Journal of Cellular Physiology

Exogenous Hydrogen Sulfide Induces Functional Inhibition and Cell Death of Cytotoxic Lymphocytes Subsets

PRISCO MIRANDOLA,[1] GIULIANA GOBBI,[1] IVONNE SPONZILLI,[1] MAURIZIA PAMBIANCO,[2] CHIARA MALINVERNO,[1] ANTONIO CACCHIOLI,[3] GIUSEPPE DE PANFILIS,[4] AND MARCO VITALE[1]*

[1]*Department of Anatomy, Pharmacology & Forensic Medicine, Human Anatomy Section, University of Parma, Ospedale Maggiore, via Gramsci, Parma, Italy*

[2]*Terme di Sirmione, Cell Biology Laboratory, Terme di Sirmione, Brescia, Italy*

[3]*Department of Animal Health, University of Parma, Italy*

[4]*Department of Surgery, Section of Dermatology, University of Parma, Italy*

The toxic effects of exogenous hydrogen sulfide on peripheral blood lymphocytes have been investigated in detail. Hydrogen sulfide is now considered as a gasotransmitter with specific functional roles in different cell types, like neurons and vascular smooth muscle. Here we show that exogenous hydrogen sulfide induces a caspase-independent cell death of peripheral blood lymphocytes that depends on their intracellular glutathion levels, with a physiologically relevant subset specificity for $CD8^+$ T cells and NK cells. Although lymphocyte activation does not modify their sensitivity to HS^-, after 24 h exposure to hydrogen sulfide surviving lymphocyte subsets show a dramatically decreased proliferation in response to mitogens and a reduced IL-2 production. Overall, our data demonstrate that HS^- reduces the cellular cytotoxic response of peripheral blood lymphocytes as well as their production of IL-2, therefore de-activating the major players of local inflammatory responses, adding new basic knowledge to the clinically well known anti-inflammatory effects of sulfur compounds.

J. Cell. Physiol. 213: 826–833, 2007.

Hydrogen sulfide (H_2S)—known for decades as a toxic gas—is endogenously generated from cysteine, in reactions catalyzed by cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) (Finkelstein and Martin, 1984; Navarra et al., 2000; Moore et al., 2003). Mounting data on endogenously generated H_2S have now included this gas in the family of gasotransmitters, together with nitric oxide (NO) and carbon monoxide (CO), and its effects start to be understood both at the cellular and molecular level (Wang, 2002). While a physiological role in hippocampal neurons long term potentiation has also been demonstrated (Eto et al., 2002), perhaps the best characterized physiological effect of H_2S to date is the relaxation of vascular smooth muscle cells, generated by H_2S-mediated K_{ATP} channel opening (Zhao et al., 2001; Teague et al., 2002).

Searching for the cellular and molecular mechanisms underlying the involvement of hydrogen sulfide in inflammatory processes has been a research challenge for many years. H_2S can be exogenously administered both in vitro and in vivo. It has been demonstrated that the H_2S in vitro induces smooth muscle cell relaxation (Cheng et al., 2004), serum-independent proliferation of non-transformed epithelial cells (Deplancke and Gaskins, 2003) and ERK-mediated apoptosis of human aorta smooth muscle cells (Yang et al., 2006). We have recently demonstrated that NaHS promoted the survival of cultured granulocytes in a dose-dependent (EC_{50}, 0.5 mM) and time-dependent (6–24 h) manner under stress conditions. The delayed onset of apoptosis of granulocytes in the presence of H_2S was mediated by the inhibition of caspase 3 cleavage and p38 MAPK phosphorylation (Rinaldi et al., 2006). Thus, complex regulatory mechanisms emerge for differential apoptosis induction in different cell types, as previous studies have shown increased apoptosis of smooth muscle cells and inhibited HEK-293 cell proliferation by endogenous and exogenous H_2S. In parallel, it has been known for more than one decade that sulfurous thermal waters have inhibitory effects on the proliferative response of T cells (Valitutti et al., 1990), and we have shown that the effect of HS^- on bulk lymphocyte populations is opposite to that observed on granulocytes (Rinaldi et al., 2006).

Given the fundamental role that lymphocytes subsets and cytokine production play in pathophysiology of inflammatory processes, we decided to study in greater detail the effects of exogenous HS^- ions on the survival and functional activity of peripheral blood lymphocytes.

Contract grant sponsor: Fondazione per la ricerca scientifica termale.
Contract grant sponsor: PRIN.

*Correspondence to: Marco Vitale, Department of Anatomy, Pharmacology & Forensic Medicine, Human Anatomy Section, University of Parma, Ospedale Maggiore, Via Gramsci, 14, I-43100 Parma, Italy. E-mail: marco.vitale@unipr.it

Received 8 March 2007; Accepted 18 April 2007

DOI: 10.1002/jcp.21151

Materials and Methods

Reagents

Peripheral blood lymphocytes (PBLs) were grown in RPMI 1640 medium with or without 10% FCS (both from BioWittaker, Walkersville, MD).

The general caspase inhibitor Z-VAD, the selective caspase 9 inhibitor Z-LEHD-FMK and calcein-acetoxymethyl ester diacetate (Calcein-AM) were from Calbiochem (La Jolla, CA).

NaHS (cat. no. 161527), the glutathione synthesis inhibitor buthionine sulphoximine (BSO), Triton-X100, β-nicotinamide dinucleotide phosphate (NADPH), 5,5′-dithiobis-2-nitrobenzoic acid (DNTP), ethylenediamine tetracetic acid (EDTA), phytohemagglutinin-M (PHA), glutathione reductase and JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide), PKH26®, were from Sigma (St. Louis, MO).

Anti-CD4 fluorescein isothiocyanate (FITC)-labeled, anti-CD8 phycoerythrin (PE)-labeled, anti-CD3 FITC, anti-CD14 FITC, anti-CD16, CD56 PE, monoclonal antibodies (mAb) were purchased from Beckman Coulter (Miami, FL).

The carboxyl fluorescein succinimidyl ester (CFSE), used for cell proliferation assay, was from Molecular Probes (Eugene, OR), while human recombinant interleukin-2 (IL-2) was from Genzyme (Boston, MA).

Primary cell cultures purification

Blood was collected from buffy coats of five healthy donors. Purified human peripheral blood lymphocytes were obtained by gradient separation. Briefly, 25 ml of 1:4 PBS-diluted blood was layered upon 20 ml of Lympholyte-H (Cedarlane, Hornby, Ontario, Canada) and centrifuged at 390*g* for 35 min. Plasma and platelets were discarded, while lymphocytes were collected for subsequent experiments in this step. The $CD4^+$ and $CD8^+$ T cells and the $CD16^+$ NK cell subsets were isolated from lymphocytes preparations by immunomagnetic selection (Milteny, Auburn, CA). Monocytes were isolated by plastic adherence to Petri dishes for 4 h in RPMI +10% SBF at 37°C. Contaminant cells were eliminated washing adherent cells with PBS.

Purity of PBL cells was checked by anti-CD3 FITC mAb staining and flow cytometry. Purity of NK cells was checked by anti-CD16 PE and anti-CD3 FITC double labeling, while purity of $CD4^+$ and $CD8^+$ T cells was monitored by anti-CD4 PE/CD3 FITC or anti-CD8 PE/CD3 FITC, respectively. Purity of monocytes cultures was evaluated by anti-CD14 PE/CD3 FITC double labeling. Only samples with a purity exceeding 95% were used.

Cell cultures and treatment

Purified human PBLs were cultured up to 24 h, at an optimal cell density of 1×10^6 cells/ml, in serum-free RPMI medium. NaHS working solutions were prepared immediately before the use, diluting in RPMI a 150 mM stock solution. Stock solution, stored at +4°C, was reconstituted monthly dissolving NaHS salt in RPMI medium. NaHS was immediately added to the cell cultures at concentrations ranging from 0.20 to 4.0 mM. Cell were cultured in flask or multiwells plates in a volume of 0.5 ml/cm^2 to ensure HS^- storage with low H_2S gas escape. Cultures were kept at 37°C, under 5% CO_2-enriched, H_2O-satured atmosphere. Control cultures were treated as above in the absence of NaHS. In some experiments, purified PBLs were pretreated with: (i) 30 μM Z-VAD or 30 μM Z-LEHD-FMK; (ii) BSO at concentrations ranging from 200 to 1,000 μM.

The NaHS solution was prepared immediately before use at 1 M concentration in RPMI medium, and added to the cell cultures to the indicated final concentration (Zhao et al., 2003).

Cell viability and apoptosis

Cell culture viability was assessed by trypan blue exclusion. Apoptotic cells were identified by flow cytometry either as subdiploid peaks generated by DNA fragmentation or by Annexin V/propidium iodide (PI) staining. In the first case, cells were either permeabilized by ethanol in the presence of RNAse H buffer and stained with 50 μg/ml of PI. Apoptotic cells appeared as a subdiploid peak. In the second case, membrane phosphatidylserine was stained by FITC conjugated Annexin V (ACTIPLATE, Valter Occhiena, Torino, Italy) in Ca^{2+} and PI staining buffer, following manufacturer's protocol. Apoptotic cells were identified as Annexin $V^+/PI^{-/dim}$ cells.

NaHS-induced modifications of mitochondrial membrane potential (Ψm) were measured using the fluorescent dye JC-1, a ratiometric cationic carbocyanine dye whose uptake into mitochondria is driven by their transmembrane potential (Reers et al., 1995). The green fluorescence ($\lambda_{em} = 527$ nm) of JC-1 monomers increases as Ψm decreases (Shenker et al., 1999). Cell suspensions were incubated with JC-1 (5 μg/ml) for 15 min at 37°C and washed twice with PBS; green and red fluorescences were measured by flow cytometry.

All the flow cytometric analyses were performed by an Epics XL flow cytometer (Beckman Coulter) and the Expo ADC software (Beckman Coulter). Data collected from 10,000 cells were reported as either percentage of positive cells or mean fluorescence intensity (MFI) values.

Cell morphology was evaluated by transmission electron microscopy (TEM). Briefly, PBL cultures were pelleted at 200*g* for 10 min, washed and fixed in 2.5% glutaraldehyde in 0.1 M PBS, pH 7.4. Pellets were then fixed in 2% osmic acid in 0.1 M PBS, pH 7.4, and embedded in Durcupan (Fluka Chemie, Buchs, Switzerland). Semithin sections were stained with Toluidine blue −0.5% sodium carbonate, while ultra-thin sections were stained with uranyl acetate and lead citrate according to Reynolds (Reynolds, 1963) and observed with a Philips 300 electron microscope (Philips, Eindhoven, The Netherlands).

Cell proliferation

To study cell proliferation, PBL were cultured in RPMI +10% FBS in the presence of 5 μg/ml of PHA for 24 h, and subsequently grown in RPMI +10% FBS and 30 U/ml IL-2. Complete medium was added twice a week (Secchiero et al., 2001; Mirandola et al., 2006).

To quantify cell proliferation, CFSE was added to the cell suspensions at the final concentration of 10 μM and incubated for 10 min at room temperature in the dark. Labeling was terminated by adding the same volume of 100% fetal calf serum for 5 min at room temperature to quench free CFSE (Kolumam et al., 2005). The labeled cells were then washed 5 times with sterile PBS containing 10% FCS, counted and resuspended into complete medium at 1×10^6/ml. Living unstimulated or PHA-stimulated cells, treated with or without 1.83 mM NaHS, were analyzed at day 3 and at day 6 of culture by flow cytomety.

Cellular cytotoxicity and IL-2 production

NK effector cells were cultured for 24 h in serum free RPMI medium with or without 1.83 mM of NaHS. To test natural cytotoxicity 2.5×10^6 or 4.6×10^6 NK cells were mixed with 2.5×10^5 target cells—the human colon carcinoma cell lines HT-29 and DLD2, and the human erythroleukemic cell line K562—labeled with PKH26® and loaded with Calcein-AM (Mirandola et al., 2006). Briefly, target cells were labeled with 1:1,000 dilution of PKH26® in the supplied buffer for 30 min at 37°C, then washed three times with complete medium to eliminate the residual dye. PKH26® labeled target cells were then loaded with 12.75 nM Calcein-AM in complete medium for 30 min at 37°C, then washed again three times with complete medium to eliminate the residual dye. After 6 h of co-incubation of target and effector cells at 37°C, cells were washed in PBS and analyzed by flow

cytometry. Control samples were treated as above but incubated in the absence of effector cells.

IL-2 production was evaluated by the ELISA assay. Supernatants from 2×10^6 of purified $CD4^+$ and $CD8^+$ T cells, stimulated 48 h with 5 μg/ml PHA in the presence or absence of 1.83 mM NaHS, were collected for the ELISA assay, according to manufacturer's protocol (Bender MedSystems, Inc., Burlingame, CA).

Intracellular total glutathione levels

Reduced (GSH) and oxidized (GSSG) glutathione levels were determined as previously described (Allen et al., 2000; Beier et al., 2004). In detail, cells were collected by centrifugation at 600*g* for 3 min at 4°C, resuspended in 80 μl of Triton-X100 solution (0.1% Triton-X100 in 143 mM potassium phosphate buffer), and lysed by freezing and thawing (4×). Samples were then deproteinized in a 5% 5-sulfosalicylic acid (SSA) solution: 100 μl of 5% SSA were added to the cell suspension, and total mix was centrifuged at 16,000*g* for 20 min. Precipitated proteins were discarded, while supernatants were transferred to a new tube, and used for the total glutathione assay: 100 μl of sample were mixed with 125 μl of a reaction mix containing 123 mM potassium phosphate, pH 7.0, 5.4 mM EDTA, 268 μM NADPH, 160 μM DNTP and 2 U of glutathione reductase. The yellow product, 5-thio-2-nitrobenzoic acid, was measured spectrophotometrically at 405 nm. Standard curves of reduced glutathione were produced to determine the amount of glutathione in the biological sample. Total glutathione levels were reported as ng/10^6 cells.

Statistical analysis

All values in the figures and text are expressed as means ± standard error (SE) of n observation (with $n \geq 3$). Data sets were examined by analysis of variance (ANOVA) and Dunnett's test (when required). A P value <0.05 was considered statistically significant.

Results

HS^- selectively induces lymphocyte cell death

The effects of HS^- on lymphocytes were assessed in vitro culturing isolated PBLs from 5 different healthy donors for 24 h with increasing doses of NaHS (ranging from 0.20 to 4.0mM). While the viability of monocytes was not affected at all by the treatment with NaHS, the viability of PBL was progressively reduced (Fig. 1A). To see if cell activation could modify the response to HS^-, we pre-activated the lymphocytes by PHA and then cultured them for 7 days in the presence of 30 U/ml of IL-2. Figure 1B shows that activated PBLs are as sensitive to HS^- as the resting ones, although a slightly higher resistance at higher NaHS doses was observed. In fact, 2 mM NaHS reduced the viability of activated PBL from 76 ± 6% to 50 ± 9% (−34 ± 7%) while cell viability of resting PBL was reduced from 93 ± 9% (control) to 36 ± 10% (−61 ± 6%; $P < 0.05$) in the treated cell culture.

Some apoptosis was present in the cell cultures treated with NaHS, but most lymphocytes died by necrosis. The Annexin V-FITC/PI flow cytometry analysis (Fig. 2A) of resting PBL cultures treated with 2.0 mM NaHS for 24 h, showed that most cells became PI^+, with a very small presence of Annexin $V^+/PI^{-/dim}$ apoptotic cells. This was confirmed by the morphological analysis of cell cultures by TEM, that essentially showed necrotic cells, with rare apoptotic figures (Fig. 2B).

Looking deeper in the mechanism of HS^--induced cell death, we studied the mitochondrial potential (Ψm) of lymphocytes by staining cell cultures with JC-1, a dye whose green fluorescence increases as mitochondria loose electrical potential (Smiley et al., 1991). Interestingly, HS^--treated lymphocytes largely loose their Ψm in 4 h, while it takes 20 h to observe a similar effect in the control cultures (Fig. 3A). In particular (Fig. 3B), the effect of HS^- is dramatically evident between 3 and 4 h of

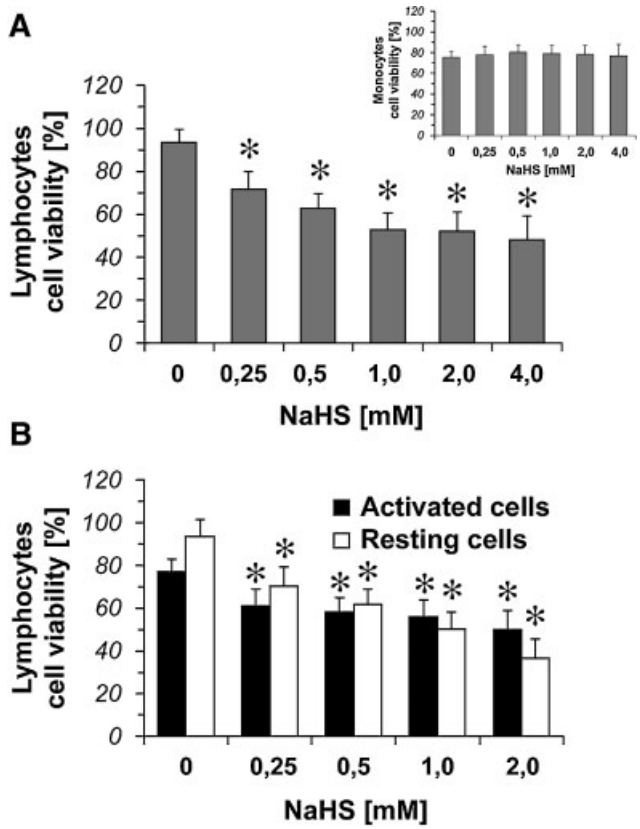

Fig. 1. HS^- induces lymphocyte cell death. Cells, seeded in serum free medium, were treated 24 h with NaHS and the residual cell viability was quantified by staining with Annexin V-FITC/PI and flow cytometry analysis. Cell viability is expressed as % of Annexin V and PI negative cells. The means ± SE of five independent experiments are reported; *$P < 0.05$ compared with the control (0 NaHS). Part A: Analysis of PBL and monocyte viability upon treatment with the indicated concentrations of NaHS. Monocyte viability is not affected by NaHS and therefore is shown as internal negative control. Part B: Sensitivity to HS^- of resting and activated PBLs. Lymphocytes were pre-activated by PHA and then cultured for 7 days in the presence of 30 U/ml IL-2. NaHS at the indicated concentrations was given to the cells during the last 24 h of culture.

treatment. The percentage of cells with physiological Ψm after 4 h of NaHS treatment was 48 ± 9% versus the 80 ± 8% of the control untreated cells ($P < 0.05$). After 20 h of NaHS treatment all (96 ± 8%) lymphocytes lost their Ψm.

To formally prove that lymphocytes essentially die by necrosis, and not by apoptosis, we then cultured our cells in the presence of 30 μM Z-VAD-FMK, a known general caspase inhibitor, or in the presence of 30 μM Z-LEHD-FMK, a specific caspase-9 inhibitor. Figure 4 shows that there are no significant differences in the percentages of HS^--induced cell death in the presence or absence of either inhibitor.

HS^- impairs lymphocyte proliferation

Living PBLs can be easily distinguished by flow cytometry from necrotic and apoptotic cells based on their different scattering properties (Zamai et al., 1993). By gating therefore on living cells, we were able to study the effect of HS^- on the proliferative capacity of PBLs cultured for 3–6 days in the presence of 30 U/ml of IL-2. To measure the number of cell replications, we stained the lymphocytes with CFSE at the

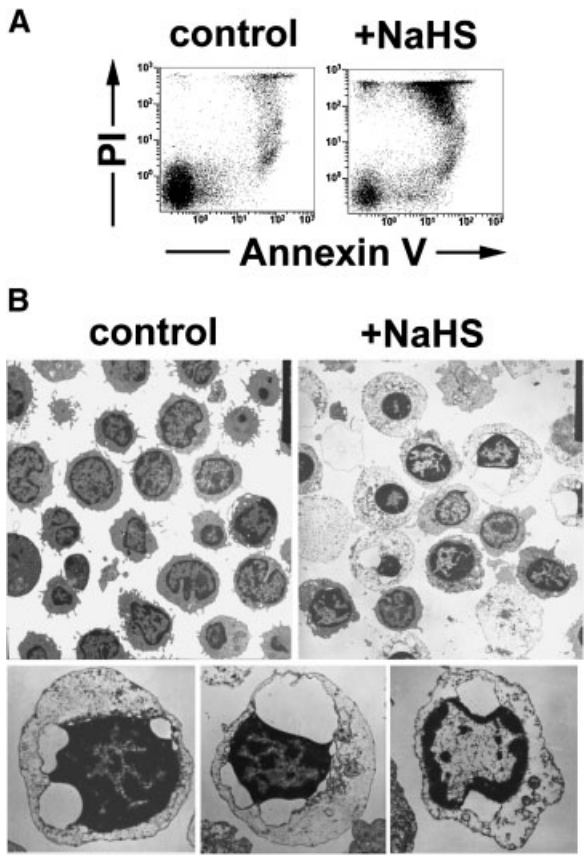

Fig. 2. HS⁻ induces lymphocyte necrosis. Lymphocytes were cultured in serum free medium and treated for 24 h with 2.0 mM NaHS. Part A: Annexin V-FITC/PI flow cytometric analysis of PBL cultures treated with (+NaHS) or without (control) NaHS. Results shown are representative of five independent experiments; at least 10,000 cells/sample were analyzed. Part B: Morphological analysis by TEM of PBL cultures treated with (+NaHS) or without (control) NaHS. Parts at the top: low magnification (3,192×). Parts at the bottom show three examples of necrotic lymphocytes after NaHS treatment, at higher magnification (7,980×). Results shown are representative of three independent experiments.

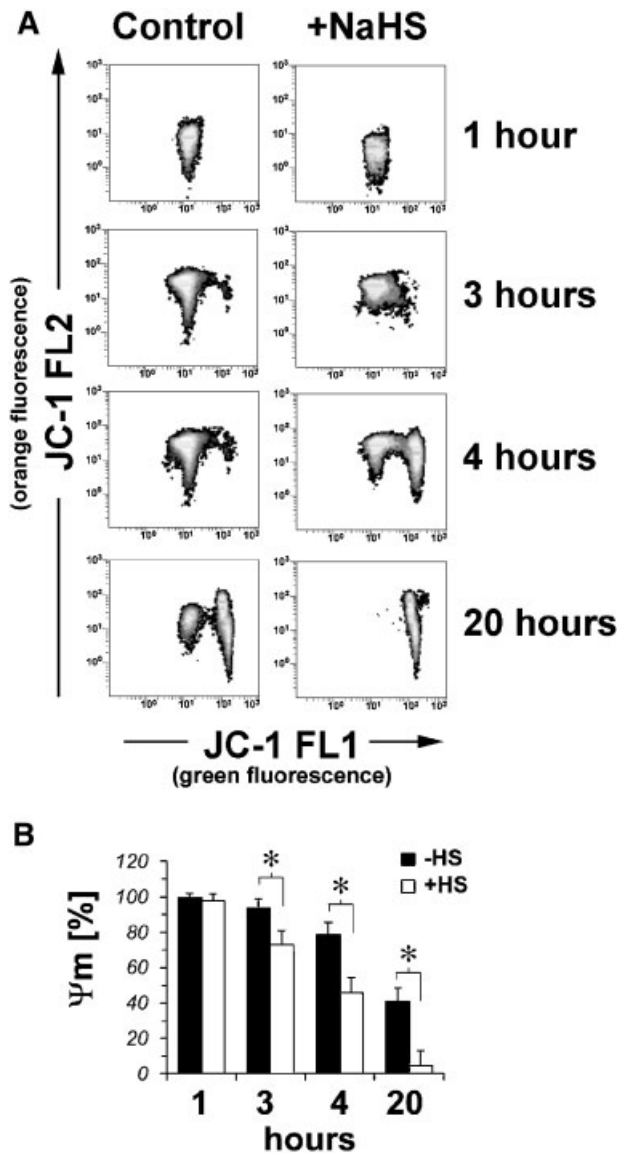

Fig. 3. HS⁻ induces the loss of mitochondrial potential in lymphocytes. Effect of NaHS on lymphocyte Ψm. Cells, seeded in serum free medium, were incubated with (+NaHS) or without (control) 2.0 mM of NaHS for 1, 3, 4, 20 h, stained with JC-1 and then analyzed by flow cytometry for fluorescence emission at 530 nm (JC-1 FL1) and 575 nm (JC-1 FL2). Freshly isolated cells exhibit bright orange fluorescence (575 nm) indicative of high Ψm. In contrast, NaHS-treated cells (3, 4 and 20 h) and control cells cultured for 20 h show an increased green fluorescence, that is the result of the formation of the JC-1 monomers at decreased Ψm. Part A: Flow cytometry dot plots of one representative experiment. Ten thousand cells per sample were analyzed. Part B: The amount of residual cells with normal Ψm is reported as % of controls (cells with bright orange and dim green fluorescence). The means ± SE of three independent experiments are reported; *$P < 0.05$ compared with the control NaHS untreated cells (white bar).

beginning of the cultures. At every cell division, CFSE fluorescence is reduced by half (Weston and Parish, 1990). After 3 days of culture in the presence of HS^-, 66 ± 5% of living PBL did not duplicate versus 20 ± 5% of the control cultures in the absence of HS^-. The cells that were able to make three duplications at day 3 were 20 ± 5% in the control cultures versus 4 ± 2% in the treated cultures (Fig. 5, parts A and B). After 6 days, virtually all the cells in the control cultures had proliferated, while 20 ± 8% of the residual living cells in the HS^- treated cultures were however unable to duplicate (Fig. 5C).

The toxic effect of HS^- on lymphocytes is subset-specific

Given that HS^- is toxic to PBL in vitro, we next asked whether the sensitivity of the different lymphocyte subsets to HS^- was the same or not. We therefore purified $CD8^+$ T cells, $CD4^+$ T cells and $CD16^+$ NK cells by immunomagnetic selection, and cultured them separately under the same conditions described above for the bulk PBL population. Interestingly, 24 h culture in the presence of 2.0 mM NaHS kills most $CD8^+$ T cells and $CD56^+$ NK cells, sparing $CD4^+$ T cells (Fig. 6A). We therefore performed dose–response experiments on each purified lymphocyte subset (Fig. 6B) that showed IC_{50} doses of NaHS of 0.092 ± 0.025 mM for NK cells, 0.18 ± 0.03 mM for $CD8^+$ T cells and 0.34 ± 0.03 mM for $CD4^+$ T cells.

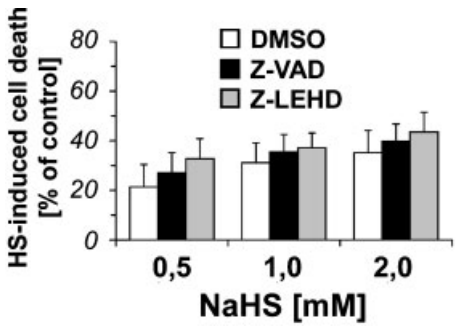

Fig. 4. HS^--induced cell death is caspase-independent. HS^--induced cell death in the presence of the pan-caspase inhibitor (Z-VAD) or the caspase-9 inhibitor (Z-LEHD). Cells, cultured in serum free medium, were treated for 1 h with the caspase inhibitors or with DMSO alone (control) and then were grown with or without NaHS at the indicated concentrations for 24 h. Cell viability was evaluated by Annexin-V/PI staining and flow cytometry. Means are calculated as 100 × (living untreated cells − living HS-treated cells)/(living untreated cells). The means ± SE of three independent experiments are reported. Caspase inhibitors did not significantly increases cell viability.

Functional impairment of natural cytotoxicity and IL-2 production

Given the specific loss of NK and $CD8^+$ T cells upon treatment with HS^-, we functionally assayed our cell cultures for natural cytotoxicity and cytokine production.

Figure 7A shows a significant reduction of the spontaneous cytotoxicity of purified $CD16^+$ NK cells treated with HS^- against both the human erythroleukemic cell line K562 and the human colon carcinoma cell lines HT-29 and DLD2. To restore the cytotoxicity levels of NaHS-treated NK cell cultures to the control levels, we had to increase the number of effector cells by two folds.

We then measured by ELISA the production of IL-2 in the supernatants of both purified $CD8^+$ T cells and of $CD4^+$ T cells, cultured with PHA for 48 h with or without 2.0 mM NaHS. Results showed that HS^- induced a significant reduction of IL-2 production in both cell populations (Fig. 7B).

Glutahtione protects cells from HS^- toxicity

It is well known that oxidized glutathione (GSSG) has a general role in the protection of cells from oxidative stress (Chakravarthi et al., 2006). We therefore measured the levels of glutathione in resting and activated PBLs as well as in the tumor cell lines HT-29 and DLD2.

The two cell lines tested showed high basal levels of GSH while, on the contrary, primary PHA-activated PBLs showed very low levels of GSH. BSO, as expected, reduced the levels of glutathione both in primary cells and in the cell lines (Fig. 8A).

Accordingly, HS^- was only moderately toxic for the tumor cell lines HT-29 and DLD2, while pretreatment of cell cultures with BSO increased their sensitivity to HS^- (Fig. 8B). Similarly, BSO sensitized activated PBLs to HS^--induced cell death (IC_{50} activated PBL $= 0.41 \pm 0.06$ mM; IC_{50} activated PBL + BSO $= 0.23 \pm 0.04$ mM; $P < 0.05$) (Fig. 8C).

Discussion

Hydrogen sulfide is a reducing agent whose production and biological utilization has been best known for prokaryotic organisms (Lloyd, 2006). Nevertheless, it is now clear that H_2S is also endogenously generated in several human tissues—the nervous system and the cardiovascular system, as well as the liver and ileum, for example (Kamoun, 2004)—where its levels are carefully regulated for different physiological roles. However, hydrogen sulfide is a lipophilic compound, and therefore it is well permeable to plasma membranes. It can therefore be exogenously administered to cells, frequently as a component of more complex sulfurous compounds, such as sulfurous thermal waters. The presence of millimolar plasma concentrations of sulfur ions has been reported in mice (Buzaleh et al., 1990) and humans (Westley and Westley, 1991). We have recently shown, indeed, that

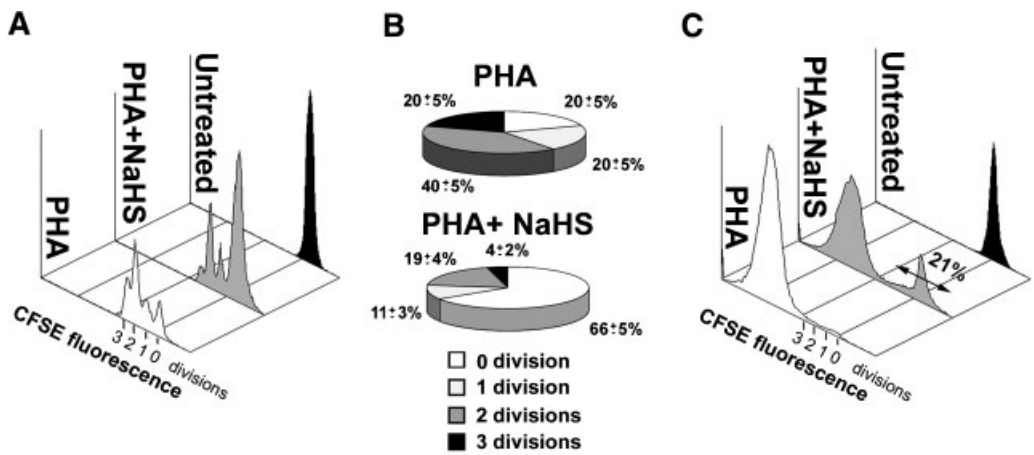

Fig. 5. HS^- impairs lymphocyte proliferation. PBLs were stained with CFSE and then stimulated with PHA + IL-2, in the presence (PHA + NaHS) or absence (PHA) 2.0 mM of NaHS. Fluorescence of CFSE-labeled resting (untreated) and activated lymphocytes was detected by flow cytometry and reported in a logarithmic scale (CFSE fluorescence). The number of cell divisions (0, 1, 2, 3) are also indicated. Cultures were analyzed after 3 days (Parts A and B) and 6 days of culture (Part C). One typical experiment is shown in parts (A and C). The percentages of lymphocytes that had performed 0, 1, 2 or 3 duplications at day 3 of culture, are reported in Part (B) as the means ± SD of three independent experiments are reported.

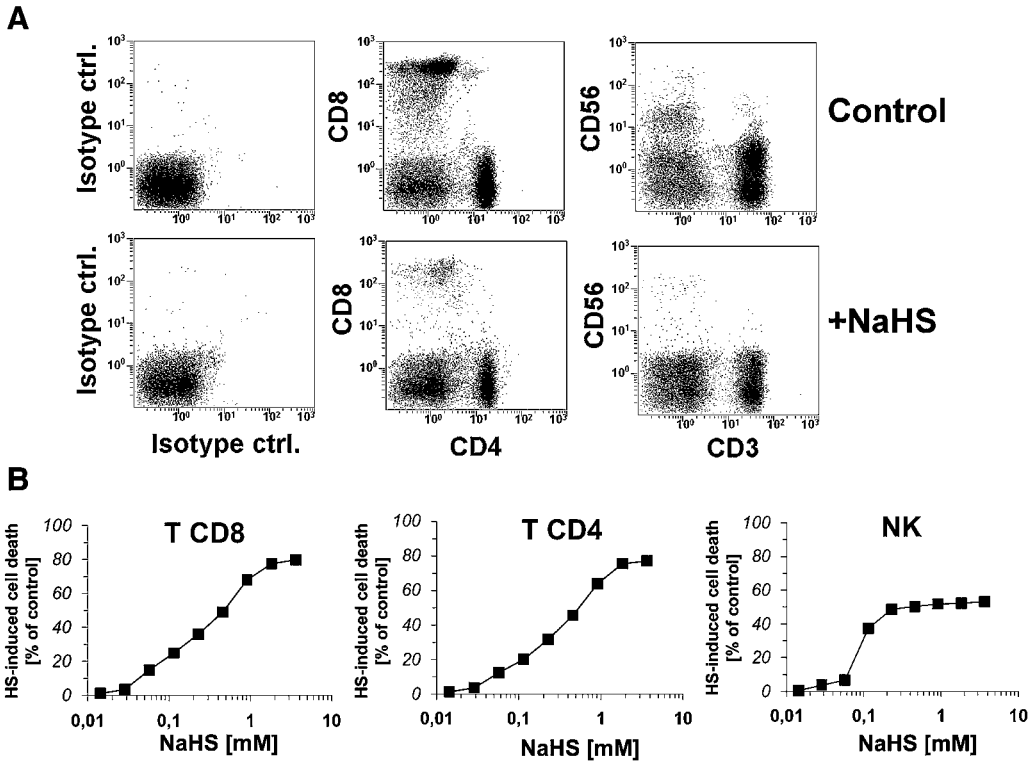

Fig. 6. The cytotoxic effects of HS^- are lymphocyte subset-specific. Flow cytometric analysis of PBL subsets treated with (+NaHS) or without (Control) NaHS. Cell were cultured in serum free medium with or without 2.0 mM of NaHS for 24 h. Part A: Flow cytometric analysis of T $CD4^+$, T $CD8^+$ and NK lymphocyte subsets. PBL subsets were stained with anti-CD8, anti-CD4, anti-CD3 and anti-CD56 mAbs. Reported dot plots are generated gating on living lymphocytes in the scatter (FSC vs. SSC) dot plot (not shown). Results shown are representative of five independent experiments; at least 10,000 cells/sample were analyzed. Part B: Lymphocyte subset cell viability upon 24 h treatment with different concentrations of NaHS, as evaluated by Annexin-V/PI staining and flow cytometry. Means are calculated as 100 × (living untreated cells − living HS-treated cells)/(living untreated cells). The means of three independent experiments are reported; SE values ranged from 5 to 6%.

exogenous H_2S can delay the onset of apoptosis of granulocytes in vitro, initially contributing to acute inflammatory and bactericidal defence and subsequently preventing the development of chronic inflammatory processes (Rinaldi et al., 2006). Here we demonstrate that—differently from what observed on granulocytes—H_2S exerts a subset-specific toxicity on peripheral blood lymphocytes. The generally accepted mechanism for H_2S cellular toxicity is the inhibition of oxidative enzymes by sulfide (Reiffenstein et al., 1992). Accordingly, we show that lymphocytes die by a caspase-independent mechanism of necrosis after a rapid loss of mitochondrial membrane potential, and not by apoptosis.

In terms of cell survival, $CD8^+$ T lymphocytes and NK cells are far more sensitive to the toxic effects of sulfide than $CD4^+$ T cells. The surviving cells—however—show a decreased proliferative capacity in response to PHA and IL-2. NK cells show a decreased spontaneous cytotoxicity against tumor target cells and both CD8 and CD4 T cells produce less IL-2 during activation. These observations may be relevant to understand some previously unexplained biological effects

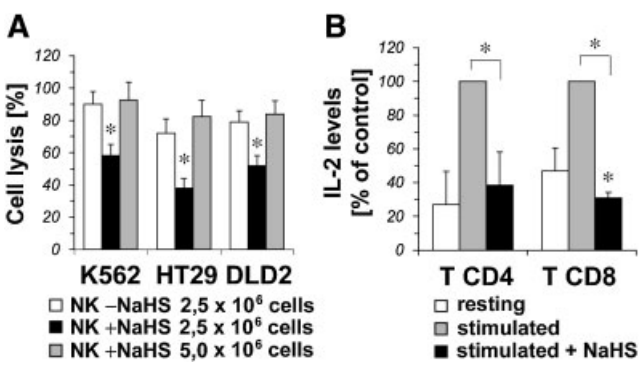

Fig. 7. Functional impairment of natural cytotoxicity and IL-2 production. Purified $CD8^+$, $CD4^+$ T cells and NK cells were treated for 24 h with or without NaHS (2.0 mM). Part A: NK-mediated cytotoxicity against HT-29, DLD2 and K562 target cell lines. Values are normalized to control target cells in the absence of effector cells. White bars, T/E = 1:1 (2.5×10^6 NK:2.5×10^6 targets); gray bars, T/E = 1:1 (2.5×10^6 NK:2.5×10^6 targets); black bars, T/E = 1:2 (5×10^6 NK:2.5×10^6 targets). T/E = target/effector ratio. Values are expressed as means ± SE of three independent experiments; $^*P < 0.05$ as compared with controls (white bars). Part B: IL-2 production by activated $CD4^+$ and $CD8^+$ purified T cells measured by ELISA. Cells were stimulated with PHA in RPMI + 10% FBS medium for 48 h (gray and black bars). The amount of IL-2 secreted by activated cells, treated with (black bars) or without (gray bars) 2.0 mM NaHS, and by resting cells (empty bars) is reported as percentage of maximal IL-2 release by fully activated lymphocytes. Values are means ± SE of three independent experiments; $^*P < 0.05$.

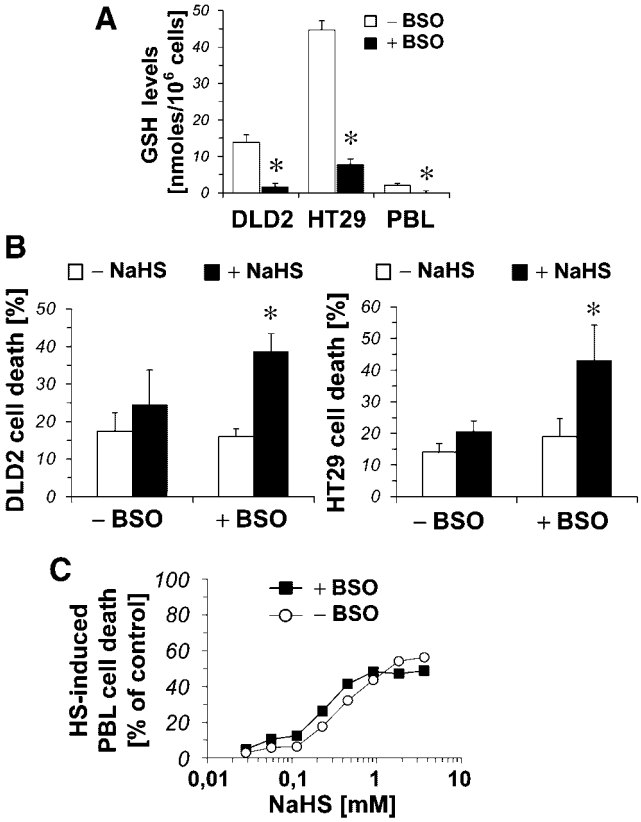

Fig. 8. Glutathione protects cells from HS^- toxicity. Cell lines and PBLs were treated with (+BSO) or without (−BSO) the glutathione synthesis inhibitor BSO (200 μM) and with NaHS. Cell viability was evaluated by Annexin-V/PI staining and flow cytometry. Means are calculated as 100 × (living untreated cells − living HS-treated cells)/(living untreated cells). The means of three independent experiments are reported. Part A: Quantification of total glutathione content (GSH) in DLD2, HT-29 cell lines and in PHA + IL-2 activated PBLs. Cells were treated with (filled bars) or without (empty bars) 200 μM BSO. Twenty-four hours later GSH content was quantified as described in Materials and Methods. Means ± SE of three independent experiments; *$P < 0.05$ compared with the control (−BSO). Part B: DLD2 and HT-29 sensitivity to 2.0 mM of NaHS in the presence or absence of BSO. Cells were treated for 24 h with 2.0 mM NaHS and cell death evaluated by PI staining and flow cytometry. Means ± SE of three independent experiments; *$P < 0.05$ compared with the control (BSO and HS untreated cells). Part C: Effect of GSH on the sensitivity of activated PBLs to NaHS. Activated PBLs were treated for 24 h with the reported concentrations of NaHS. The means of three independent experiments are shown.

exerted by sulfur therapy. For example, sulfur is able to penetrate the skin, and a sulfur-rich balneotherapy is known to be effective in the treatment of psoriasis (Matz et al., 2003). Indeed, psoriasis is now considered a genetically programmed, immune-mediated, skin-specific inflammatory disease, in which intralesional T lymphocytes trigger keratinocytes to proliferate and perpetuate the disease process (Gaspari, 2006). Specifically, epidermal T cells are chiefly $CD8^+$ T cells (Rottman et al., 2001), whereas dermal T lymphocytes are a mixture of $CD4^+$ and $CD8^+$ cells, with a $CD4^+$ predominance (De Panfilis et al., 1989; Ferenczi et al., 2000). In addition, the psoriatic plaque is characterized by the presence of Th1-type cytokines, including IL-2 (Austin et al., 1999). As a matter of fact, sulfur balneotherapy is known to have indication in the management of psoriasis (Matz et al., 2003), whilst both highly concentrated salt water and tap water have no effect (Gambichler et al., 2001).

The sensitivity of lymphocytes to the toxic effect of sulfur ions can be—as expected in the case of a reducing agent, like HS^-—modulated by intracellular gluthatione levels as H_2S can be scavenged by metallo—or disulfide—containing molecules such as oxidized glutathione.

The HS^--induced decrease of lymphocyte proliferation is opposite to what Deplancke and Gaskins (2003) described in a different cellular model of non-transformed rat intestinal epithelial cells, but parallels what Valitutti et al. (1990) on a bulk PBL population treated with sulfurous thermal waters.

Overall, our data demonstrate that HS^- reduces the cellular cytotoxic response of peripheral blood lymphocytes as well as their production of IL-2, therefore de-activating the major players of local inflammatory responses, adding new basic knowledge to the clinically well known anti-inflammatory effects of sulfur compounds.

Acknowledgments

We are grateful to Instrumentation Laboratory, Italy, to Vincenzo Palermo, Domenico Manfredi and Davide Dallatana and to Terme di Sirmione for technical support.

Literature Cited

Allen S, Shea JM, Felmet T, Gadra J, Dehn PF. 2000. A kinetic microassay for glutathione in cells plated on 96-well microtiter plates. Methods Cell Sci 22:305–312.
Austin LM, Ozawa M, Kikuchi T, Walters IB, Krueger JG. 1999. The majority of epidermal T cells in psoriasis vulgaris lesions can produce type 1 cytokines, interferon-gamma, interleukin-2, and tumor necrosis factor-alpha, defining TC1 (cytotoxic T lymphocyte) and TH1 effector populations: A type 1 differentiation bias is also measured in circulating blood T cells in psoriatic patients. J Invest Dermatol 113:752–759.
Beier J, Beeh KM, Kornmann O, Buhl R. 2004. Induced sputum methodology: Validity and reproducibility of total glutathione measurement in supernatant of healthy and asthmatic individuals. J Lab Clin Med 144:38–44.
Buzaleh AM, Vazquez ES, del Carmen Batlle AM. 1990. Cyanide intoxication. III. On the analogous and different effects provoked by non-lethal and lethal challenged doses. Gen Pharmacol 21:27–32.
Chakravarthi S, Jessop CE, Bulleid NJ. 2006. The role of glutathione in disulfide bond formation and endoplasmic-reticulum-generated oxidative stress. EMBO Rep 7: 271–275.
Cheng Y, Ndisang JF, Tang G, Cao K, Wang R. 2004. Hydrogen sulfide-induced relaxation of resistance mesenteric artery beds of rats. Am J Physiol Heart Circ Physiol 287:H2316–H2323.
De Panfilis G, Manara GC, Ferrari C, Torresani C, Zucchi A, Devoto RM. 1989. Further characterization of the "incipient lesion of chronic stationary type psoriasis vulgaris in exacerbation". Acta Dermatovenereol (Stockh) 146:26–30.
Deplancke B, Gaskins HR. 2003. Hydrogen sulfide induces serum-independent cell cycle entry in nontransformed rat intestinal epithelial cells. FASEB J 17:1310–1312.
Eto K, Ogasawara M, Umemura K, Nagai Y, Kimura H. 2002. Hydrogen sulfide is produced in response to neuronal excitation. J Neurosci 22:3386–3391.
Ferenczi K, Burack L, Pope M, Krueger JG, Austin LM. 2000. CD69, HLA-DR and the IL-2R identify persistently activated T cells in psoriasis vulgaris lesional skin: Blood and skin comparisons by flow cytometry. J Autoimmun 14:63–78.
Finkelstein JD, Martin JJ. 1984. Methionine metabolism in mammals. Distribution of homocysteine between competing pathways. J Biol Chem 259:9508–9513.
Gambichler T, Rapp S, Senger E, Altmeyer P, Hoffmann K. 2001. Balneophototherapy of psoriasis: Highly concentrated salt water versus tap water—A randomized, one-blind, right/left comparative study. Photodermatol Photoimmunol Photomed 17:22–25.
Gaspari AA. 2006. Innate and adaptive immunity and the pathophysiology of psoriasis. J Am Acad Dermatol 54:S67–S80.
Kamoun P. 2004. Endogenous production of hydrogen sulfide in mammals. Amino Acids 26:243–254.
Kolumam GA, Thomas S, Thompson LJ, Sprent J, Murali-Krishna K. 2005. Type I interferons act directly on CD8 T cells to allow clonal expansion and memory formation in response to viral infection. J Exp Med 202:637–650.
Lloyd D. 2006. Hydrogen sulfide: Clandestine microbial messenger? Trends Microbiol 14:456–462.
Matz H, Orion E, Wolf R. 2003. Balneotherapy in dermatology. Dermatol Ther 16:132–140.
Mirandola P, Sponzilli I, Solenghi E, Micheloni C, Rinaldi L, Gobbi G, Vitale M. 2006. Down-regulation of human leukocyte antigen class I and II and beta 2-microglobulin expression in human herpesvirus-7-infected cells. J Infect Dis 193:917–926.
Moore PK, Bhatia M, Moochhala S. 2003. Hydrogen sulfide: From the smell of the past to the gas of the future? Trends Pharmacol Sci 24:609–611.
Navarra P, Dello Russo C, Mancuso C, Preziosi P, Grossman A. 2000. Gaseous neuromodulators in the control of neuroendocrine stress axis. Ann NY Acad Sci 917:638–646.
Reers M, Smiley ST, Mottola-Hartshorn C, Chen A, Lin M, Chen LB. 1995. Mitochondrial membrane potential monitored by JC-1 dye. Methods Enzymol 260:406–417.
Reiffenstein RJ, Hulbert WC, Roth SH. 1992. Toxicology of hydrogen sulfide. Annu Rev Pharmacol Toxicol 32:109–134.

Reynolds ES. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J Cell Biol 17:208–212.
Rinaldi L, Gobbi G, Pambianco M, Micheloni C, Mirandola P, Vitale M. 2006. Hydrogen sulfide prevents apoptosis of human PMN via inhibition of p38 and caspase 3. Lab Invest 86:391–397.
Rottman JB, Smith TL, Ganley KG, Kikuchi T, Krueger JG. 2001. Potential role of the chemokine receptors CXCR3, CCR4, and the integrin alphaEbeta7 in the pathogenesis of psoriasis vulgaris. Lab Invest 81:335–347.
Secchiero P, Mirandola P, Zella D, Celeghini C, Gonelli A, Vitale M, Capitani S, Zauli G. 2001. Human herpesvirus 7 induces the functional up-regulation of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) coupled to TRAIL-R1 down-modulation in CD4(+) T cells. Blood 98:2474–2481.
Shenker BJ, Guo TL, O I, Shapiro IM. 1999. Induction of apoptosis in human T-cells by methyl mercury: Temporal relationship between mitochondrial dysfunction and loss of reductive reserve. Toxicol Appl Pharmacol 157:23–35.
Smiley ST, Reers M, Mottola-Hartshorn C, Lin M, Chen A, Smith TW, Steele GD, Jr., Chen LB. 1991. Intracellular heterogeneity in mitochondrial membrane potentials revealed by a J-aggregate-forming lipophilic cation JC-1. Proc Natl Acad Sci USA 88:3671–3675.
Teague B, Asiedu S, Moore PK. 2002. The smooth muscle relaxant effect of hydrogen sulfide in vitro: Evidence for a physiological role to control intestinal contractility. Br J Pharmacol 137:139–145.
Valitutti S, Castellino F, Musiani P. 1990. Effect of sulfurous (thermal) water on T lymphocyte proliferative response. Ann Allergy 65:463–468.
Wang R. 2002. Two's company, three's a crowd: Can H_2S be the third endogenous gaseous transmitter? FASEB J 16:1792–1798.
Westley AM, Westley J. 1991. Biological sulfane sulfur. Anal Biochem 195:63–67.
Weston SA, Parish CR. 1990. New fluorescent dyes for lymphocyte migration studies. Analysis by flow cytometry and fluorescence microscopy. J Immunol Methods 133:87–97.
Yang G, Wu L, Wang R. 2006. Pro-apoptotic effect of endogenous H2S on human aorta smooth muscle cells. FASEB J 20:553–555.
Zamai L, Falcieri E, Zauli G, Cataldi A, Vitale M. 1993. Optimal detection of apoptosis by flow cytometry depends on cell morphology. Cytometry 14:891–897.
Zhao W, Zhang J, Lu Y, Wang R. 2001. The vasorelaxant effect of H_2S as a novel endogenous gaseous K_{ATP} channel opener. EMBO J 20:6008–6016.
Zhao W, Ndisang JF, Wang R. 2003. Modulation of endogenous production of H_2S in rat tissues. Can J Physiol Pharmacol 81:848–853.