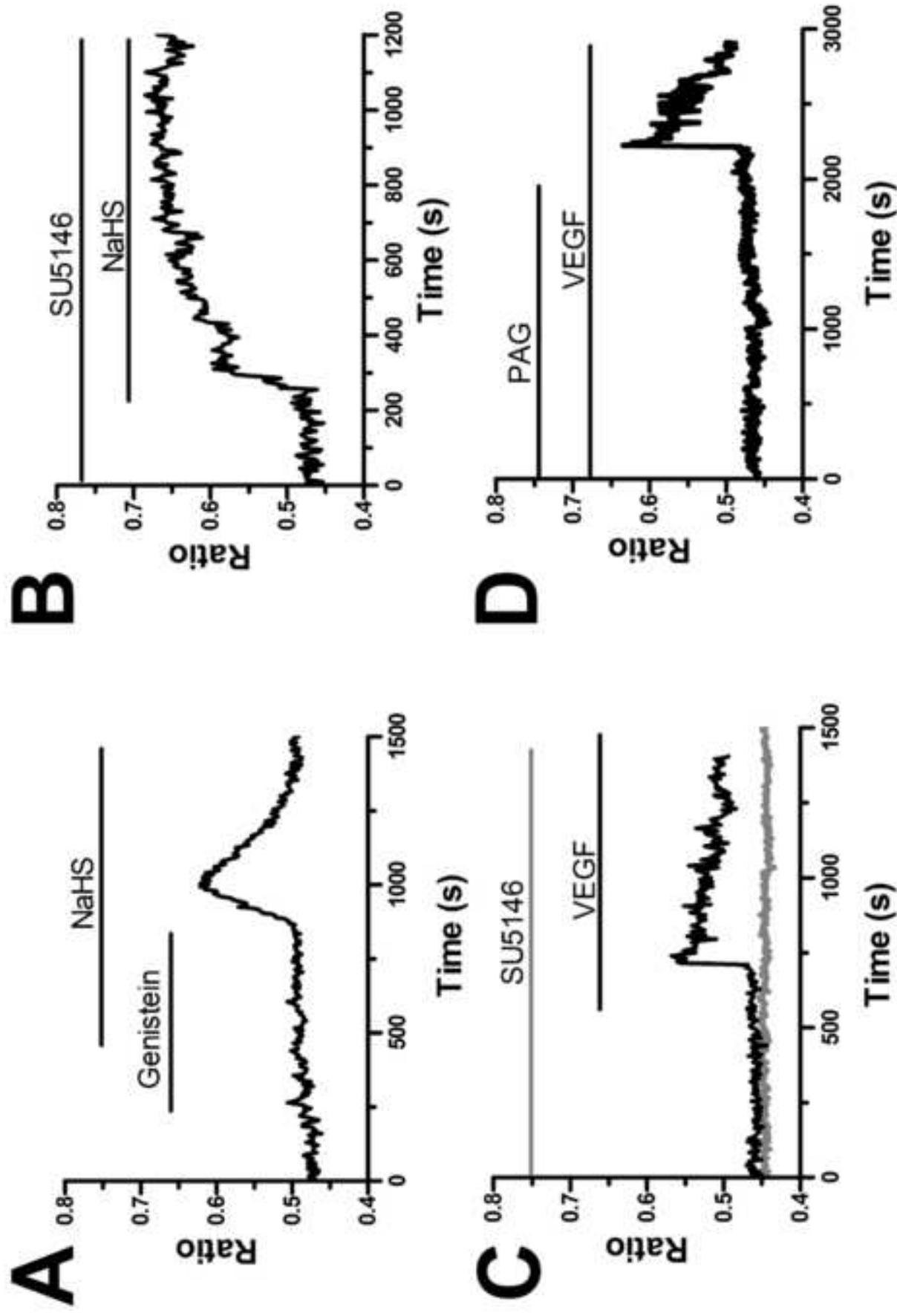
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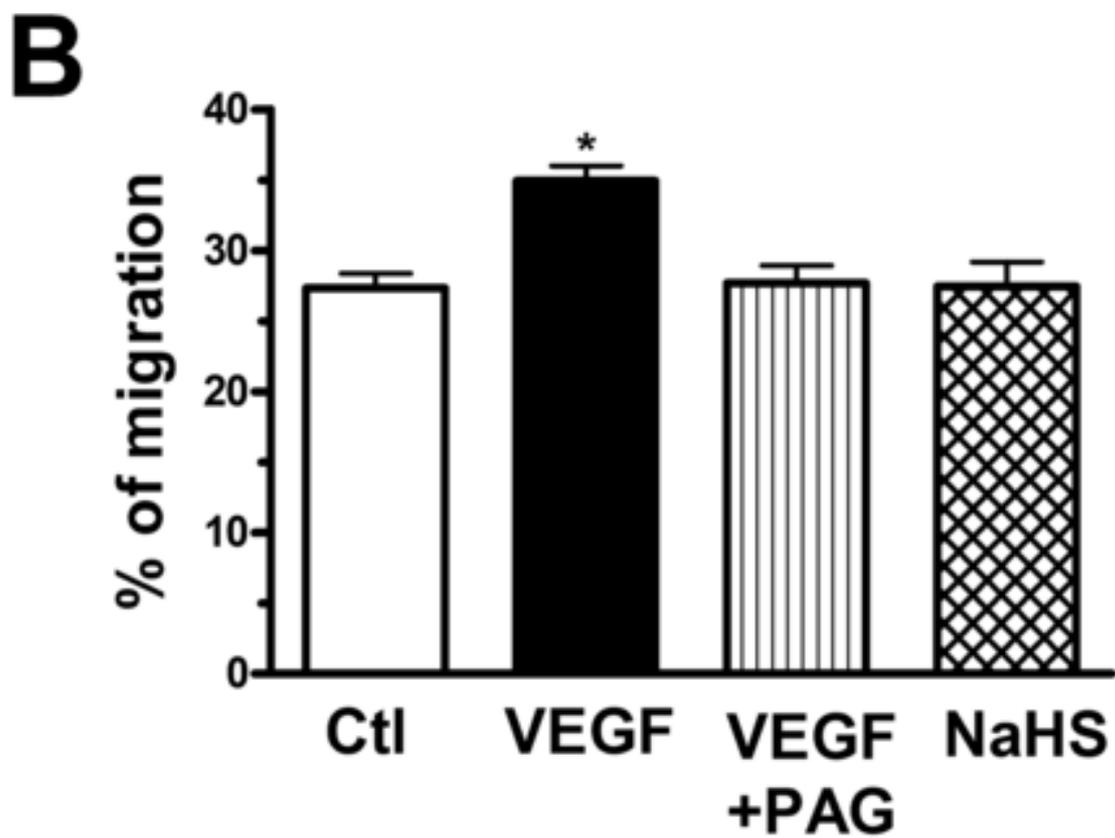
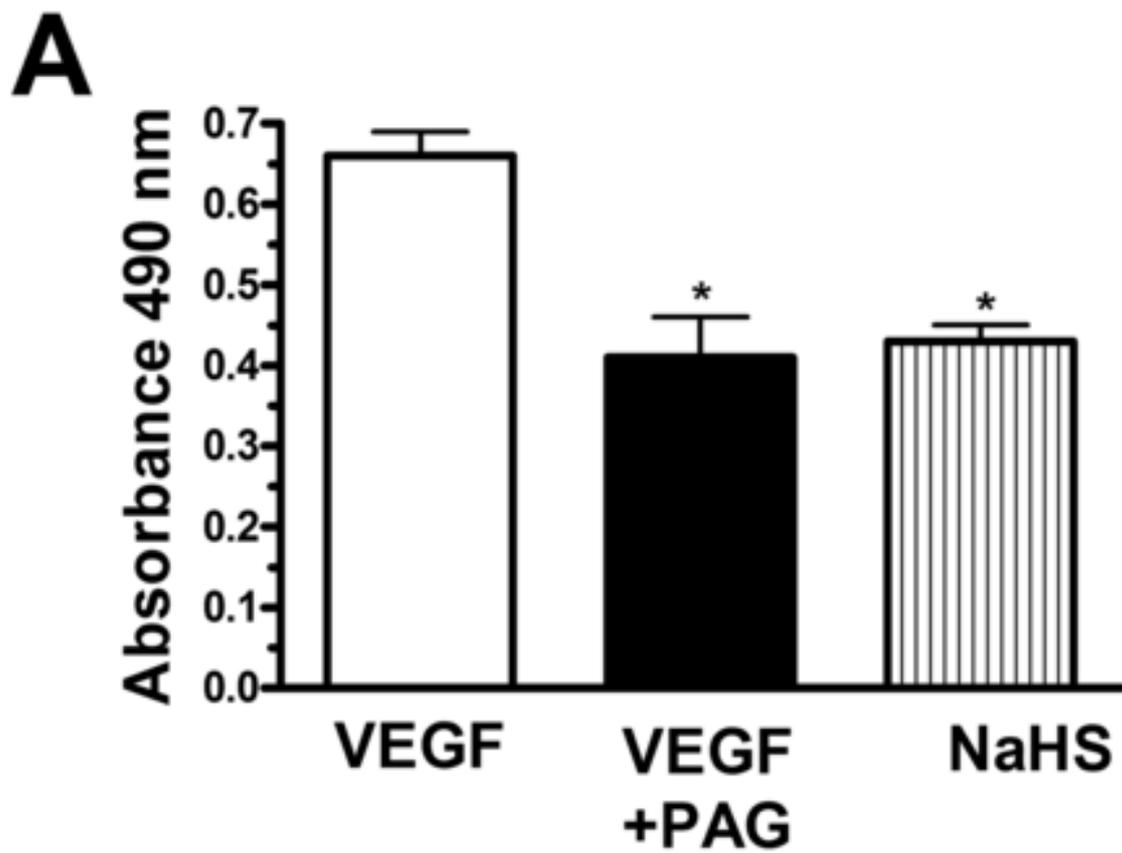
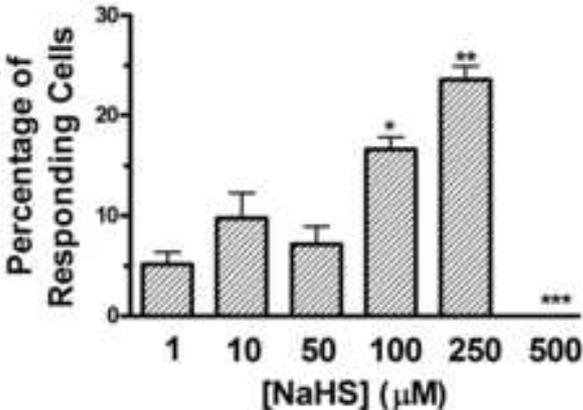
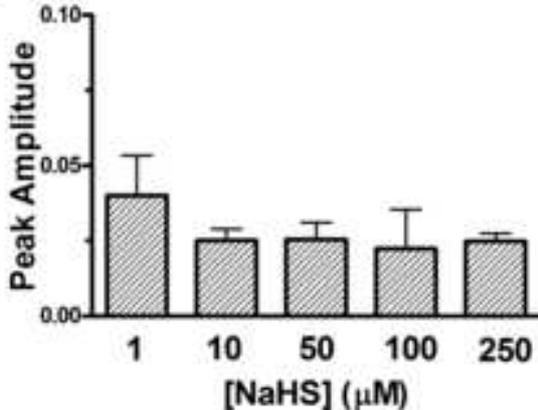


Figure
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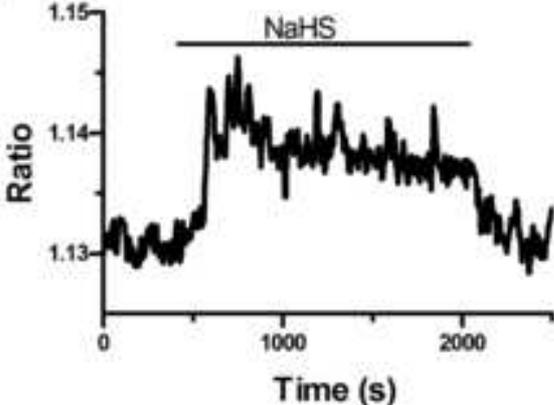
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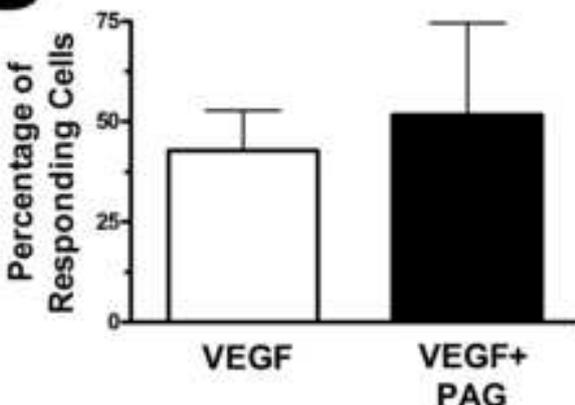
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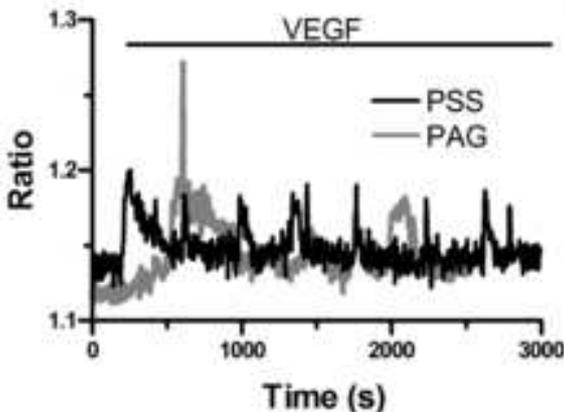
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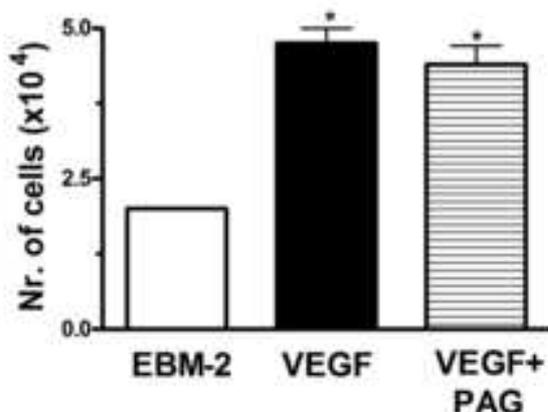
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F



Approval statement form

All the authors have materially participated in the research and/or article preparation. Their roles have been described below as well as their signed approval of the manuscript.

Duilio Michele Potenza: performed Ca^{2+} imaging experiments.

Germano Guerra: analyzed Ca^{2+} imaging data and drafted the manuscript.

Daniele Avanzato: carried out proliferation and migration assays in Ea.hy926 cells.

Valentina Poletto: isolated EPCs and carried out the related proliferative assays.

Sumedha Pareek: performed Ca^{2+} imaging experiments.

Daniele Guido: performed Ca^{2+} imaging experiments.

Angelo Gallanti: provided Ea.hy926 cells and participated to Ca^{2+} imaging experiments.

Vittorio Rosti: isolated EPCs and carried out the related proliferative assays.

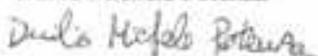
Luca Munaron: carried out proliferation and migration assays in Ea.hy926 cells, contributed to draft the manuscript.

Franco Tanzi: drafted the manuscript and analyzed Ca^{2+} imaging data.

Francesco Moccia: conceived the study, analyzed the data and wrote the manuscript.

Signed by all authors as follows:

Duilio Michele Potenza



Germano Guerra



Daniele Avanzato



Valentina Poletto



Sumedha Pareek



Daniele Guido



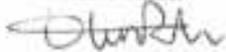
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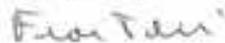
Vittorio Rosti



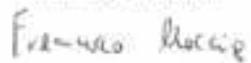
Luca Munaron



Franco Tanzi



Francesco Moccia



Conflict of interest form

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

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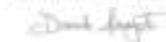
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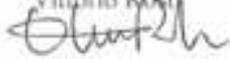
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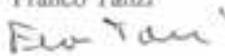
Vittorio Rosti



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Dear Referee #1,

Thank you very much for your nice comments on our manuscript entitled “Hydrogen sulphide triggers VEGF-induced intracellular Ca^{2+} signals in human endothelial cells but not in their immature progenitors”. We amended the manuscript according to your suggestions and addressed all your criticisms you raised. We do believe that your suggestions did improve the quality of the paper and do hope that you will now find it suitable for publication on Cell Calcium.

Major comments

It was reported that NaHS stock solution can be oxidized by O_2 under air to polysulfides (ABS at 290 nm), which can have biological effects (see Yuka Kimura, et al. FASEB J. 2013; † doi: 10.1096/fj.12-226415), and the commercial product, NaHS, mostly contains impurities and/or oxidation products, those can have also biological effects.

In this context, it is not clear how your H_2S stock solution was prepared, history of the solution and handling of the samples.

How was H_2S concentration determined? (See publication - + Supplementary data Greiner R. et al. ARS 2013).

This assertion is certainly true and we thank you for the advice to illustrate the preparation of NaHS stock and final solutions. The use of NaHS in the current experiments does not only depend on our past experience with this H_2S donor. We aimed at investigating for the first time the relationship, if any, between H_2S , Ca^{2+} signalling and angiogenesis. Most of the studies conducted to assess whether H_2S promotes angiogenesis both *in vitro* (Altaany et al., J Cell Mol Med 2013; 17(7):879-88; Cai et al., Cardiovasc Res 2007; 76:29-40; Coletta et al., Proc Natl Acad Sci U S A 2012; 109(23):9161-6; Papapetropoulos et al., Proc Natl Acad Sci USA 2009; 106(51):21972-7; tao et al., Antioxid Redox Signal 2013; 19(5):448-64) and *in vivo* (Coletta et al., Proc Natl Acad Sci



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USA 2012; 109(23):9161-6; Papapetropoulos et al., Proc Natl Acad Sci USA 2009; 106(51):21972-7; Qipshidze et al., Int J Biol Sci 2012; 8:(4), 430-441; Wang et al., Clin Exp Pharmacol Physiol 2010; 37(7):764-71), utilized NaHS as a donor. Therefore, we reasoned that it was more appropriate to exploit this same compound in our experiments.

Prof. Tanzi's comments here.

Minor comments

1.) Can you conclude from your study that RyR calcium channel is not involved in your observed effects?

As now discussed on Page 13 Line 28, one earlier report suggests that this endothelial cell line does not express ryanodine receptors.

2.) Some abbreviations (e.g. 2-APB page 10) should have full name somewhere.

You are right. We introduced in the text all the full names missing in the previous version of the manuscript.

3.) P. 11: Fig. 6A; Can you discuss no effect of NaHS at 500 μ M?

Thank you for this suggestion. This issue deserved more attention and we discussed it on page 16 Line 4.

4.) Fig. 6B; Can you discuss the same peak amplitude for different NaHS concentrations?

Again, thank you very much for this observation. We discussed this feature on Page 15, Line 31.



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5.) Can you include into the discussion some inconsistency of H₂S effect on IP₃R activity (e.g. inhibition effect (Castro-Piedras I, J Physiol. 2013) and your activation effects or others (e.g. L. Lencesova, et al. Acta Physiol 2013).

Absolutely so. We discussed this rather interesting point on Page 14, Line 3.

With my kindest regards,

Francesco Moccia

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Dear Referee #2,

We were happy to read your comments on our manuscript entitled “Hydrogen sulphide triggers VEGF-induced intracellular Ca^{2+} signals in human endothelial cells but not in their immature progenitors”. We amended the manuscript according to your suggestions and addressed all your criticisms you raised. We do believe that your suggestions did improve the quality of the paper and do hope that you will now find it suitable for publication on Cell Calcium.

Specific points

* If VEGF receptor is simultaneously stimulated by its selective agonist and by NaSH treatment, are the effects on pro-angiogenic Ca^{2+} response additive or mutually exclusive? What is the dominant mechanism?

Hydrogen sulphide is utilized by VEGF to trigger the Ca^{2+} response. Therefore, it is a key hub in the signalling pathway initiating the pro-angiogenic cascade elicited by this growth factor. If VEGF requires hydrogen sulphide to augment intracellular Ca^{2+} levels, the mitogen and the gasotransmitter operate in series rather than in parallel; this implies that their responses are neither additive nor mutually exclusive since they target the same machinery (i.e. the $\text{PLC}\gamma/\text{InsP}_3$ receptors axis) to exert the same effect. Indeed, our preliminary data show that neither the kinetics nor the amplitude of the Ca^{2+} response to VEGF are different when administrated along with NaHS. We will be glad to add this data if you require it.

* Did the Authors consider S-sulphydrilation of specific target as alternative mechanism of action of H_2S ?



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Thank you for this very interesting observation. Yes, we did it and we address this issue on Page 13, Line 27. Moreover, upon request of Referee #1, we discussed the possible effect of InsP3R reduction by hydrogen sulphide on Page 14, Line 8.

* In Materials and Methods the specific statistical approach used for determination difference in Fig. 1 and Fig 2 should be indicated. One way ANOVA is appropriate.

You are absolutely right and we are grateful for this observation. Actually, we used One way Anova followed by Student's t-test.

* Introduction. The authors should insert more information regarding physiological cardiovascular effects of H₂S, citing original article rather than reviews.

We were happy to follow your advice and to quote the original articles describing the effects of hydrogen sulphide on cardiovascular system. In this view, we added more effects exerted by this gasotransmitter on vascular endothelial cells.

* Discussion needs to be strongly reduced.

We shortened the Discussion, but had to address several additional issues upon kind request of Referee #1.

* There are several typos in the text.

Thank you for this observation. We amended all the typos in the text.

With my kindest regards,

Franco Maccè



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