ARTICLE
Journal of Cellular Biochemistry 114:1536–1548 (2013)

Journal of Cellular Biochemistry

Hydrogen Sulfide Reduces Cell Adhesion and Relevant Inflammatory Triggering by Preventing ADAM17-Dependent TNF-α Activation

Alessandra F. Perna,[1*] Immacolata Sepe,[1] Diana Lanza,[1] Rosanna Capasso,[2] Silvia Zappavigna,[2] Giovambattista Capasso,[1] Michele Caraglia,[2] and Diego Ingrosso[2]

[1] *First Division of Nephrology, Department of Cardio-thoracic and Respiratory Sciences, Second University of Naples, School of Medicine and Surgery, via Pansini 5, 80131 Naples, Italy*

[2] *Department of Biochemistry, Biophysics and General Pathology, Second University of Naples, School of Medicine and Surgery, via De Crecchio 7, 80138 Naples, Italy*

ABSTRACT

H_2S is the third endogenous gaseous mediator, after nitric oxide and carbon monoxide, possessing pleiotropic effects, including cyto-protection and anti-inflammatory action. We analyzed, in an in vitro model entailing monocyte adhesion to an endothelial monolayer, the changes induced by H_2S on various potential targets, including cytokines, chemokines, and proteases, playing a crucial role in inflammation and cell adhesion. Results show that H_2S prevents the increase in monocyte adhesion induced by tumor necrosis factor-α (TNF-α). Under these conditions, downregulation of monocyte chemoattractant protein-1 (MCP-1), chemokine C-C motif receptor 2, and increase of cluster of differentiation 36 could be detected in monocytes. In endothelial cells, H_2S treatment reduces the increase in MCP-1, inter-cellular adhesion molecule-1, vascular cell adhesion molecule-1, and of a disintegrin and metalloproteinase metallopeptidase domain 17 (ADAM17), both at the gene expression and protein levels. Cystathionine γ-lyase and 3-mercaptopyruvate sulfurtransferase, the major H_2S forming enzymes, are downregulated in endothelial cells. In addition, H_2S significantly reduces activation of ADAM17 by PMA in endothelial cells, with consequent reduction of both ADAM17-dependent TNF-α ectodomain shedding and MCP-1 release. In conclusion, H_2S is able to prevent endothelial activation by hampering endothelial activation, triggered by TNF-α. The mechanism of this protective effect is mainly mediated by down-modulation of ADAM17-dependent TNF-converting enzyme (TACE) activity with consequent inhibition of soluble TNF-α shedding and its relevant MCP-1 release in the medium. These results are discussed in the light of the potential protective role of H_2S in pro-inflammatory and pro-atherogenic processes, such as chronic renal failure. J. Cell. Biochem. 114: 1536–1548, 2013.

KEY WORDS: HYDROGEN SULFIDE; MONOCYTE ADHESION; MCP-1; ADAM17; TNF-α

Hydrogen sulfide, H_2S, the third endogenous gas with cardiovascular properties, after nitric oxide, and carbon monoxide, is a newly recognized vasorelaxant, whose deficiency is involved in the pathogenesis of hypertension and atherosclerosis [Qiao et al., 2010; Lavu et al., 2011]. Three enzymes catalyze the formation of H_2S: CSE, 3-mercaptopyruvate sulfurtransferase (MPST, EC 2.8.1.2) and cystathionine β-synthase (CBS, EC 4.2.1.22). In general, H_2S in the cardiovascular system is mainly produced by CSE [Chen et al., 1999; Whiteman and Moore, 2009].

Abbreviations used: H_2S, hydrogen sulfide; NaHS, sodium hydrosulfide; MCP-1, monocyte chemoattractant protein-1; ICAM-1, inter-cellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; ADAM17, a disintegrin and metalloproteinase metallopeptidase domain 17; TNF-α, tumor necrosis factor alpha; TACE, TNF-α converting enzyme; CD36, cluster of differentiation 36; CCR2, chemokine (C-C motif) receptor 2; CSE, cystathionine γ-lyase; MST, 3-mercaptopyruvate sulfurtransferase; PMA, phorbol 12-myristate 13-acetate; LFA-1, lymphocyte function-associated antigen 1; VSMC, vascular smooth muscle cells; FBS, fetal bovine serum; EDTA, ethylenediaminetetraacetic acid; PCR, polymerase chain reaction; qPCR, real-time quantitative PCR; ELISA, enzyme linked immunosorbent assay; FITC, fluorescein isothiocyanate.

The authors declared that no competing interests exist.

*Correspondence to: Alessandra F. Perna, MD, PhD, Associate Professor of Nephrology, First Division of Nephrology, Department of Cardio-thoracic and Respiratory Sciences, Second University of Naples, School of Medicine and Surgery, via Pansini 5, Ed. 17, 80131 Naples, Italy. E-mail: alessandra.perna@unina2.it

Manuscript Received: 28 September 2012; Manuscript Accepted: 18 December 2012

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 7 January 2013

DOI 10.1002/jcb.24495 • © 2013 Wiley Periodicals, Inc.

We demonstrated that, in hemodialysis patients, H_2S levels are decreased through transcriptional downregulation of cystathionine γ-lyase (CSE, EC 4.4.1.1), the major enzyme implicated in H_2S formation [Perna et al., 2009].

Hemodialysis patients are at high risk for cardiovascular disease [Collins et al., 2010]. The burden of cardiovascular disease refers to "accelerated atherosclerosis," arteriosclerosis with vascular stiffening, and cardiomyopathy [Drüeke and Massy, 2010; Sarnak and Foley, 2011]. The complex events taking place in these interrelated pathologies are initiated by the subendothelial retention of apolipoprotein (apo)B-containing lipoproteins. These modified lipoproteins trigger a series of maladaptive inflammatory responses, including attraction of monocytes to activated endothelial cells, followed by monocyte differentiation into macrophages and foam cell formation. As lesions develop, the inflammatory response amplifies, inducing the expression and release of cytokines and chemokines [Ait-Oufella et al., 2011]. Among the latter, a description of those playing a role in the development of atherosclerosis, which are studied in the present work, follows.

Monocyte chemoattractant protein-1 (MCP-1) is both an endothelial and monocyte-secreted chemokine with a central role in monocyte recruitment towards endothelial lesions, along with its receptor, chemokine (C-C motif) receptor 2 (CCR2), expressed on monocyte surface. Inter-cellular adhesion molecule 1 (ICAM-1), expressed by the vascular endothelium, macrophages, and lymphocytes, can be induced by tumor necrosis factor-α (TNF-α). ICAM-1 is a ligand for integrin lymphocyte function-associated antigen 1 (LFA-1), a receptor found on leukocytes. When activated, leukocytes bind to endothelial cells via ICAM-1/LFA-1 and then transmigrate into tissues. Vascular cell adhesion molecule 1 (VCAM-1) also mediates the adhesion of lymphocytes, monocytes, eosinophils, and basophils to vascular endothelium. It also functions in leukocyte-endothelial cell signal transduction.

A disintegrin and metalloproteinase metallopeptidase domain 17 (ADAM17), a member of the ADAM family of proteases, cleaves, and releases the active form of many inflammatory cell surface proteins. In particular, ADAM17 [Reiss et al., 2011] catalyzes the release (shedding) of various inflammatory adhesion molecules, cytokines, and their receptors involved in each step of leukocyte recruitment and activation, including ICAM-1, VCAM-1, and TNF-α, thus shedding their active forms. In the presence of inflammation or tumors, it amplifies the pathological process itself [Pruessmeyer and Ludwig, 2009]. ADAM17 fulfills a key role in diverse processes and pathologies, making it a prime target for developing therapies [Arribas and Esselens, 2009]. In fact, inhibiting ADAM17 activity can be beneficial for preventing complications of acute myocardial infarction, cardiac remodeling in hypertension and kidney disease progression, by interfering with kidney fibrosis [Gooz, 2010; Therrien et al., 2012].

Therefore, we explored the modifications induced by a H_2S donor, sodium hydrosulfide (NaHS), on monocyte adhesion to an activated endothelial cell monolayer in vitro, also evaluating MCP-1, ICAM-1, VCAM-1, and ADAM17 in endothelial cells, MCP-1, CCR2, and CD36 in monocytes, along with the expression of the relevant enzymes.

MATERIALS AND METHODS

CELL CULTURES AND REAGENTS

All cell lines were purchased from American Type Culture Collection (ATCC), Manassas, VA, USA. Human endothelial cells (EAhy926) were grown in Dulbecco's modified Eagle's medium (GIBCO® DMEM, Invitrogen™, Carlsbad, CA) with 10% fetal bovine serum (FBS, GIBCO®). Human monocytoid U937 cells were grown in RPMI 1640 (GIBCO®, Invitrogen™), 10% FBS, and 1% amphotericin B antifungal (Lonza BioWhittaker®, Walkersville, MD). Hepatocarcinoma cell line HepG2 was grown in DMEM, 10% FBS, and 1% non-essential amino acids (Lonza BioWhittaker®). All cell culture media were supplemented with 100 U of penicillin/ml, 100 μg of streptomycin/ml, and 2 mM L-glutamine. Cell lines are grown at 37°C in a humidified atmosphere with 5% CO_2.

Human lymphocytes were isolated by isopicnic centrifugation using Histopaque®-1077 (Sigma-Aldrich, St. Louis, MO), from whole blood withdrawn, in EDTA, from healthy donors.

Sodium hydrosulfide, NaHS, was purchased from Sigma-Aldrich (St. Louis, MO). TNF-α was purchased from BD (Becton-Dickinson & Co, NJ).

The farnesyl transferase inhibitor R115777 (FTI), Zarnestra®, was provided by one of us, Professor Michele Caraglia (Department of Biochemistry, Biophysics, and General Pathology, S.U.N. Naples, Italy).

TREATMENTS

Eahy926 cells (2×10^6) were grown to confluence in 6- or 24-well plates, or 60 mm dishes (Falcon BD, Becton-Dickinson & Co), as appropriate. Cells were serum-starved for 12 h and then incubated with TNF-α (10 ng/ml) for various times (2, 6, 9, 18 h), in the presence of H_2S at various concentrations as appropriate, in the bulk of experiments optimal incubation time was set at 2 h.

Unless otherwise indicated, cell samples were incubated, in parallel for 2 h, with medium alone (negative control), or with TNF-α (10 ng/ml, positive control), or co-incubated with TNF-α (10 ng/ml) and NaHS (100 μM) or pre-incubated for 30 min with TNF-α (10 ng/ml) before stimulation with NaHS (100 μM).

After treatments, cell samples were utilized for mRNA and protein extraction, for the evaluation of adhesion molecules, and for the cell adhesion assays.

To demonstrate the role of H_2S as a ADAM17 inhibitor, a different cell treatment scheme was employed, by stimulating Eahy926 monolayers with phorbol 12-myristate 13-acetate (PMA) at 50 ng/ml (81 nM) concentration, for 2 h as previously described [Burke-Gaffney and Hellewell, 1996; Gräf et al., 2001; Xu and Derynck, 2010], alone and/or upon pre-treatment with NaHS for various times. TNF-α and MCP-1 concentrations in cell culture media were determined by enzyme-linked immunosorbent assay (ELISA).

Samples of the cell culture media used for cell growth were stored at −20°C for ELISA.

CYTOFLUORIMETRIC ANALYSES

Cell viability and cytotoxicity was monitored using flow cytometry, after PI staining. For cell cycle distribution monitoring, cells were collected by centrifugation and resuspended in 500 μl of a

hypotonic buffer (0.1% NP-40, 0.1% sodium citrate, 50 μg/ml PI, RNAse A) and incubated (protected from light) for 30 min.

Annexin V staining flow cytometric analysis of apoptosis was accomplished basically as according to Caraglia et al. [2007]. Briefly, cells were incubated with Annexin-V-FITC (BD Pharmigen) and propidium iodide (Applichem) in a binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) for 30 min in the dark at room temperature, washed and resuspended in the same buffer. Cells were tracked as Annexin-V-FITC and PI negative (viable, no measurable apoptosis), Annexin-V-FITC negative and PI positive (necrosis), Annexin-V-FITC positive and PI negative (early apoptosis), Annexin-V-FITC positive and PI positive (late apoptosis).

The analyses were performed with a FACS-Calibur instrument (FACSCalibur, BD Biosciences, Milan, Italy) using the Cell Quest Pro software (Becton-Dickinson & Co) and ModFit LT version 3 software (Verity). For each sample, 2×10^4 events were acquired. Each experiment was performed in duplicate.

ADHESION ASSAY

Adhesion assays were accomplished according to Capasso et al. [2012]. Endothelial cells were starved for 12 h with serum-free medium, and then subjected to 2 h treatment: (a) TNF-α (10 ng/ml), or (b) TNF-α with NaHS (100 μM), or (c) TNF-α and, after 30 min NaHS, or (d) NaHS alone. U937 cells were counted and seeded on TNF-α-activated Eahy926 monolayers and incubated for 1 h at 37°C. Non-adhering U937 cells were then removed and washed with PBS three times. Eahy926 were fixed with 1% glutaraldehyde in PBS for 10 min and adherent U937 cells were counted on three randomly selected high magnification microscopic fields per well, for five independent experiment (Zeiss optical inverted photomicroscope system, Carl Zeiss, Jena, Germany).

PCR EXPERIMENTS

Total RNA was extracted from cells using TRIzol® Reagent (Invitrogen™). After removing the cell culture medium, cells were washed twice with PBS and lysed with 1 ml Trizol. RNA was obtained by chloroform extraction, and subsequent centrifugation and precipitation with isopropanol. The pellet was washed in 70% ethanol, and suspended in H_2O DEPC. RNA concentration was measured with a NanoDrop (ND-1000; NanoDrop Technologies, Wilmington, DE).

Total RNA (1 μg) of each sample was reverse-transcribed into cDNA and amplified using a SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen™) according to the manufacturer's directions in a Veriti® 96-Well Thermal Cycler (Applied Biosystems). qPCR experiments were performed for 35 cycles, using an iQ real-time RT-PCR detection System (Bio-Rad Laboratories S.r.l., Segrate Milan, Italy) in a total volume of 25 μl reaction mixture containing 1 μl cDNA, 12.5 μl iQ SYBR Green Supermix (Bio-Rad Laboratories S.r.l.), and 1 μl of each primer. GAPDH was used as an internal control. The primers used were the ones reported by Capasso et al. [2012] (MCP-1, ICAM-1, ADAM17, CCR2, GAPDH), Perna et al. [2009] (CSE, MPST) and Boulares et al. [1999] for VCAM-1, while, for CD36, primers were: 5′-GTGCTGTCCTGGCTGTGTTTG-3′ (sense); 5′-TTGCTGCTGTTCATCATCACTTCC-3′ (antisense).

Amplifying conditions were 35 cycles of denaturation for 30 s at 95°C; annealing for 30 s at 58°C for MCP-1, 62°C for ICAM-1, 55°C for ADAM17, 57°C for CSE, 53°C for MPST, 64°C for CCR2, 57°C for CD36; and elongation for 30 s at 72°C.

Expression levels after treatment were evaluated, for each gene, by comparing samples treated with 100 μM NaHS with negative control treatment. Values were obtained utilizing the means of triplicate measurements for the gene of interest (GOI) in each sample, normalized for the PCR product levels of the housekeeping reference gene (GAPDH). Relative expression was calculated using the ΔCt method. The value of $2^{-\Delta\Delta Ct} > 1$ reflects increased expression of the relevant gene, and a value of $2^{-\Delta\Delta Ct} < 1$ points to a decrease in gene expression [Cimmino et al., 2008].

VCAM-1 gene is not constitutively expressed in Eahy926, and its transcriptional levels at basal conditions were not detectable. The presence of VCAM-1 encoding transcript was evaluated by PCR in a Veriti® 96-Well Thermal Cycler. The amplification protocol was: 35 cycles of 95°C for 30 s, 63°C for 30 s, and 72°C for 30 s. GAPDH was amplified in parallel samples, using adequate primers and used as a housekeeping reference gene loading control.

ELISA DOSAGES

MCP-1, ICAM-1, VCAM-1, and TNF-α concentrations in the treated endothelial cell culture medium were determined utilizing the relevant ELISA kits (R&D Systems, Minneapolis, MN) according to the supplier's protocols.

PREPARATION OF WHOLE CELL PROTEIN EXTRACTS

Cells were washed twice with ice-cold PBS and lysed in RIPA buffer with protease and phosphatase inhibitor. Human lymphocytes were isolated by isopicnic centrifugation using Histopaque®-1077 (Sigma-Aldrich), from whole blood withdrawn, in EDTA, from healthy donors. Lymphocytes were washed twice with 1% ice-cold PBS and lysed in RIPA buffer after isolation.

After centrifugation (4°C, 10 min, 10,000*g*), samples were stored at −20°C in preparation for Western blot analysis. Protein concentration was determined using the Bio-Rad Protein Assay Kit II (Bio-Rad Laboratories S.r.l.).

WESTERN BLOTTING

Fifty micrograms of proteins were separated on 12–15% sodium dodecyl sulfate–polyacrylamide gels and transferred to polyvinyl difluoride membrane (Millipore, USA). After blocking with 5% non-fat dry milk, membranes were incubated overnight at 4°C with each of the following primary antibodies against: p21 Waf1/Cip1, Caspase-8 (Cell Signaling Technology), Goat anti-PARP (R&D Systems); MCP-1, VCAM-1 (Santa Cruz Biotechnology); CD54 (ICAM-1), anti-actin Ab-5 (BD); ADAM17, CSE, CCR2, CD36 (Abcam); MPST (Sigma). Secondary antibodies used were: donkey anti-goat, goat anti-rabbit, goat anti-mouse (R&D Systems). After incubation with appropriate secondary antibodies conjugated with horseradish peroxidase for 1 h at room temperature, immunocomplex visualization was obtained by chemiluminescence, utilizing the ECL-Plus kit (GE Healthcare). Signal intensity is quantified with the ChemiDoc™ (Bio-Rad Laboratories S.r.l.) with the Bio-Rad Quantity

One® software version 4.6.3. Densitometry was normalized for the corresponding β-actin band.

STATISTICAL ANALYSIS

Data are presented as mean (SE). A paired t Student test was performed, or a two-way ANOVA to assess the timed effects of treatments, as appropriate [Dawson-Saunders and Trapp, 1990]. All calculations were performed using the software package GraphPad Prism, Version 5.0 for Windows (GraphPad Software, San Diego, CA). All experiments were done at least in triplicate except otherwise stated. Within the same assay session, each sample of a set relevant to individual treatments was run in triplicate. Statistical significance is considered at $P < 0.05$, except where otherwise stated.

RESULTS

CYTOTOXICITY EXPERIMENTS

Sodium hydrosulfide, NaHS, an established and reliable H_2S donor in culture media [Li and Moore, 2008], was used at concentrations of 1 mM, 500 μM, and 100 μM and results compared with the TNF-α (10 ng/ml) treatment and untreated control. EAhy926 cell viability was monitored by cell cycle analysis with propidium iodide staining (PI) using flow cytometry. Hypodiploid peaks, indicating cell death, were negligible in all samples, allowing us to conclude for no significant cytotoxicity induced by the various treatments (Table I).

TABLE I. Cytofluorimetric Analysis of Cell Cycle of Endothelial Cells Treated With NaHS at Various Concentrations

	G0/G1	S	G2/M	dth
CTRL	67.2	22.79	10.02	0.15
TNF-α	66.49	22.47	11.03	0.12
H_2S, 1 mM	69.67	21.46	8.87	0.36
H_2S, 500 μM	57.96	27.27	14.77	0.27
H_2S, 100 μM	67.26	22.86	9.88	0.14

Values are expressed as percentage of total events counted; dth: dead cells (%).

The extent of apoptosis was monitored at various NaHS concentrations during cell treatments: double cell labeling with propidium iodide and Annexin V-FITC and subsequent cytofluorimetric assay were performed. As a positive apoptosis control, R115777 farnesyl transferase inhibitor (FTI) at a 10 μM concentration for 48 h was used, as according to Caraglia et al. [2007]. Results showed (Fig. 1A,B) that considering the early, late, and total apoptosis, 100 μM NaHS treatment determines a significant lower amount of apoptotic cells, compared to the higher NaHS concentrations employed, as well as to the FTI positive control.

The extent of poly(ADP-ribose) polymerase (PARP) cleavage, triggered by caspase 3 protease activation, is a marker of ongoing apoptosis [Boulares et al., 1999]. In EAhy926 treated with 100 μM NaHS, we could detect significantly lower levels of 23 kDa PARP

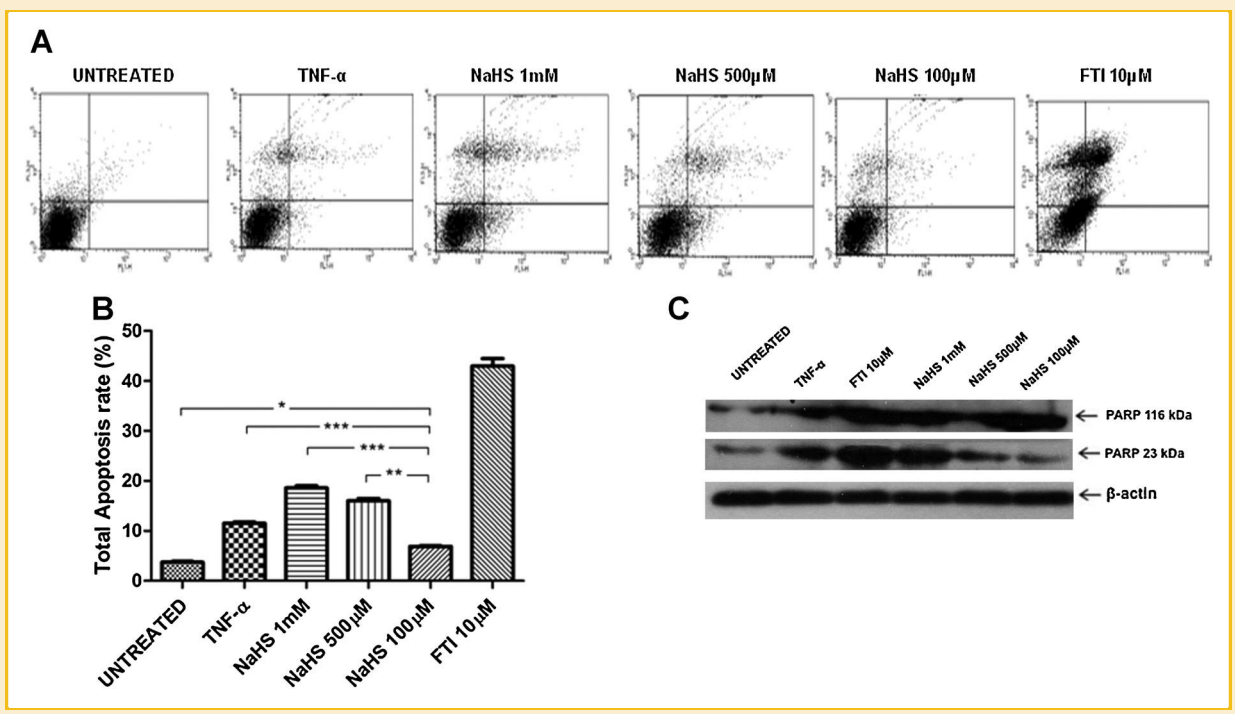

Fig. 1. Evaluation of apoptosis in endothelial cells upon treatment with NaHS. A: Dot plot of cytofluorimetric analysis of Annexin V staining on EAhy926 untreated (negative control) and on EAhy926 treated with: TNF-α, NaHS 1 mM, NaHS 500 μM, NaHS 100 μM, FTI 10 μM (apoptosis positive control). B: Total apoptosis rate defined as the sum of early + late apoptosis (see the Materials and Methods Section). Results are given as the mean (SE) of triplicates. Asterisks indicate: $^{*}P < 0.05$, $^{**}P \leq 0.01$, and $^{***}P < 0.001$ compared with NaHS 100 μM. C: Western blotting of PARP (intact 116 and the 23 kDa fragments, the latter generated during apoptosis), and the relevant β-actin loading control.

fragment compared with the positive FTI-treated cells positive control, as well as with both the cell samples treated with TNF-α or NaHS at the highest concentrations (Fig. 1C), thus further confirming that 100 μM NaHS was a rather safe concentration to be used for the in vitro cell treatments.

All the above data allowed as to identify 100 μM NaHS as the optimal, non-toxic, concentration of this salt to be used in the subsequent experiments. It is worth noting that 100 μM NaHS was also the concentration used in previous studies [Oh et al., 2006].

H_2S INHIBITS MONOCYTE CELL ADHESION AND EXPRESSION OF ADHESION MOLECULES

We measured U937 monocyte adhesion onto a EAhy926 endothelial monolayer, treated as under the Materials and Methods Section, as recently described [Capasso et al., 2012]. As shown in Figure 2, maximal monocyte-endothelial adhesion, observed in the TNF-α treated cells (positive control), was prevented by NaHS co-treatment. Adhesion upon TNF-α + NaHS treatment was indistinguishable from the negative control (vehicle alone) (Fig. 2A). NaHS alone elicited levels of adhesion not different from the negative control, and, with respect to TNF-α, was significantly reduced. NaHS, applied after 30 min from TNF-α, when adhesion induction is fully established, was not able to reverse maximal adhesion. Examples of cell co-cultures morphology in the adhesion assays are presented for each set of experiments (Fig. 2B).

To additionally corroborate these results, we evaluated the expression of adhesion molecules, induced by treatments, through quantitative real-time PCR (qPCR), Western blotting on cell extracts and confirmation by ELISA in order to detect molecules released in the medium. Figure 2C shows that ICAM-1 mRNA was significantly reduced in EAhy926 treated with both NaHS and TNF-α, compared with TNF-α alone. This effect was more pronounced when NaHS and TNF-α were added simultaneously. Expression levels of ICAM-1 upon treatment with NaHS alone were lower than baseline (Fig. 2C), as confirmed by Western blot (Fig. 2F). H_2S also reduced significantly ICAM-1 released in the culture medium of TNF-α treated cells (Fig. 2D). The same pattern could be observed for VCAM-1, both at transcriptional and protein levels (Fig. 2E–F).

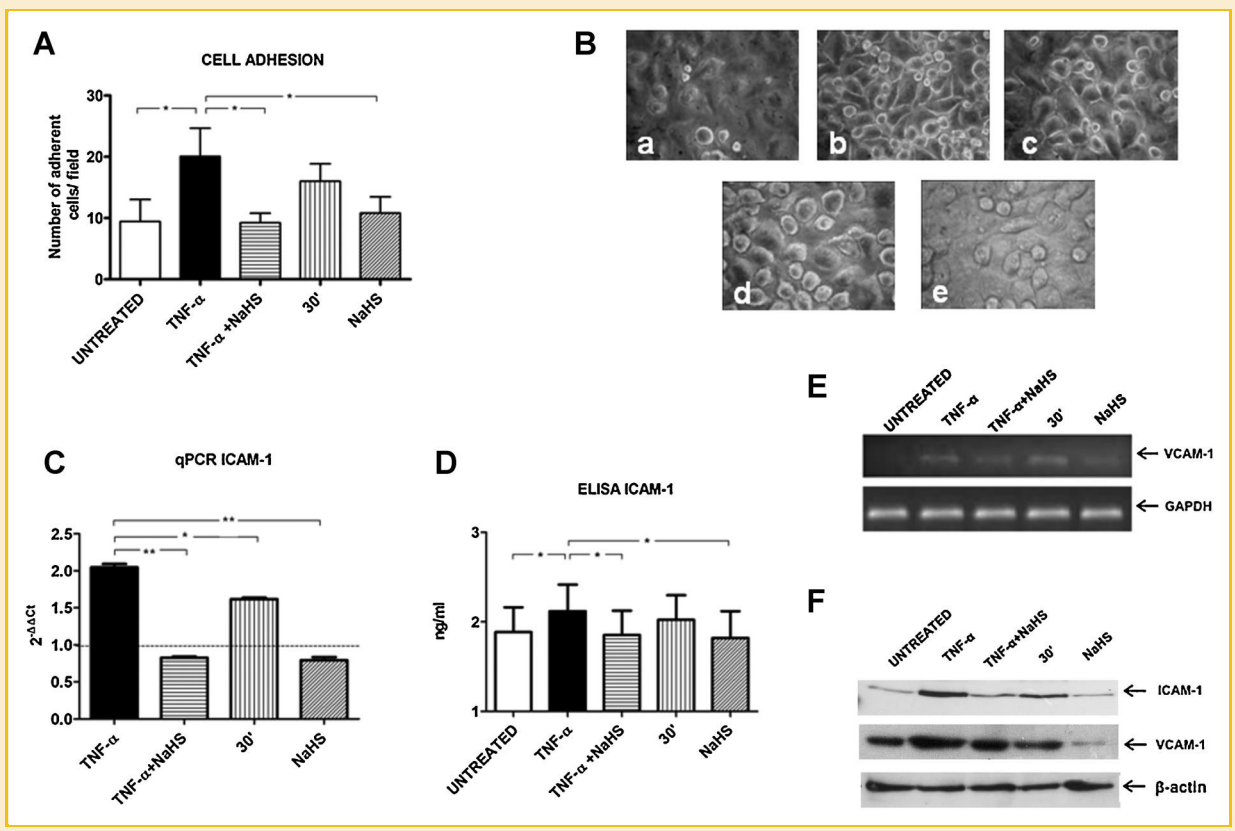

Fig. 2. Effects of H_2S on monocyte cell adhesion to an endothelial monolayer. Adherent U937 monocyte cells to endothelial cells Eahy926 were counted on three randomly selected high magnification microscopic fields for each well. A: Number of adherent cells/field. $^{*}P < 0.05$ compared with untreated cells, $^{*}P < 0.05$ compared with TNF-α stimulated cells. B: Examples of microscopic fields of adhesion assays carried out on Eahy926 treated as follows: (a) untreated cells (b) TNF-α (10 ng/ml), or (c) TNF-α with NaHS (100 μM), or (d) TNF-α and, after 30 min NaHS, or (e) NaHS only. C: mRNA levels of ICAM-1 were analyzed by qPCR. ICAM-1 levels were normalized for the mRNA levels of the constitutive gene GAPDH. $^{*}P < 0.05$; $^{**}P \leq 0.01$ compared with TNF-α stimulated cells. The untreated sample taken as a reference to calculate the $2^{-\Delta\Delta Ct}$ value, this reference baseline level is indicated by a dotted line. D: ELISA assay of ICAM-1 released in the culture medium of treated cells. $^{*}P < 0.05$ compared with untreated cells, $^{*}P < 0.05$ compared with TNF-α stimulated cells. E: Electrophoresis analysis of traditional PCR VCAM-1 products. GAPDH is the internal loading control amplified in parallel. F: Western blot analysis of adhesion molecule ICAM-1 and VCAM-1 in Eahy926 treated with NaHS (100 μM) or/and stimulated with TNF-α (10 ng/ml) for 2 h. All data were presented as the mean (SE) of results from least at three independent experiments.

These results as a whole showed that H_2S, generated by cell incubation with its donor NaHS, is able to prevent monocyte adhesion onto an endothelial monolayer induced by TNF-α, by reducing the expression of cell adhesion molecules.

H_2S PREVENTS TNF-α-INDUCED MCP-1 EXPRESSION IN BOTH ENDOTHELIAL CELLS AND MONOCYTES

MCP-1 is secreted by endothelial cells, but also by monocytes, and is responsible for the direct migration of the latter cells toward the endothelium and into the intima at sites of lesion formation.

The effects of H_2S on TNF-α-induced MCP-1 expression were monitored on both EAhy926 and U937 cells at mRNA and protein levels.

Both in EAhy926 endothelial cells (Fig. 3A) and U937 monocytes (Fig. 3B), MCP-1 mRNA levels were significantly reduced, in cells co-incubated with TNF-α and NaHS, compared to TNF-α alone, while NaHS, applied 30 min after TNF-α addition, was apparently less effective, particularly in the EAhy926. MCP-1 mRNA levels were significantly lower upon treatment with NaHS alone with respect to TNF-α alone, in both cell types (Fig. 3A,B). The Western blot analysis provided a clear-cut interpretation of results, in that it showed that NaHS treatment was always able to reduce MCP-1 protein levels in both EAhy926 and U937 compared to TNF-α alone and also with the untreated sample (Fig. 3C). However, NaHS applied 30 min after TNF-α was less effective in the reduction of MCP-1 protein upon stimulation with TNF-α. Consistently, NaHS significantly reduced also MCP-1 release in the culture medium of TNF-α treated EAhy926 cells (Fig. 3D).

These set of experiments led to the conclusion that the H_2S donor NaHS, particularly when used alone, induced a significant decrease of MCP-1 expression in both endothelial and monocyte cell types.

H_2S INVERSELY MODULATES CCR2 AND CD36 EXPRESSION INDUCED BY TNF-α IN MONOCYTES

CCR2 is the MCP-1 receptor on monocyte surface. In U937 treated with NaHS, the expression of CCR2 was significantly decreased with respect to cells treated with TNF-α alone both at mRNA (Fig. 4A) and protein (Fig. 4B,C) levels. Human lymphocytes were used as a positive control.

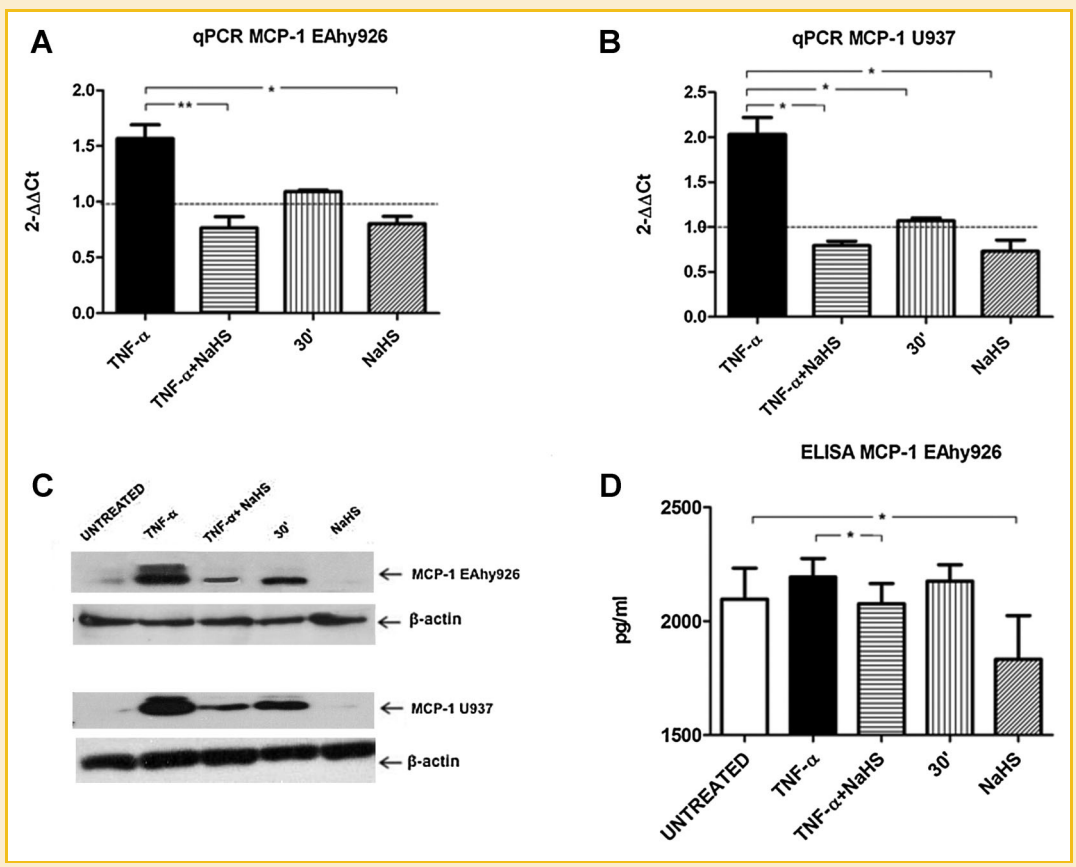

Fig. 3. Effects of H_2S on MCP-1 expression in endothelial cells and monocytes. Eahy926 and U937 were treated in parallel with NaHS (100 μM) and/or stimulated with TNF-α (10 ng/ml) for 2 h. A: mRNA levels of MCP-1 on EAhy926, analyzed by qPCR. MCP-1 levels were normalized for the mRNA levels of the constitutive gene GAPDH. $^{*}P < 0.05$ and $^{**}P \leq 0.01$ compared with TNF-α treated cells. The untreated sample (baseline level) was taken as a reference to calculate the $2^{-\Delta\Delta Ct}$ value and is indicated by a dotted line. B: mRNA levels, analyzed by qPCR, of MCP-1 on U937. MCP-1 levels were normalized for the mRNA levels of the constitutive gene GAPDH. The untreated sample (baseline level) was taken as a reference to calculate the $2^{-\Delta\Delta Ct}$ value and is indicated by a dotted line. $^{*}P < 0.05$ compared with TNF-α stimulated cells. C: Western blot analysis of MCP-1 in Eahy926 and in U937 cell lysates. D: ELISA assay of MCP-1 released in the culture medium of Eahy926 treated cells. $^{*}P < 0.05$ compared with untreated cells, $^{*}P < 0.05$ compared with TNF-α stimulated cells. All data were presented as the mean (SE) of results from least at three independent experiments.

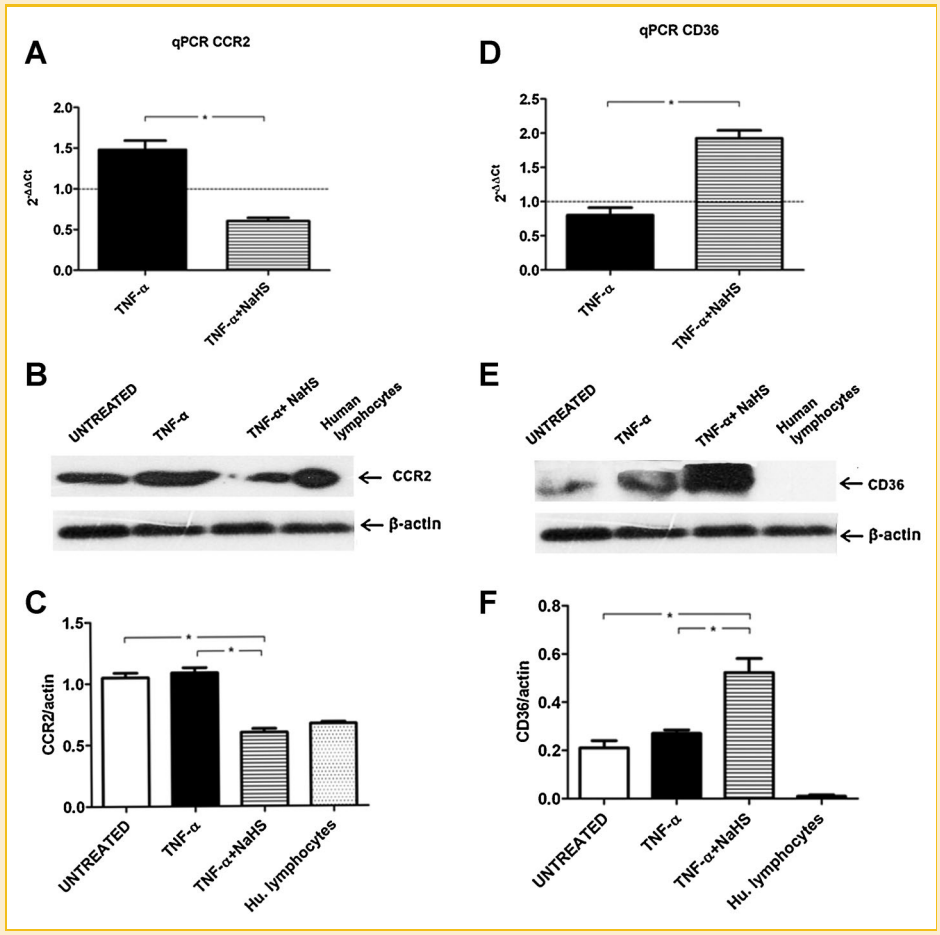

Fig. 4. Effects of H_2S on CCR2 and CD36 expression in monocyte cells. U937 were treated with NaHS (100 μM) and/or stimulated with TNF-α (10 ng/ml) for 2 h. A: CCR2 mRNA levels were analyzed by qPCR. CCR2 levels were normalized for the mRNA levels of the constitutive gene GAPDH. $^*P < 0.05$ compared with TNF-α stimulated cells. The untreated sample (baseline level) was taken as a reference to calculate the $2^{-\Delta\Delta Ct}$ value and is indicated by a dotted line. B: Western blot analysis of CCR2 and the relevant β-actin loading control. C: Densitometry analysis of the quantitative difference in expression of CCR2. $^*P < 0.05$ compared with untreated cells, $^*P < 0.05$ compared with TNF-α stimulated cells. All data were presented as the mean (SE) of results from at least three independent experiments. Human lymphocytes extracts were utilized as a positive control for CCR2 expression. The effects of H_2S on CD36 expression were analyzed in parallel U937 treated with NaHS (100 μM) and/or stimulated with TNF-α (10 ng/ml) for 2 h. D: CD36 mRNA levels were analyzed by qPCR. CD36 levels were normalized for the mRNA levels of the constitutive gene GAPDH. $^*P < 0.05$ compared with TNF-α stimulated cells. The untreated sample (baseline level) was taken as a reference to calculate the $2^{-\Delta\Delta Ct}$ value and is indicated by a dotted line. E: Western blot analysis of CD36 and the relevant β-actin loading control. F: Densitometry analysis of the quantitative difference in expression of CD36. $^*P < 0.05$ compared with untreated cells, $^*P < 0.05$ compared with TNF-α stimulated cells. All data were presented as the mean (SE) of results from at least three independent experiments. Human lymphocytes were utilized as a negative control.

These results demonstrated that NaHS treatment effectively prevents the increase of CCR2 levels induced by TNF-α stimulation on U937 cells. Functionally, this effect is consistent with the decrease in MCP-1 expression and release shown in the previous paragraph.

We also evaluated in parallel CD36 expression in monocyte cells treated with NaHS and co-stimulated with TNF-α. Results showed that CD36 was significantly increased both at the mRNA and protein levels (Fig. 4 panels D–F), with respect to the samples treated with TNF-α alone. Human lymphocytes were used as a negative control.

Results as a whole showed that H_2S treatment exerts an opposite action on CCR2 (downregulation) and CD36 (upregulation) levels; these effects reverse those induced by TNF-α stimulation on U937 cells.

H_2S REDUCES CSE AND MPST LEVELS

CSE and MPST are the enzymes involved in H_2S synthesis in both vascular smooth muscle cells (VSMC) and endothelial cells. Endogenous H_2S production plays a significant role in the regulation of several cellular functions in which H_2S is involved. It has also been reported that *S*-propargyl-cysteine, a substrate donor for H_2S biosynthesis, increases CSE in vitro and in vivo [Ma et al., 2011].

In Eahy926 stimulated with TNF-α, we observed maximal CSE expression both at the mRNA and protein levels (Fig. 5A–C), while CSE decreased in cells co-stimulated with TNF-α and NaHS.

Figure 5 (D–F) shows the expression of MPST both at mRNA and protein levels. MPST decreased in cells stimulated with NaHS. This also occurred in EAhy926 stimulated with TNF-α and NaHS after 30 min.

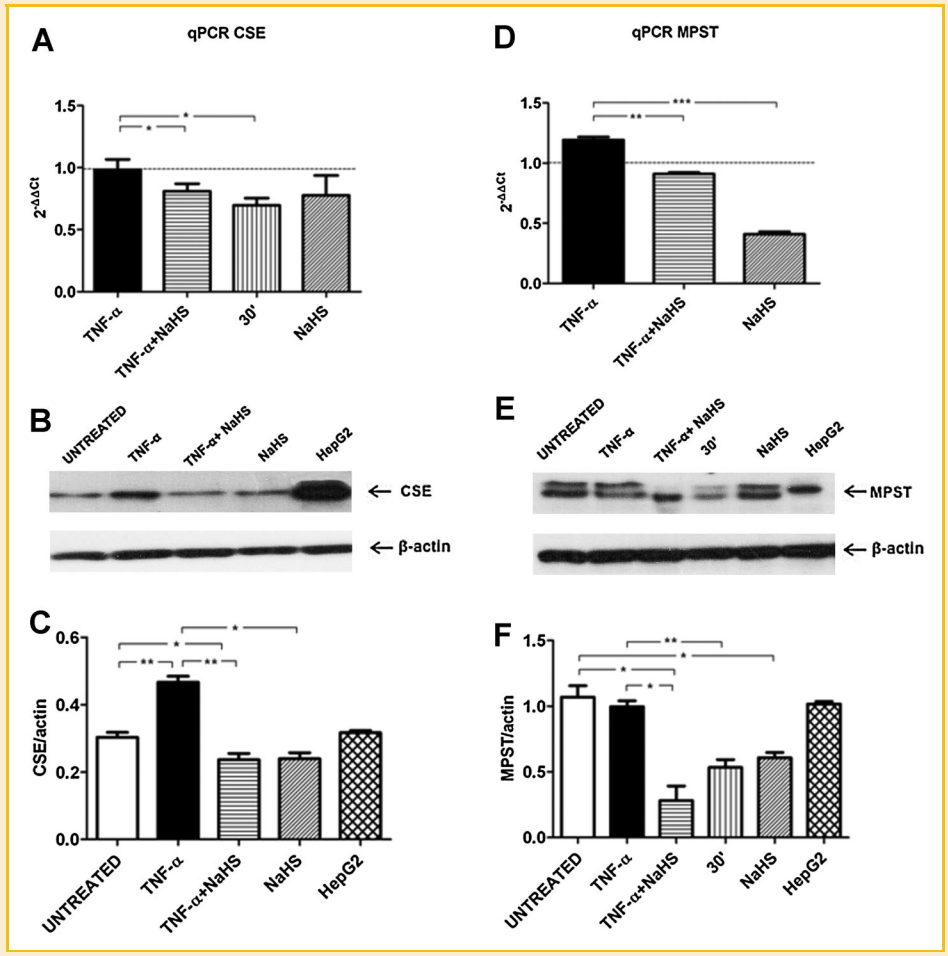

Fig. 5. CSE and MPST expression upon H_2S treatment in endothelial cells. CSE and MPST were evaluated in Eahy926 treated with NaHS (100 μM) and/or stimulated with TNF-α (10 ng/ml) for 2 h. A: mRNA levels of CSE were analyzed by qPCR. CSE levels were normalized for the mRNA levels of the constitutive gene GAPDH. $^{*}P < 0.05$ compared with TNF-α stimulated cells. The untreated sample (baseline level) was taken as a reference to calculate the $2^{-\Delta\Delta Ct}$ value and is indicated by a dotted line. B: Western blot analysis of CSE and the relevant β-actin loading control. C: Densitometry analysis of the quantitative difference in expression of CSE. $^{*}P < 0.05$ and $^{**}P \leq 0.01$ compared with untreated cells, $^{*}P < 0.05$ and $^{**}P \leq 0.01$ compared with TNF-α stimulated cells. D: mRNA levels of MPST were analyzed by qPCR. MPST levels were normalized for the mRNA levels of the constitutive gene GAPDH. $^{**}P \leq 0.01$ and $^{***}P < 0.001$ compared with TNF-α stimulated cells. The untreated sample (baseline level) was taken as a reference to calculate the $2^{-\Delta\Delta Ct}$ value and is indicated by a dotted line. E: Western blot analysis of MPST and the relevant β-actin loading control. F: Densitometry analysis of the quantitative difference in expression of MPST. $^{*}P < 0.05$ compared with untreated cells, $^{*}P < 0.05$ and $^{**}P \leq 0.01$ compared with TNF-α stimulated cells. All data were presented as the mean (SE) of results from at least three independent experiments. HepG2 cell lysate was used as a positive control for CSE and MPST protein expression.

HepG2 cell lysate was used as a positive control for CSE and MPST protein expression.

H_2S REDUCES ADAM17 EXPRESSION AND PREVENTS ITS ACTIVATION

ADAM17 is a membrane-related protease, synthesized as a zymogen, which needs to be later cleaved and its prodomain removed, in order to be active [Scheller et al., 2011]. ADAM17 plays a crucial role in the activation of several inflammatory molecules and is involved in the shedding of TNF-α and adhesion molecules, thus releasing their soluble ectodomains in the extracellular space [Gooz, 2010]. TNF-α in turn is responsible for MCP-1 release from vascular endothelial cells [Murao et al., 2000]. ADAM17 is therefore currently investigated as a potential therapeutical target [Arribas and Esselens, 2009].

Figure 6 demonstrates that H_2S suppresses the TNF-α-induced ADAM17 expression both at mRNA (Fig. 6A) and protein levels (Fig. 6B,C). When NaHS was applied after 30 min incubation, ADAM17 expression significantly decreased, with respect to the samples where NaHS was applied from the start of incubation. Moreover, ADAM17 gene and protein expression levels in cells stimulated with NaHS alone appeared significantly lower than basal control levels. Therefore, NaHS alone decreases basal ADAM17 expression.

In order to test the ability of H_2S to counteract ADAM17 activation, we used a different cell treatment model in which EAhy926 were treated with PMA which has been shown to be a powerful stimulator of ADAM17 TNF-α converting enzyme (TACE) activity with consequent release of TNF-α soluble ectodomain in the medium [Bell et al., 2007; Scheller et al., 2011]. We then treated

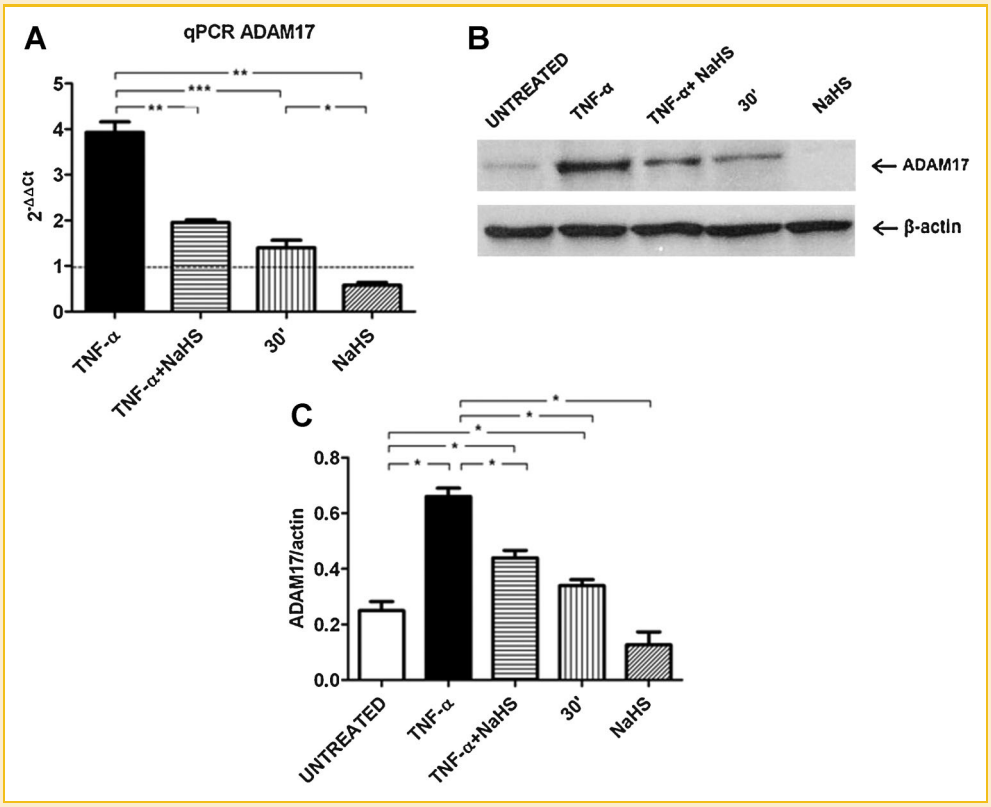

Fig. 6. Effects of H_2S on ADAM17 expression in endothelial cells. Eahy926 were treated with NaHS (100 μM) and/or stimulated with TNF-α (10 ng/ml) for 2 h. A: ADAM17 mRNA levels were analyzed by qPCR. ADAM17 levels were normalized for the mRNA levels of the constitutive gene GAPDH. $^{**}P \leq 0.01$ and $^{***}P < 0.001$ compared with TNF-α stimulated cells, $^{*}P < 0.05$ compared with NaHS treated cells. The untreated sample (baseline level) was taken as a reference to calculate the $2^{-\Delta\Delta Ct}$ value and is indicated by a dotted line. B: Western blot analysis of ADAM17 and the relevant β-actin loading control. C: Densitometry analysis of the quantitative difference in expression of ADAM17. $^{*}P < 0.05$ compared with untreated cells, $^{*}P < 0.05$ compared with TNF-α stimulated cells. All data were presented as the mean (SE) of results from at least three independent experiments.

endothelial cell monolayers with PMA, as under the Materials and Methods Section. This treatment alone caused a significant release of soluble TNF-α in the cell medium (Fig. 7, panel A). When cells were then pre-treated with NaHS before PMA addition, such TNF-α release did not occur (Fig. 7, panel A) and this effect was particularly evident when NaHS treatment was prolonged for 1 h. This result was further corroborated by the ability of NaHS to hamper the TNF-α-dependent release of MCP-1 in the PMA stimulated endothelial cells (Fig. 7B).

These results, as a whole, demonstrate the ability of H_2S to both downregulate ADAM17 and downmodulate ADAM17 TACE-dependent activity in activated endothelial cells.

DISCUSSION

Our results show that H_2S, a gaseous mediator of vascular responses, which is decreased in uremia [Perna et al., 2009], prevents the increase in monocyte adhesion, induced by TNF-α, to an endothelial cell culture monolayer, a model of the initial events in atherosclerosis. In addition, cell treatment with NaHS, as a H_2S donor, significantly prevents the TNF-α-induced increase in MCP-1, ICAM-1, VCAM-1, and ADAM17, in endothelial cells, both at the gene and protein levels. Moreover, upon addition of NaHS to endothelial cell cultures, both CSE and MPST expressions are downregulated, at gene and protein levels. H_2S also reduces MCP-1 and CCR2, while it increases CD36 in monocyte cells. Therefore, H_2S is able to significantly decrease monocyte-endothelial adhesion, a pro-atherogenic process. This occurs through the modulation of the production of molecular mediators involved in the early stages of atherosclerosis.

H_2S is an endogenous gas endowed with anti-oxidant, anti-inflammatory, anti-hypertensive, and other regulatory activities on the vasculature. CBS, CSE, and MPST catalyze its production, in several tissues. In chronic kidney disease, low plasma H_2S levels have been established in humans and in animal models [Perna et al., 2009; Aminzadeh and Vaziri, 2012]. These low levels can be explained by the downregulation of CSE expression.

H_2S is involved in atherosclerosis, as shown in studies underscoring its anti-inflammatory and anti-oxidative properties in this pathological process [Wang et al., 2009]. H_2S also inhibits proliferation of VSMC, but promotes endothelial cell proliferation through a K (ATP) channel/MAPK-dependent pathway [Papapetropoulos et al., 2009; Wang, 2011].

Our present study shows that 100 μM NaHS is not toxic in terms of both cell viability and activation of proteins involved in cell cycle

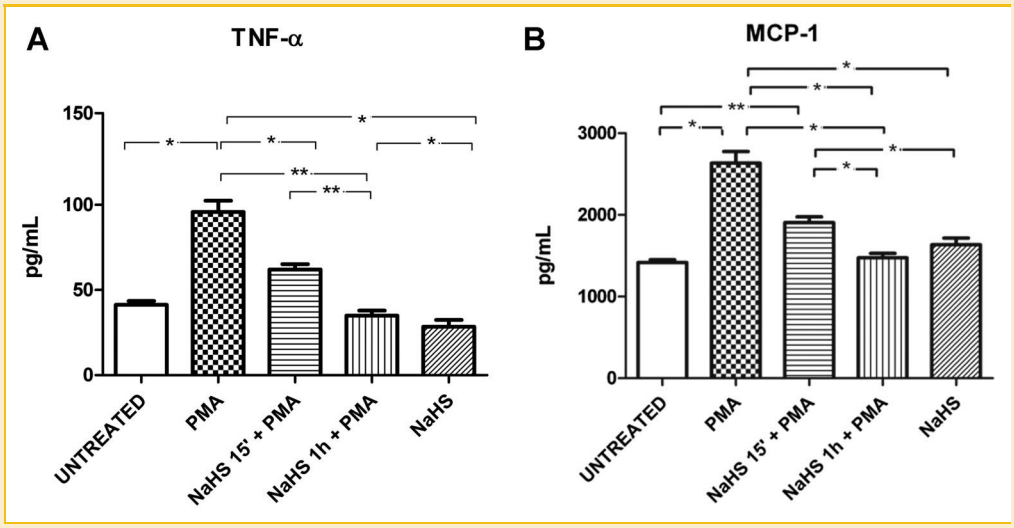

Fig. 7. Inhibition of H_2S on PMA-dependent ADAM17 activation effects. PMA treatment was performed, as under the Materials and Methods Section, in endothelial cells EAhy926 monolayers. NaHS pre-treatment was performed at 15′ or 1 h before PMA addition. TNF-α (panel A) and MCP-1 (panel B) concentrations were measured in the medium.

and apoptosis. On the basis of these data, consistently with previous reports, H_2S prevents the adhesion of monocytes to an endothelial cell monolayer.

The molecules involved in monocyte adhesion are MCP-1, ICAM-1, and VCAM-1. H_2S treatment induces a decreased expression of MCP-1, and of the adhesion molecules ICAM-1 and VCAM-1 on endothelial cells, as well as a decreased release of these proteins in the culture medium. The underlying mechanisms consist in the transcriptional downregulation of MCP-1, ICAM-1, and VCAM-1 encoding genes. NaHS, applied after 30 min after TNF-α treatment, also blocks this process, although not reversing it completely. These results are somewhat in line with previous findings showing that, in HUVEC, H_2S is able to suppress TNF-α-induced ICAM-1 and VCAM-1 expression [Pan et al., 2011] and U937 monocyte adhesion [Oh et al., 2006].

Moreover, treatment with NaHS alone on Eahy926 involves the reduction of adhesion molecules below the level of control cells, implying a beneficial effect of this gaseous mediator even under unstimulated conditions. In monocytes, H_2S suppresses both MCP-1 and its receptor CCR2, thus exerting a similar effect to that found in the endothelial cells. The recorded effect is substantial; in fact, the reduction is more than half the levels found after TNF-α stimulation. Therefore, H_2S prevents the initial steps of monocyte activation and adhesion consisting in MCP-1/CCR2 interaction. It is worth noting, in this respect, that high serum levels of ICAM-1, VCAM-1, and MCP-1 have been detected in hemodialysis patients and are associated with inflammation, dyslipidemia, and cardiovascular events [Papayianni et al., 2002].

Therefore our present results may provide an interpretative model for the role, in these processes, of decreased H_2S detected in end-stage renal disease patients.

The enzymes catalyzing H_2S production in the endothelium, CSE and MPST, are markedly reduced at the gene and protein level. This finding can be explained by hypothesizing that exogenous H_2S, applied to the culture system in the form of NaHS, may modulate its endogenous production through down-regulation of its producing enzymes. This hypothesis deserves further investigations, however, it is to notice that CSE protein expression is increased when cells are stimulated by TNF-α compared to untreated cells. This may be interpreted as a compensatory response aimed to circumvent the pro-inflammatory action of TNF-α and is in agreement with a very recent report demonstrating that TNF-α treatment significantly increases CSE protein levels in mouse liver [Sen et al., 2012]. This view appears even more likely, since such an effect could not be evidenced for MPST. In fact, it has been reported that MPST contributes only in a limited manner to H_2S production, while it functions more as a detoxifying agent under special circumstances such as uremia, underscoring the different role of this enzyme [Perna et al., 2009].

The most extensively studied class B scavenger receptors encompass CD36 and SR-BI and have been found to bind to native and modified LDL [Ashraf and Gupta, 2011]. It has also been reported that macrophage scavenger receptor CD36 is the major receptor for LDL modified by monocyte-generated reactive nitrogen species [Podrez et al., 2000]. In our monocyte model, H_2S induces, both at gene and protein levels, a significant increase of CD36. Although apparently in contrast with a previous report about CD36 reduction induced by NaHS in Ox-LDL-stimulated macrophages [Zhao et al., 2011], our results are in line with previous findings, showing that anti-TNF-α monoclonal antibody, adalimumab, increases CD36 expression [Boyer et al., 2007]. It should be pointed out, in this respect, that CD36 presents differential responses to atherosclerosis. In fact, its activation may favor formation of foam cells, but its inhibition may in turn dampen it. This is the demonstration of its important scavenging function, which, as an adaptive response to the excess of oxidized LDLs, helps the tissue to get rid of them, even if, unavoidably to some extent, it facilitates, as the reverse side of the coin, foam cell formation.

ADAM17 is involved in the cleavage and the consequent shedding of several molecules (among which TNF-α, ICAM-1, and VCAM-1), thus exerting a regulatory role in inflammation, atherosclerosis, etc. In the present study, we observed slightly differential responses: (i) when NaHS is added from the start of the incubation, a significant reduction in ADAM17 is induced; (ii) when NaHS is added after 30 min, a reduction in ADAM17 is elicited. These two conditions may reflect the ability of NaHS to modify ADAM17 expression and activity through direct sulfhydration and/or other mechanisms, such as those related to its antioxidant activity, variously combined [Mustafa et al., 2009; Chen et al., 2011; Wang, 2011]. Moreover, NaHS alone is able to significantly reduce ADAM17, even with respect to its basal levels, indicating that H_2S is capable to suppress ADAM17 even under TNF-α un-stimulated conditions.

Even more importantly, our results demonstrate that, in a different cell system, H_2S is also able to inhibit the activation of ADAM17 induced by PMA, a powerful ADAM17 activator [Willems et al., 2010; Scheller et al., 2011]. The relevant effects include the reduction of soluble TNF-α ectodomain release in the medium, which is clearly the direct result of H_2S ability to downmodulate TACE activity of ADAM17. The inhibition of release of TNF-α soluble form is, in turn, directly related to the H_2S-induced inhibition of MCP-1 release, which could be also detected in PMA-stimulated cells. ADAM17 inhibitors represent a burgeoning research field aiming at preventing complications of heart pathologies and kidney disease and interfering with renal fibrosis [Gooz, 2010]. Therefore, these findings as a whole provide the rationale to use H_2S releasing compounds as a new potential tool to suppress ADAM17. Relatively to the early events in atherosclerosis, it has been shown that H_2S also inhibits hemin-induced oxidative modification of LDL through a mechanism involving a reduction of lipid hydroperoxide (LOOH) content. Downstream processes induced by LOOH, like endothelial cytotoxicity or induction of heme oxygenase-1 (HO-1), are also abolished by H_2S [Jeney et al., 2009]. H_2S antioxidant activity is likely to be also involved in the mechanism counteracting PMA-induced ADAM17 activation, which is, in turn, thought to be dependent on the formation of reactive oxygen species [Willems et al., 2010; Scheller et al., 2011]. Therefore, we propose that H_2S represents a novel tool to inhibit TNF-α and in turn to curb its inflammatory action (Fig. 8). If H_2S is able to prevent ADAM17 activity, as we have clearly shown in this article, the inflammation attached to TNF-α stimulation, with its noxious consequences, could be dampened, at least in part, through this mechanism, and/or its insurgence prevented [Paul and Snyder, 2012]. A number of H_2S slow-releasing compounds have been recently developed, and could be therefore prospectively utilized for this purpose.

In conclusion, H_2S interferes with the pro-atherogenic inflammatory processes typical of the early stages of atherosclerosis, by

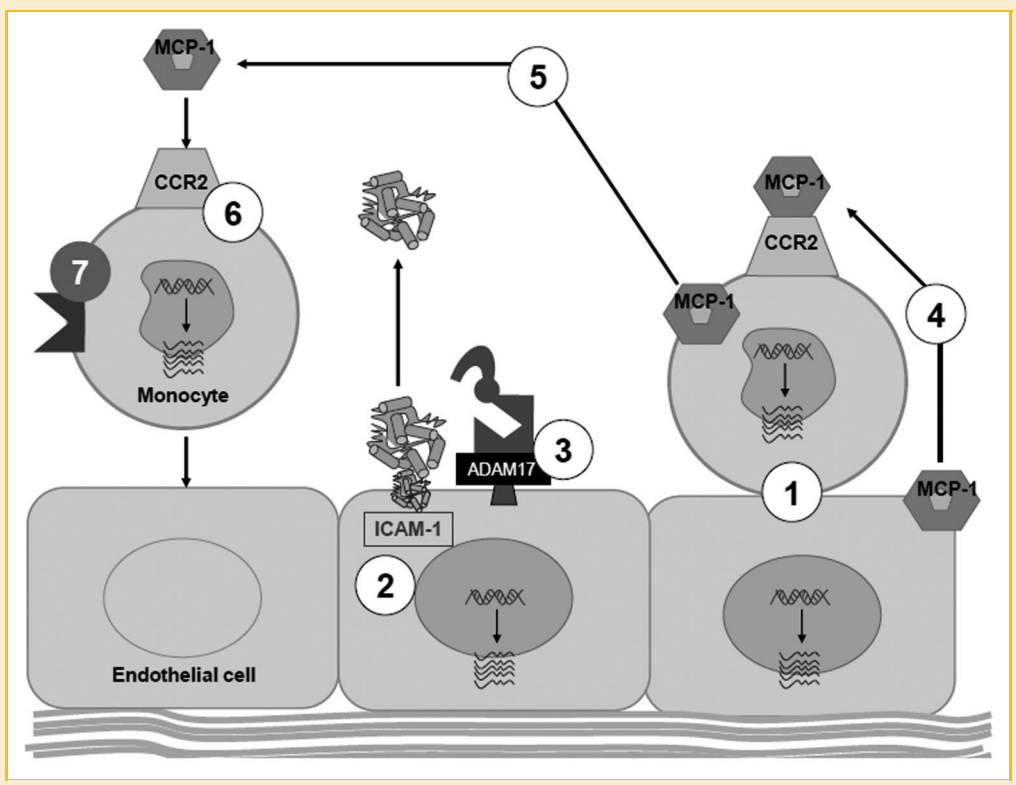

Fig. 8. A summary of the molecular targets and effects of H_2S. Chemokine/cytokine/receptor/protease targets and relevant functions were identified as the ability of NaHS to inhibit effects of TNF-α or PMA in endothelial and/or monocyte cells. Cell adhesion (1); ICAM-1 expression and release (2); ADAM17 expression (3); MCP-1 expression and release both at endothelial (4) and monocyte (5) levels; CCR2 expression (6); CD36 expression (7). Dark numbers in white circles indicate decrease; white numbers in dark circles indicate increase.

influencing the levels of key molecules involved in this process, such as ADAM17 and, through this, TNF-α and MCP1. Acting on H_2S levels may thus represent a novel putative strategy to limit cardiovascular risk in the patient population affected, for example, by chronic kidney disease [Therrien et al., 2012].

ACKNOWLEDGMENTS

This study was entirely financed by public sources, no private profit institutions provided any funding. This work was supported in part by Grants PRIN2005-prot.2005062199_003 and PRIN2007-prot.2007EBCYYW_004 (to D.I.) and Grant PRIN2007 prot. 2007EBCYYW_001 (to A.F.P.). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

REFERENCES

Ait-Oufella H, Taleb S, Mallat Z, Tedgui A. 2011. Recent advances on the role of cytokines in atherosclerosis. Arterioscler Thromb Vasc Biol 31(5):969–979.

Aminzadeh MA, Vaziri ND. 2012. Downregulation of the renal and hepatic hydrogen sulfide (H_2S)-producing enzymes and capacity in chronic kidney disease. Nephrol Dial Transplant 27(2):498–504.

Arribas J, Esselens C. 2009. ADAM17 as a therapeutic target in multiple diseases. Curr Pharm Des 15(20):2319–2335.

Ashraf MZ, Gupta N. 2011. Scavenger receptors: Implications in atherothrombotic disorders. Int J Biochem Cell Biol 43(5):697–700.

Bell JH, Herrera AH, Li Y, Walcheck B. 2007. Role of ADAM17 in the ectodomain shedding of TNF-alpha and its receptors by neutrophils and macrophages. J Leukoc Biol 82(1):173–176.

Boulares AH, Yakovlev AG, Ivanova V, Stoica A, Wang G, Iyer S, Smulson M. 1999. Role of poly(ADP-ribose) polymerase (PARP) cleavage in apoptosis. Caspase 3-resistant PARP mutant increases rates of apoptosis in transfected cells. J Biol Chem 274(33):22932–22940.

Boyer JF, Balard P, Authier H, Faucon B, Bernad J, Mazières B, Davignon JL, Cantagrel A, Pipy B, Constantin A. 2007. Tumor necrosis factor alpha and adalimumab differentially regulate CD36 expression in human monocytes. Arthritis Res Ther 9(2):R22.

Burke-Gaffney A, Hellewell PG. 1996. Tumour necrosis factor-ac-induced ICAM-1 expression in human vascular endothelial and lung epithelial cells: Modulation by tyrosine kinase inhibitors. Brit J Pharmacol 119:1149–1158.

Capasso R, Sambri I, Cimmino A, Salemme S, Lombardi C, Acanfora F, Satta E, Puppione DL, Perna AF, Ingrosso D. 2012. Homocysteinylated albumin promotes increased monocyte-endothelial cell adhesion and up-regulation of MCP1, Hsp60 and ADAM17. PLoS ONE 7(2):e31388.

Caraglia M, Marra M, Viscomi C, D'Alessandro AM, Budillon A, Meo G, Arra C, Barbieri A, Rapp UR, Baldi A, Tassone P, Venuta S, Abbruzzese A, Tagliaferri P. 2007. The farnesyltransferase inhibitor R115777 (ZARNESTRA) enhances the pro-apoptotic activity of interferon-alpha through the inhibition of multiple survival pathways. Int J Cancer 121(10):2317–2330. Erratum in: Int J Cancer. 2008 Apr 15;122(8): 1922.

Chen P, Poddar R, Tipa EV, Dibello PM, Moravec CD, Robinson K, Green R, Kruger WD, Garrow TA, Jacobsen DW. 1999. Homocysteine metabolism in cardiovascular cells and tissues: Implications for hyperhomocysteinemia and cardiovascular disease. Adv Enzyme Regul 39:93–109.

Chen MJ, Peng ZF, Manikandan J, Melendez AJ, Tan GS, Chung CM, Li QT, Tan TM, Deng LW, Whiteman M, Beart PM, Moore PK, Cheung NS. 2011. Gene profiling reveals hydrogen sulphide recruits death signaling via the N-methyl-D-aspartate receptor identifying commonalities with excitotoxicity. J Cell Physiol 226(5):1308–1322.

Cimmino A, Capasso R, Muller F, Sambri I, Masella L, Raimo M, De Bonis ML, D'Angelo S, Zappia V, Galletti P, Ingrosso D. 2008. Protein isoaspartate methyltransferase prevents apoptosis induced by oxidative stress in endothelial cells: Role of Bcl-Xl deamidation and methylation. PLoS ONE 3(9):e3258.

Collins AJ, Foley RN, Herzog C, Chavers BM, Gilbertson D, Ishani A, Kasiske BL, Liu J, Mau LW, McBean M, Murray A, St Peter W, Guo H, Li Q, Li S, Li S, Peng Y, Qiu Y, Roberts T, Skeans M, Snyder J, Solid C, Wang C, Weinhandl E, Zaun D, Arko C, Chen SC, Dalleska F, Daniels F, Dunning S, Ebben J, Frazier E, Hanzlik C, Johnson R, Sheets D, Wang X, Forrest B, Constantini E, Everson S, Eggers PW, Agodoa L. 2010. Excerpts from the US Renal Data System 2009 Annual Data Report. Am J Kidney Dis 55(Suppl. 1):S1–S420, A6–A7.

Dawson-Saunders B, Trapp RG. 1990. Basic and clinical biostatistics. East Norwalk, CT, USA: Appleton & Lange. 329 pp.

Drüeke TB, Massy ZA. 2010. Atherosclerosis in CKD: Differences from the general population. Nat Rev Nephrol 6(12):723–735.

Gooz M. 2010. ADAM-17: The enzyme that does it all. Crit Rev Biochem Mol Biol 45(2):146–169.

Gräf P, Förstermann U, Closs EI. 2001. The transport activity of the human cationic amino acid transporter hCAT-1 is downregulated by activation of protein kinase C. Br J Pharmacol 132:1193–1200.

Jeney V, Komódi E, Nagy E, Zarjou A, Vercellotti GM, Eaton JW, Balla G, Balla J. 2009. Suppression of hemin-mediated oxidation of low-density lipoprotein and subsequent endothelial reactions by hydrogen sulfide (H(2)S). Free Radic Biol Med 46(5):616–623.

Lavu M, Bhushan S, Lefer DJ. 2011. Hydrogen sulfide-mediated cardioprotection: Mechanisms and therapeutic potential. Clin Sci 120(6):219–229.

Li L, Moore PK. 2008. Putative biological roles of hydrogen sulfide in health and disease: A breath of not so fresh air? Trends Pharmacol Sci 29(2):84–90.

Ma K, Liu Y, Zhu Q, Liu CH, Duan JL, Tan BK, Zhu YZ. 2011. H_2S donor, S-propargyl-cysteine, increases CSE in SGC-7901 and cancer-induced mice: Evidence for a novel anti-cancer effect of endogenous H_2S? PLoS ONE 6(6):e20525.

Murao K, Ohyama T, Imachi H, Ishida T, Cao WM, Namihira H, Sato M, Wong NC, Takahara J. 2000. TNF-alpha stimulation of MCP-1 expression is mediated by the Akt/PKB signal transduction pathway in vascular endothelial cells. Biochem Biophys Res Commun 276:791–796.

Mustafa AK, Gadalla MM, Sen N, Kim S, Mu W, Gazi SK, Barrow RK, Yang G, Wang R, Snyder SH. 2009. H_2S signals through protein S-sulfhydration. Sci Signal 2(96):ra72.

Oh GS, Pae HO, Lee BS, Kim BN, Kim JM, Kim HR, Jeon SB, Jeon WK, Chae HJ, Chung HT. 2006. Hydrogen sulfide inhibits nitric oxide production and nuclear factor-kappaB via heme oxygenase-1 expression in RAW264.7 macrophages stimulated with lipopolysaccharide. Free Radic Biol Med 41:106–119.

Pan LL, Liu XH, Gong QH, Wu D, Zhu YZ. 2011. Hydrogen sulfide attenuated tumor necrosis factor-α-induced inflammatory signaling and dysfunction in vascular endothelial cells. PLoS ONE 6(5):e19766.

Papapetropoulos A, Pyriochou A, Altaany Z, Yang G, Marazioti A, Zhou Z, Jeschke MG, Branski LK, Herndon DN, Wang R, Szabó C. 2009. Hydrogen sulfide is an endogenous stimulator of angiogenesis. Proc Natl Acad Sci USA 106(51):21972–21977.

Papayianni A, Alexopoulos E, Giamalis P, Gionanlis L, Belechri AM, Koukoudis P, Memmos D. 2002. Circulating levels of ICAM-1, VCAM-1, and MCP-1 are increased in haemodialysis patients: Association with inflammation, dyslipidaemia, and vascular events. Nephrol Dial Transplant 17(3):435–441.

Paul BD, Snyder SH. 2012. H_2S signalling through protein sulfhydration and beyond. Nat Rev 13:499–507.

Perna AF, Luciano MG, Ingrosso D, Pulzella P, Sepe I, Lanza D, Violetti E, Capasso R, Lombardi C, De Santo NG. 2009. Hydrogen sulphide-generating pathways in haemodialysis patients: A study on relevant metabolites and transcriptional regulation of genes encoding for key enzymes. Nephrol Dial Transplant 24:3756–3763.

Podrez EA, Febbraio M, Sheibani N, Schmitt D, Silverstein RL, Hajjar DP, Cohen PA, Frazier WA, Hoff HF, Hazen SL. 2000. Macrophage scavenger receptor CD36 is the major receptor for LDL modified by monocyte-generated reactive nitrogen species. J Clin Invest 105(8):1095–1108.

Pruessmeyer J, Ludwig A. 2009. The good, the bad and the ugly substrates for ADAM10 and ADAM17 in brain pathology, inflammation and cancer. Semin Cell Dev Biol 20(2):164–174.

Qiao W, Chaoshu T, Hongfang J, Junbao D. 2010. Endogenous hydrogen sulfide is involved in the pathogenesis of atherosclerosis. Biochem Biophys Res Commun 396(2):182–186.

Reiss K, Cornelsen I, Husmann M, Gimpl G, Bhakdi S. 2011. Unsaturated fatty acids drive ADAM-dependent cell adhesion, proliferation and migration by modulating membrane fluidity. J Biol Chem 286(30):26931–26942.

Sarnak MJ, Foley NR. 2011. Cardiovascular mortality in the general population versus dialysis: A glass half full or empty? Am J Kidney Dis 58(1):4–6.

Scheller J, Chalaris A, Garbers C, Rose-John S. 2011. ADAM17: A molecular switch to control inflammation and tissue regeneration. Trends Immunol 32:380–387.

Sen N, Paul BD, Gadalla MM, Mustafa AK, Sen T, Xu R, Kim S, Snyder SH. 2012. Hydrogen sulfide-linked sulfhydration of NF-κB mediates its anti-apoptotic actions. Mol Cell 45:13–24.

Therrien FJ, Agharazii M, Lebel M, Larivière R. 2012. Neutralization of tumor necrosis factor-alpha reduces renal fibrosis and hypertension in rats with renal failure. Am J Nephrol 36(2):151–161.

Wang R. 2011. Signaling pathways for the vascular effects of hydrogen sulfide. Curr Opin Nephrol Hypertens 20(2):107–112.

Wang Y, Zhao X, Jin H, Wei H, Li W, Bu D, Tang X, Ren Y, Tang C, Du J. 2009. Role of hydrogen sulfide in the development of atherosclerotic lesions in Apolipoprotein E knockout mice. Arterioscler Thromb Vasc Biol 29:173–179.

Whiteman M, Moore PK. 2009. Hydrogen sulfide and the vasculature: A novel vasculoprotective entity and regulator of nitric oxide bioavailability? J Cell Mol Med 13:488–507.

Willems SH, Tape CJ, Stanley PL, Taylor NA, Mills IG, Neal DE, McCafferty J, Murphy G. 2010. Thiol isomerases negatively regulate the cellular shedding activity of ADAM17. Biochem J 428:439–450.

Xu P, Derynck R. 2010. Direct activation of TACE-mediated ectodomain shedding by p38 MAP kinase regulates EGF receptor-dependent cell proliferation. Mol Cell 37:551–566.

Zhao ZZ, Wang Z, Li GH, Wang R, Tan JM, Cao X, Suo R, Jiang ZS. 2011. Hydrogen sulfide inhibits macrophage-derived foam cell formation. Exp Biol Med (Maywood) 236(2):169–176.