Annexin A1 mediates hydrogen sulfide properties in the control of inflammation

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Running title page

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Abstract

Hydrogen sulfide (H₂S) is a gaseous mediator synthesized in mammalian tissues by three main enzymes, cystathionine-β-synthase (CBS), cystathionine-γ-lyase (CSE) and 3-mercaptoppyruvate-sulfurtransferase (3MST), and its levels increase under inflammatory conditions or sepsis. Since H₂S and H₂S-releasing molecules afford inhibitory properties on leukocyte trafficking, we tested whether endogenous AnnexinA1 (AnxA1), a glucocorticoid-regulated inhibitor of inflammation acting through formylated-peptide-receptor-2 (FPR2/ALX), could display intermediary functions in the anti-inflammatory profile of H₂S. We first investigated whether endogenous AnxA1 could modulate H₂S biosynthesis. To this end, a marked increase in CBS and/or CSE gene products was quantified by qPCR in aorta, kidney and spleen collected from AnxA1-/- mice, as compared to wild type animals. When LPS-stimulated bone marrow derived macrophages (BMDM) were studied, H₂S-donor NaHS counteracted the increased expression of iNOS and COX2 mRNA evoked by the endotoxin, yet it was inactive in macrophages harvested from AnxA1-/- mice. Next we studied the effect of in vivo administration of NaHS in a model of IL1β-induced mesenteric inflammation. AnxA1+/+ mice treated with NaHS (100 μmol/kg) displayed inhibition of IL1β-induced leukocyte adhesion/emigration in the inflamed microcirculation, not observed in AnxA1-/- animals. These results were translated by testing human neutrophils, where NaHS (10-100 μM) prompted an intense mobilization (>50%) of AnxA1 from cytosol to cell surface, an event associated with inhibition of cell/endothelium interaction under flow. Taken together, these data strongly indicate the existence of a positive interlink between AnxA1 and H₂S pathway, with non-redundant functions in the control of experimental inflammation.
**Introduction**

Inflammatory process represents a complex network of events in the host response to infections and insults. This network is tightly regulated by engagement of a series of lipid mediators, cytokines and adhesion molecules promoting leukocyte extravasation, immune cell trafficking and removal of pathogen (Nathan, 2002; Nathan and Ding, 2010). Such a chain of events is somehow paralleled by release/biosynthesis of different mediators stopping cell trafficking, promoting apoptosis and efferocytosis of extravasated cells and leading to tissue repair (Serhan, 2011), known as effectors of resolution. The latter events are temporally and spatially regulated within the inflamed tissue site, in line with the appreciation that “the beginning programs the end” (Serhan and Savill, 2005). Annexin A1 (AnxA1), a 37kDa calcium binding protein is involved within the resolution process together with other mediators regulating the pro-resolving response such as lipoxin A₄ (LXA₄) or resolvins (Serhan, 2007). In particular, AnxA1 and LXA₄ are prototypes of pro-resolving mediators in view of their nature (protein vs lipid), mode of production/release (cytoplasmic pool mobilization vs enzymatic biosynthesis) and engagement by widely used therapeutics (glucocorticoids for AnxA1 vs. aspirin for LXA₄). Interestingly, these two mediators share the activation of the same G-protein-coupled receptor, namely formyl-peptide receptor 2 (FPR2/ALX), through which they exert antinflammatory actions (Perretti et al., 2002; Brancaleone et al., 2011). AnxA1 is very abundant in human polymorphonuclear cells (PMN), representing between 2% and 4% of total intracellular proteins (Rosales and Ernst, 1997). In resting PMN, a proportion (~60%) of intracellular AnxA1 is localized in gelatinase and azurophilic granules (Perretti et al., 2000; Lominadze et al., 2005), with the
remaining stored as "free" cytosolic pool. PMN activation triggers a rapid mobilization of AnxA1 and its exposure onto the cell surface, which in turn curtails the extent of neutrophil trafficking leading to a reduction of inflammation (Gastardelo et al., 2009). In particular, AnxA1 also exerts its effect by provoking the detachment of leukocytes from vessel wall when administered during inflammation (Gavins et al., 2003; Brancaleone et al., 2011).

Among the wide range of signaling mediators that regulate organ and cell function including inflammatory processes, hydrogen sulfide (H₂S) represents a novel gasotransmitter. It is mainly synthesized by pyridoxal-phosphate dependent enzymes cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CSE) from physiological precursor L-cysteine (Stipanuk, 2004). Conversely, 3-mercaptoppyruvate sulfurtransferase (3MST) is a zinc-dependent enzyme mainly localized in mitochondria that also accounts for hydrogen sulfide biosynthesis (Shibuya et al., 2009).

Recent findings support involvement of hydrogen sulfide in maintenance of cardiovascular homoeostasis, as it exhibits vasodilator activity both in vitro and in vivo (Zhong et al., 2003; Ali et al., 2006; Qu et al., 2006) and modulates apoptosis in vascular smooth muscle cells (Zhao et al., 2001; Yang et al., 2004). Although hydrogen sulfide functions in cardiovascular system have been widely studied and diverse mechanisms have been addressed, there is still a lack of univocal information on its role in inflammation. Indeed, administration of hydrogen sulfide donors mitigates leukocyte-endothelium interaction in the inflamed microcirculation (Zanardo et al., 2006; Zhang et al., 2007b) and promotes tissue healing (Wallace et al., 2007; Wallace et al., 2009); although there are reports for hydrogen sulfide increasing in septic shock conditions and
promoting organ injury in endotoxemia (Hui et al., 2003; Collin et al., 2005). Consistently, lipopolysaccharide (LPS)-induced inflammation displays an increase in hydrogen sulfide levels and CSE expression, which is reverted by glucocorticoids (Zhu et al., 2010). Despite recent literature seems to address hydrogen sulfide as a pro-resolutive mediator (Wallace et al., 2012), scientific evidence does not unambiguously clarify whether hydrogen sulfide biosynthesis is elevated to drive the inflammatory response or to limit tissue inflammation (Bhatia et al., 2005; Zanardo et al., 2006; Sivarajah et al., 2009; di Villa Bianca et al., 2010; Whiteman and Winyard, 2011). Here we addressed this aspect by testing whether hydrogen sulfide modulates vascular inflammatory processes and determining whether the AnxA1 pathway might display intermediary functions in its signaling.
Materials and Methods

Animals. Male AnxA1+/+ or AnxA1-/- littermate mice (C57Bl/6 background, 3-4 week old) were maintained on a standard chow pellet diet and had free access to water, with a 12h light-dark cycle. Animals were used according to the guidelines laid down by the Ethical Committee for the Use of Animals, Barts and The London School of Medicine on the basis of ARRIVE protocol. Animal work was performed according to Home Office Regulations (Guidance on the Operation of Animals, Scientific Procedures Act, 1986).

Bone Marrow-Derived Macrophages (BMDM) Preparation. BMDM were obtained from femurs and tibias of 4-6 week old AnxA1+/+ or AnxA1-/- mice, using ≥ three mice per genotype. The marrow was flushed from the bone, washed, re-suspended (2-3x10^6 cells/ml) in DMEM supplemented with L-glutamine, penicillin/streptomycin (Lonza Biologics, Slough, U.K.), 20% FCS, and 30% L929 conditioned medium, and incubated at 37°C over 5 days. Macrophages were treated with *E.coli* LPS (0111:B4, 100ng/ml, 6h; Sigma-Aldrich) alone or in presence of sodium hydrosulfide (NaHS,100μM, -1h). Cells were then collected and used for RNA extraction and real time PCR analysis.

Hydrogen sulfide Quantification Assay. H₂S determination in plasma samples was performed as described (d’Emmanuele di Villa Bianca et al., 2013). Briefly, samples (200μL) were added to eppendorf tubes containing trichloroacetic acid (TCA, 10%, 300μL), in order to allow protein precipitation. Supernatant was collected after centrifugation and zinc acetate (ZnAc, 1%, 150μL) was then added. Subsequently, N,N-diphenylenediamine (DPD, 20mM, 100μL) in 7.2M HCl
and iron chloride (III) (FeCl₃, 30mM, 133µL) in 1.2M HCl were added to the reaction mixture and absorbance was measured after 20 min at a wavelength of 668nm. All samples were assayed in duplicate and H₂S concentration was calculated against a calibration curve of NaHS (3.12–250µM).

**Quantitative Real-Time PCR.** Quantization of the expression level of selected genes (CBS, CSE, COX2, iNOS) was performed by quantitative real-time PCR. Total RNA was isolated form liver, aorta, kidney and spleen collected from AnxA1+/+ or AnxA1-/- mice by using TRizol reagent (Invitrogen, Carlsbad, CA, USA). For real-time PCR, 10ng template was used in combination with each primer solution (Qiagen, Hilden, Germany) and Fast SYBR Green Master Mix solution (Applied Biosystem, Paisley, UK). All reactions were performed in a 7900HT Fast Real-Time PCR System instrument (Applied Biosystem). Relative expression (vs housekeeping gene GAPDH) was reported in graphs as relative quantity (RQ) (n=3).

**Intravital Microscopy in Mouse Mesenteric Microcirculation.** Intravital microscopy was performed as previously reported (Gavins et al., 2003; Leoni et al., 2008). Mice were treated with IL1β (10ng/mouse i.p., 2h; eBioscience, Hatfield, UK) alone or in combination with NaHS (100µmol/kg s.c., 1h before IL1β injection; Sigma-Aldrich), L-cysteine (1000µmol/kg s.c. 1h before IL1β injection; Sigma-Aldrich) or D,L-propargylglycine (PAG, 10mg/kg i.p., 30 minutes before IL1β injection; Sigma-Aldrich). In another set of experiments, NaHS was given at the same time as IL1β or 1h after IL1β injection. In all cases, AnxA1-/- or wild type mice were anesthetized and placed in the supine position on a heating pad.
(37°C). A cautery incision was made along the abdominal region and the vascular bed was exposed and positioned under the microscope while superfused with thermostated (37°C) bicarbonate-buffered solution gassed with 5%CO2/95%N2, at a rate of 2 ml/min; recording started after a 5-minute equilibration period, followed by offline analyses, as reported (Gavins et al., 2003). These were made in 1 to 3 randomly selected postcapillary venules (diameter, 20-40µm; visible length >100µm) for each mouse. Thus, rolling leukocyte flux was obtained by the number of leukocytes passing a reference point in the venule per minute (cells/minute); leukocyte adhesion reflected cells stationary for 30 seconds or longer; leukocyte emigration was calculated by the number of cells in a 100x50µm² area on both sides of the 100µm vessel segment. For all vessels, red blood cell centerline velocity was measured with an optical Doppler velocimeter (Microcirculation Research Institute, Texas A&M University, Dallas, TX) and venular wall shear rate was determined based on the Newtonian definition: wall shear rate = 8000 [(red blood cell velocity/1.6)/venular diameter].

**Human PMN Isolation and Membrane/Cytosolic Fractions Preparation.**
Peripheral blood was collected from male healthy volunteers by intravenous withdrawal in 3.2% sodium citrate solution (1:10). PMNs were isolated from blood by density centrifugation on Histopaque 1119/1077 (Sigma-Aldrich, Poole, UK) gradient according to the manufacturer’s instructions and suspended in PBS containing 0.5% BSA. All healthy volunteers gave oral and written consent and cell separation was covered by ethical approval 05/Q0603/34 (East London and The City Research Ethics Committee 1). Cells (3x10⁶) from 4 different male volunteers were treated with NaHS (10-100µM) or rolipram (1-100µM; Tocris,
Bristol, UK) for 30 minutes. After incubation, PMN were centrifuged to separate cell pellet from supernatant and cytosolic and membrane fractions were obtained for western blot analysis. In a separate experiment, PMN were also screened for cAMP levels upon NaHS and/or rolipram administration (10 or 100µM). In another set of experiments, PMN were therefore treated with fMLP (0.1µM, 30 minutes; Sigma-Aldrich) in presence of vehicle, NaHS (1-100µM, -10min) or L-cysteine (1-100µM, -10min) and cells were then analysed to check CD11b surface expression by flow cytometry. After treatment, cells (4x10^6) were re-suspended in lysis buffer and processed. Lysates were subsequently centrifuged for 2 min at 300xg, and supernatants centrifuged again for 45 min at 800xg. The resultant supernatant (cytosolic fraction) was collected and the remaining pellet re-suspended in lysis buffer supplemented with 1% Triton X-100, for 15 minutes (membrane fraction) (Vong et al., 2007).

**Western Blot Analysis.** Samples boiled in 6x Laemmlili buffer were subjected to standard SDS-polyacrylamide gel electrophoresis (12%) and electrophoretically blotted onto polyvinylidene difluoride membranes (PVDF, Millipore, Watford, UK). Membranes were incubated with mouse monoclonal antibodies (mAb) anti-human AnxA1 (clone 1B; dilution 1:1,000, (Pepinsky et al., 1990)) in Tris-buffer saline solution containing 0.1% Tween-20 (TBST) and 5% (w/v) non-fat dry milk overnight at 4°C. Membranes were washed for 30 minutes with TBST with the solution being changed at 10 minutes intervals; membranes were then incubated with secondary antibody (HRP-conjugated goat anti-mouse 1:5000, Dako, Cambridge, UK), for 2h at room temperature. Proteins were then detected using...
the ECL detection kit and visualized on Hyperfilm (Amersham Biosciences, Amersham, UK).

**Cyclic AMP Determination.** Samples collected after NaHS and/or rolipram treatment were processed according to the manufacturer's instructions to measure intracellular cAMP levels (Cayman, Ann Arbor, MI, US).

**Flow Cytometry.** Human PMN flow cytometry was performed to quantify the extent of CD11b expression on neutrophil as a marker of cell activation. Following staining with anti-CD11b (clone CBRM1/5, eBioscience, Hatfield, UK), cell pellet was washed and fixed before sample analysis with a FACScalibur flow cytometer (BD Bioscience, Oxford, UK), acquiring >10000 events. Results are reported as fluorescence intensity (MFI) units of CD11b positive events.

**Data analysis.** All values are given as the mean±S.E.M., with n indicating the number of animals for each experiment or the number of times that the experiments were repeated. Statistical differences were determined by Student’s t-test or repeated-measurement analysis of variance followed by the Dunnett post-test. P<0.05 was considered to be significant. Analysis of the data and plotting of the figures were aided by GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA).
Results

Differential Levels of CBS/CSE mRNA and hydrogen sulfide in AnxA1-/- Mice. We first analyzed different tissues harvested from AnxA1-/- and AnxA1+/+ mice and compared their CBS and CSE mRNA content. In particular, we found an increase in mRNA expression for both CBS and CSE genes in aorta (figure 1A), while only CBS was upregulated in spleen and kidney (figure 1B and C). No changes in mRNA levels were measured in liver samples (supplemental figure 1). Next, we evaluated circulating hydrogen sulfide levels in both AnxA1+/+ and AnxA1-/- mice. Our data revealed that H_{2}S plasma levels were higher in AnxA1-/- compared to AnxA1+/+ mice (figure 1D), as a plausible outcome for higher CBS/CSE mRNA expression.

Hydrogen Sulfide Reverts LPS-induced Inflammation in BMDMs. Based on differences in CBS/CSE expression and H_{2}S levels observed in AnxA1-/- compared to AnxA1+/+ mice, we decided to use macrophages derived from bone marrow to investigate on a possible anti-inflammatory effect mediated by hydrogen sulfide in this setting. LPS administration to cells derived from both AnxA1+/+ or AnxA1-/- mice showed a significant increase in COX2 (figure 2A and D) and iNOS (figure 2B and E) mRNA levels, used as established markers for inflammation. Furthermore, LPS treatment also increased CSE expression, in line with current literature (figure 2C) (Collin et al., 2005; Zhu et al., 2010). However, this effect was only observed in AnxA1+/+ derived macrophages. Exposure of the cells to exogenous hydrogen sulfide (100µM NaHS) 1h prior to LPS administration, significantly dampened the increase of COX2, iNOS, and CSE mRNA induced by LPS in AnxA1+/+ (figure 2A-C), while administration of NaHS
alone to unstimulated BMDMs was unappreciable in any of the gene products studied (supplemental figure 2). Furthermore, opposite to AnxA1+/+ cells, administration of hydrogen sulfide in AnxA1-/- derived macrophages did not affect COX2, iNOS, and CSE mRNA levels (figure 2D-F). These data implicate AnxA1 in the effects driven by hydrogen sulfide.

Hydrogen Sulfide Exerts Antinflammatory Properties Through AnxA1 Pathway. In order to better define the role of AnxA1 in the anti-inflammatory action of H$_2$S, we performed intravital microscopy analysis in mouse mesenteric microcirculation and the number of emigrated cells was evaluated. In untreated animals, baseline cell number is within 0-1 range. Inflammation was induced by injecting IL1$\beta$ (10ng/mouse) into mice peritoneal cavity 1h after vehicle, NaHS (100µmol/kg) or L-cysteine (1000µmol/kg) treatment. Pre-treatment of AnxA1+/+ mice with hydrogen sulfide significantly reduced cell emigration in post-capillary venules compared to vehicle treated group (figure 3A and B). Similar effects were also observed for cell adhesion (supplemental figure 3). Conversely, the same protocol, when performed in AnxA1-/- mice, did not display any antinflammatory effect following IL1$\beta$ injection (figure 3C). Same results were obtained by using L-cysteine (figure 3D). Furthermore, pretreatment with CSE inhibitor PAG significantly exacerbated the inflammatory pattern observed in AnxA1+/+ animals, confirming a tonic role for H$_2$S in control of inflammation (figure 3D) (Asimakopoulou et al., 2013). In order to establish whether H$_2$S could also revert inflammation, besides preventing it, we carried out a different approach where NaHS was injected in wild type mice at the same time of (time 0) or 1 hour after (time +1) IL1$\beta$. In both cases the systemic administration of exogenous hydrogen
sulfide significantly reduced cell emigration (figure 3E). It is noteworthy to underline that NaHS, L-cysteine and PAG only exerted their effects in presence of inflammation, since their administration did not alter per se baseline levels of leukocyte (supplemental figure 4).

**Hydrogen Sulfide activates the AnxA1 Pathway.** To provide translational impact to these findings, the last series of experiments was performed with human granulocytes. Resting PMN retain the majority of AnxA1 in the cytosol as granular or free stores, which are mobilized by agonist-induced mechanisms or cell activation (Brancaleone et al., 2011). Treatment with NaHS (10-100μM, 30min) induced externalization of intracellular AnxA1 from cytosol towards the membrane surface and its further release in the cell culture medium (figure 4A). One of the mechanisms engaged by PMN to release AnxA1 relies on the phosphorylation of AnxA1 and this involves a mechanism based on agonist-receptor interaction (Solito et al., 2003; Yazid et al., 2010). Nonetheless, hydrogen sulfide is also known as a non-specific phosphodiesterases (PDE) inhibitor (Bucci et al., 2010). Therefore, we supposed that inhibition of PDE4, the predominant PDE in granulocytes (Smolen and Geosits, 1984; Grady and Thomas, 1986; Wang et al., 1999), might have a role in AnxA1 mobilization triggered by hydrogen sulfide. In order to test this hypothesis, we stimulated PMN with PDE4 inhibitor rolipram (1-100μM, 30min) and monitored for both AnxA1 mobilization and cAMP levels. As shown in figure 4B, rolipram, similarly to NaHS, significantly increased AnxA1 migration from cytosol to membrane in a dose-dependent fashion. In addition, cAMP levels were increased upon treatment with NaHS (100μM), rolipram (100μM) or their combination (both at 10μM) (Table 1).
Hydrogen Sulfide Reduces The Expression of CD11b. Finally, we aimed to verify whether AnxA1 exposure onto PMN cell surface triggered by H₂S, might generate a functional response underlying the reduced leukocytes transmigration observed in vivo (figure 3). For this purpose, we determined PMN surface expression of CD11b, a well-established marker for leukocyte activation, following fMLP (0.1µM) treatment in presence of NaHS or L-cysteine (1-100µM). As shown in figure 5, flow cytometry data indicated that cell incubation with either NaHS or L-cysteine reduced CD11b surface expression in a concentration dependent manner (figure 5).
Discussion

Inflammatory response relies on a complex network of chemically different modulators produced within the body (Stipanuk, 2004; Perretti and D’Acquisto, 2009). Hydrogen sulfide is one of these though it can promote inflammation in animal models of sepsis (Zhang et al., 2007a), where inhibition of its biosynthesis exerted beneficial effects (Li et al., 2005). Nevertheless it can also dampens inflammation through different pathways i.e. reducing cytokines (IL6, IL8, TNFα) production or iNOS expression (Ganster et al., 2010; Zeng et al., 2013). Furthermore, additional evidence show that H2S treatment during ongoing inflammatory reactions attenuates leukocytes infiltration by reducing adhesion molecule expression (Elrod et al., 2007; Sivarajah et al., 2009; Li et al., 2011).

Here we have further investigated on the role played by hydrogen sulfide in the control of inflammation, aiming to disclose a possible interplay with the pro-resolving protein AnxA1. To pursue our aim, we first evaluated whether lack of functional AnxA1 in mice could affect expression of H2S synthesizing enzymes CBS and CSE. Real time PCR data showed that mRNA levels of either CBS or CSE were actually augmented in aorta, spleen and kidney samples derived from AnxA1-/- mice (figure 1). Such an increased expression associated to lack of AnxA1 reflected a parallel increase in plasma H2S levels, indicating the existence of cross-talk between hydrogen sulfide and AnxA1. In order to verify this aspect in functional terms, we used LPS-challenged BMDMs collected from AnxA1+/+ or AnxA1-/- mice. We first showed that, in AnxA1+/+ derived cells, exposure to hydrogen sulfide prior to LPS challenge significantly reversed the increase in mRNA expression of COX2, iNOS, and CSE. Such an antinflammatory response resulted absent in cells derived from AnxA1-/- mice. This finding indicates that
hydrogen sulfide has no effect when AnxA1 is lacking or not functional, thereby suggesting that H₂S requires AnxA1 activation. Nevertheless, we cannot exclude that the lack of effect by H₂S in AnxA1-/- cells might derive by the fact that in AnxA1-/- mice H₂S is already overproduced, generating a possible saturated condition.

Based on these findings, we then hypothesized that H₂S could exert its *in vivo* antinflammatory effect also by engaging the AnxA1 pathway (Li et al., 2011). To validate this hypothesis, we used an *in vivo* model of inflammation, where IL1β was injected into the peritoneal cavity of both AnxA1+/+ and AnxA1-/- mice to trigger leukocyte trafficking in the mesenteric microvasculature. Administration of hydrogen sulfide to AnxA1+/+ mice significantly reduced the number of emigrated leukocytes (figure 3A and 3B). Conversely, its administration failed to counteract *in vivo* cell trafficking in AnxA1-/- animals. Therefore absence of functional AnxA1 blunts H₂S *in vivo* anti-inflammatory effect. This finding is further confirmed by the observation that PAG, used as CSE inhibitor (Asimakopoulou et al., 2013), exacerbated inflammatory process when injected before IL1β (figure 3D). These observations further confirm that AnxA1 is somehow involved in controlling PMN trafficking *in vivo* operated by H₂S (figure 3C and 3D). In addition the reduction in emigrated cells, following hydrogen sulfide treatment, observed in wild type animals was consistently independent from the administration timeframe. On this basis we propose that H₂S or H₂S-based drugs show antinflammatory efficacy only during ongoing inflammation. Indeed, there is no effect on leukocyte trafficking in absence of inflammatory stimulus (supplemental figure 4).

One of the pro-resolutive action of AnxA1 occurs through the detachment of adherent leukocytes from the vessel wall (Gavins et al., 2003; Brancaleone et al.,
2011) and we found that this effect is also shared by hydrogen sulfide (supplemental figure 5). Indeed, a similar degree of leukocyte detachment between hydrogen sulfide and AnxA1 is evident. Indeed, the observation that such detaching properties by hydrogen sulfide is lost in AnxA1-/- mice add further evidence to the hypothesis that H_2S may trigger activation, and the functional involvement, of the AnxA1 pathway.

Having assessed the importance of AnxA1 in hydrogen sulfide-associated effects, we attempted to translate these observations to human, using polymorphonuclear cells (mainly neutrophils) harvested from human healthy volunteers. We used human PMN since AnxA1 is highly abundant in these cells and it is mainly stored in the cytosol as granular or “free” pool. Studies in the past years have indicated how distinct stimuli, spanning from Lipoxin A4 (Brancaleone et al., 2011) to histone-deacetylase inhibitors (Montero-Melendez et al., 2013), from estrogen (Nadkarni et al., 2011) to glucocorticoids (Yazid et al., 2010), can externalize and/or release the protein in extracellular environment, leading to the conformational change required to activate its receptors (Perretti and D’Acquisto, 2009). Following hydrogen sulfide treatment, AnxA1 is mobilized from the cytosol onto the plasma membrane and then released in cell supernatant and this movement does not involve AnxA1 stored within PMN granules (supplemental figure 6). Thus, our data suggest that H_2S induces the release of AnxA1 from a “free” cytosolic pool, although we cannot exclude that such a mobilization might involve microparticles-incorporated AnxA1 (Dalli et al., 2008). Therefore, since H_2S has been shown to inhibit PDE (Bucci et al., 2010), we evaluated whether AnxA1 mobilization process triggered by hydrogen sulfide might involve cAMP release within the cell. Thus, we measured cAMP levels upon NaHS treatment
and, in parallel experiments, we also treated PMN with rolipram, a selective inhibitor of PDE4 (Smolen and Geosits, 1984; Wang et al., 1999). Our results demonstrated that cAMP levels raised upon NaHS stimulation and that PDE4 blockade by rolipram caused both elevation of intracellular cAMP and, similarly to NaHS, AnxA1 mobilization. In addition, we also observed a synergistic effect when NaHS and rolipram were co-administered at lower concentration (10µM). Collectively, our data suggest that hydrogen sulfide can trigger AnxA1 release in inflammatory cells, which in turn activates pro-resolving pathways in order to restore tissue homeostasis.

Generally, upon membrane exposure, AnxA1 reduces surface expression of adhesion molecules, resulting in leukocyte detachment or reduction of adherent/transmigrated inflammatory cells (Perretti and Flower, 2004). Therefore, we questioned whether hydrogen sulfide treatment could affect PMN surface expression of CD11b, an established marker for leukocyte activation and cell adhesion (Gavins et al., 2003). Interestingly, our data showed that hydrogen sulfide treatment reduced CD11b surface expression in PMN. Therefore, H2S effect on CD11b expression might occur through mobilization of AnxA1, which results in a reduced cell activation. Noteworthy, this finding was also replicated when H2S precursor L-cysteine was used. This result indicates that, when inflammation occurs, L-cysteine/H2S pathway contributes to trigger AnxA1 mobilization, which in turn controls leukocyte trafficking.

In conclusion, we propose that H2S antinflammatory response involves activation of AnxA1 pro-resolutive pathway. Therefore, the increase in H2S levels occurring during inflammation represents a response aiming to restore tissue homeostasis, also based on AnxA1 pathway activation, and to promote resolution and healing.
process (Wallace et al., 2012). This novel perspective provides new insights in understanding inflammation-resolution balance and might be crucial in approaching new therapeutic targets that utilize endogenous hydrogen sulfide to counteract tissue injury.
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None.
Authorship contributions:

Participated in research design: Brancaleone, Perretti, Cirino

Conducted experiments: Brancaleone

Performed cAMP determination: Mitidieri

Performed data analysis: Brancaleone, Perretti, Flower

Wrote the manuscript: Brancaleone

Contributed to the writing of the manuscript: Perretti, Cirino
References


Footnotes

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Figure Legends

Figure 1. Expression of mRNA levels in different tissues harvested from AnxA1+/+ and AnxA1-/- mice. mRNA levels expressed as relative quantity (RQ) in aorta (A), spleen (B) and kidney (C) respectively. Plasma H2S levels in AnxA1+/+ and AnxA1-/- mice (D). Statistical analysis was made by using Student's t-test (*p<0.05, **p<0.01; *vs AnxA1+/+; n=6).

Figure 2. Expression of mRNA levels in BMDMs from AnxA1+/+ or AnxA1-/- mice challenged with LPS (100ng/ml, 6h) alone or in presence of NaHS (100μM, -1h). Effect of LPS/NaHS treatment in AnxA1+/+ BMDMs on levels of mRNA for COX2 (A), iNOS (B) and CSE (C). Effect of LPS/NaHS treatment in AnxA1-/- BMDMs on levels of mRNA for COX2 (D), iNOS (E) and CSE (F). Statistical analysis was made by using one-way ANOVA with Dunnett's post hoc test (*p<0.05, ***p<0.001; ###p<0.001; * vs vehicle, # vs LPS; n=3).

Figure 3. Intravital microscopy analysis of postcapillary venules in AnxA1+/+ or AnxA1-/- mice showing emigrated leukocytes. Representative picture of postcapillary venule in AnxA1+/+ animal treated with IL1β (10ng/mouse i.p., 2h) (A). Representative picture of postcapillary venule in AnxA1+/+ animal treated with IL1β (10ng/mouse i.p., 2h), where NaHS (100μmol/kg s.c.) was given 1h before IL1β injection (B). Representative picture of postcapillary venule in AnxA1-/- animal treated with IL1β (10ng/mouse i.p., 2h), where NaHS (100μmol/kg s.c.) was given 1h before IL1β injection (C). Mice were pre-treated with vehicle, NaHS (100μmol/kg s.c.) or L-cysteine (L-cys, 1000μmol/kg s.c.) 1h before stimulation with IL1β (10ng/mouse i.p., 2h) and number of adherent leukocytes were
analyzed (expressed as no. cells per 50x100µm²). Pretreatment with D,L-propargylglycine (PAG, 10mg/kg i.p.) was performed 30 minutes before IL1β injection (D). Number of emigrated leukocytes in AnxA1+/+ animal treated with IL1β (10ng/mouse i.p., 2h), where NaHS (100µmol/kg s.c.) at the same time as IL1β (time 0) or 1 hour after its injection (time +1) (E). Statistical analysis was made by using two-way ANOVA (*p<0.05, **p<0.01; §p<0.05; * vs vehicle, § vs AnxA1+/+; n=6)

**Figure 4.** AnxA1 mobilization within human PMN and underlying mechanisms. AnxA1 expression in cytosolic (Cyt), membrane (Mem) or supernatant (Sup) fractions from human PMN upon NaHS (10-100µM, 30min) challenge (A). Optical density is expressed as % of total AnxA1 (A). AnxA1 expression in cytosolic (Cyt) and membrane (Mem) fractions from human PMN upon treatment with PDE4 inhibitor rolipram (1-100µM, 30min) (B). Statistical analysis was made by using one-way ANOVA (*p<0.05, **p<0.01; * vs vehicle; n=4)

**Figure 5.** Modulation of CD11b surface expression by H2S. Histograms of CD11b surface expression in human PMN pretreated with vehicle or NaHS (1-100µM) 10 minutes before challenge with fMLP (0.1µM, 30 minutes) (A). Expression of CD11b as mean fluorescence intensity (MFI) in human PMN upon NaHS treatment (B). Histograms of CD11b surface expression in human PMN pretreated with vehicle or L-cysteine (1-100µM) 10 minutes before challenge with fMLP (0.1µM, 30 minutes) (C). Expression of CD11b as mean fluorescence intensity (MFI) in human PMN upon L-cysteine treatment (D). Control (CTR)
histograms and bars represent untreated cells. Statistical analysis was made by using one-way ANOVA (*p<0.05, **p<0.01; * vs vehicle; n=4).

Figure 6. Schematic representation of inflammatory circuit driven by H₂S and triggering AnxA1 pathway.
TABLE 1

Determination of cAMP levels in PMN treated with NaHS (100µM), rolipram (100µM) or NaHS+rolipram (both at 10µM) for 30 minutes.

Data are presented as mean±SEM for n=3 replicated experiments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>cAMP (pmol/10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>2.01±0.56</td>
</tr>
<tr>
<td>NaHS</td>
<td>6.74±1.56*</td>
</tr>
<tr>
<td>Rolipram</td>
<td>10.46±2.38*</td>
</tr>
<tr>
<td>NaHS+Rolipram</td>
<td>15.66±2.02**#</td>
</tr>
</tbody>
</table>

Statistical analysis was made by using Student's t-test.

* Statistically significant (P<0.05) vs vehicle.

** Statistically significant (P<0.01) vs vehicle.

# Statistically significant (P<0.05) vs NaHS.
Figure 2
Figure 5

A. Flow cytometry histograms showing the effect of NaHS on APC-CD11b expression.

B. Bar graph showing the mean fluorescence intensity (MFI) units of CD11b in response to NaHS at different concentrations: 1μM, 10μM, and 100μM, compared to control (CTR) and vehicle.

C. Flow cytometry histograms showing the effect of L-cysteine on APC-CD11b expression.

D. Bar graph showing the mean fluorescence intensity (MFI) units of CD11b in response to L-cysteine at different concentrations: 1μM, 10μM, and 100μM, compared to control (CTR) and vehicle.