

NF-KappaB Independent Activation of a Series of Proinflammatory Genes by Hydrogen Sulfide

KARL M. STUHLMEIER,^{*,1} JOHANN BRÖLL,^{*} AND BOYAN ILIEV[†]

**Medical University of Vienna and Ludwig Boltzmann Institute for Rheumatology and Balneology, 1100 Vienna, Austria; and [†]University of Stuttgart, Institute of Organic Chemistry, 70569 Stuttgart, Germany*

A stress response has the potential to induce greater resistance to subsequent stress damage. We tested whether hydrogen sulfide (H₂S), an important signaling molecule, also used therapeutically, and known for detrimental effects, might induce a protective stress response. Therefore, the response of fibroblast-like synoviocytes (FLS) treated with sodium hydrosulfide and mice exposed to H₂S were examined. In both cases a profound and long lasting induction of the stress-response could be detected. However, despite the sustained presence of large levels of HO-1 and HSP-70, proinflammatory effects of exposure to IL-1 β or H₂S itself were not ameliorated. On the contrary, at H₂S concentrations significantly lower than 10 ppm—the current maximal allowable concentration of H₂S in many countries—COX-2, IL-8, IL-1 α , IL-1 β and TNF α were dose dependently elevated. Importantly, in FLS, short-term exposure to H₂S resulted in the activation of all three MAPK. In addition, mitochondrial activity was also significantly impaired at relatively low H₂S concentrations. The transcription factor NF- κ B is essential for the activation of most proinflammatory genes. However, the data presented imply that H₂S activates proinflammatory genes in FLS through non-NF- κ B-dependent pathways. Stress proteins reportedly act by blocking NF- κ B activation, a mechanism that would explain the inability of stress proteins to prevent H₂S mediated inflammatory processes. The presented data, showing MAPK activation, NF- κ B-independent activation of a number of proinflammatory genes and mitochondrial damage, help to provide a better understanding of the biological and pathophysiological effects of exposure to H₂S. *Exp Biol Med* 234:1327–1338, 2009

Key words: balneology; arthritis; sulfur; stress response; inflammation

Introduction

Exposing cells, tissue or whole organisms to sub-lethal stress has been shown numerous times to induce a response that in many cases results in protection from a subsequent challenge by the same or other stressors (1, 2). Short-term exposure to elevated temperature is the most commonly employed form of stress used to study the mechanisms and consequences of this type of cellular response. Fever, infections, radiation, carbon monoxide, arsenite and many other chemicals are also frequently cited as being able to elicit a profound stress response (2). The induction of such a protective response has been associated with many desirable effects, e.g. ameliorating rejection episodes in transplanted organs, protecting heart cells from ischemia-induced injury to preventing the activation of the transcription factor NF- κ B (3–11).

Various types of balneotherapy (spa therapy) for patients with arthritis and other ailments are among the oldest forms of treatment and are still practiced today in many parts of the world. One type of balneotherapy, namely sulfur-bath therapy, utilizes mineral water with high sulfur content from geothermal wells or springs. Reports about the potential beneficial effects of such spas abound and date back centuries (12–14). However, scientific reports, especially sound studies as to the effectiveness of this kind of treatment, are rare (15–17).

There have been few clinical studies investigating the effectiveness of balneological therapies and even fewer studies that explore possible mechanisms of balneotherapy at the cellular and/or molecular level. It is for this reason that we began to investigate the effects of a sulfur spa treatment, utilizing *in vitro* as well as *in vivo* study models. Our working hypothesis was based on the assumption that H₂S, or other bio-active sulfur compounds present in such spas, might induce a stress response and that it is the

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¹ To whom correspondence should be addressed at Medical University of Vienna and Ludwig Boltzmann Institute for Rheumatology and Balneology, Kurbadstrasse 10, 1100 Vienna, Austria. E-mail: karl-manfred.stuhlmeier@meduniwien.ac.at

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activation of the stress response genes that will ameliorate inflammation in patients undergoing such treatment.

In the past, possible negative health effects of H₂S have been at the center of attention (18–26). Recently, however, the focus has shifted to biological functions of endogenous H₂S, the most recently discovered gaseous signaling molecule (27, 28). Interest in H₂S has been further sparked by the demonstration of μ M concentrations (up to 160 μ M H₂S in brain tissue) of this gas in healthy individuals (29). Subsequently, a number of articles discussing an array of physiological functions of H₂S have been published (29–31). The demonstration that low to moderately elevated levels of H₂S seemingly play a role in intra- and extra cellular signaling further encouraged us to investigate effects of exogenous H₂S. Such effects might typically be expected from exposure to H₂S during sulfur bath therapy sessions.

Amid the rather large group of stress proteins, heat shock protein (HSP)-70 and heme oxygenase (HO-1) (also called HSP-32) are among the best studied. These proteins are clearly linked to a number of desirable, protective effects (8, 11). These genes are readily induced by an array of stresses and have been demonstrated to account for many of the reported beneficial effects of the stress response (32). Both HSP-70 and HO-1 have repeatedly been shown to prevent the activation of NF- κ B, a transcription factor that has been demonstrated to be essential for the activation of most proinflammatory genes (33–35).

H₂S at higher concentrations is clearly toxic (36). However, whether and to what degree the concentration of sulfur employed in a typical spa treatment is sufficient to induce a protective stress response without harming organisms/cells has, to our knowledge, never been studied.

Materials and Methods

Reagents. If not stated otherwise, reagents were from Sigma (Sigma, Vienna, Austria). The MAPK inhibitors SB-203580, PD-98059, and the JNK inhibitor II SP600125 were from Calbiochem (Merck, Darmstadt, Germany). Interleukin-1 β (IL-1 β) was purchased from Strathmann (Strathmann Biotec, Hamburg, Germany). Antibodies for p38, ERK, JNK, I κ B α and p-I κ B used in Western blots were from Cell Signaling (New England Biolabs, Frankfurt am Main, Germany). Antibodies used in EMSA supershift experiments, anti-HSP-70 and anti-HO-1, were from Santa Cruz Biotechnology (Santa Cruz, California, USA). The anti-tubulin antibody was from Neo Marker (Fremont, California). Oligonucleotides resembling consensus sequences for NF- κ B, AP-1, etc., were from Promega (Promega, Mannheim, Germany). The antibody recognizing COX-2 was from Cayman Chemical (Cayman Chemical, Ann Arbor, Michigan, USA). The cell proliferation and cytotoxicity assay EZ4U was from Biomedica (Biomedica, Vienna, Austria).

Cell Culture and Animal Model. Commercially

available human fibroblast-like synoviocytes (FLS) isolated from rheumatoid arthritis patients were used in *in vitro* experiments. Origin of cells, number of donors, passage numbers, culture conditions, etc., of FLS have been provided in detail elsewhere (37).

Dissolving NaHS results in the release of H₂S—a method that has been frequently used to study effects of H₂S in diverse projects (30, 36). This method was used in *in vitro* experiments that were set up to mimic the conditions of a spa sulfur bath treatment. At 25°C, a solution of 5 mM NaHS (3 ml per 3 cm tissue culture dish), will reach an equilibrium containing approximately 2.3 ppm gaseous H₂S (calculation made by Dr. B. Iliev, Stuttgart, Germany) (38). Treatment with NaHS was done the following way: For each experiment, a solution of NaHS was prepared in PBS immediately before the experiment. This stock solution was quickly further diluted to the desired working solution in complete medium (DMEM, 10% fetal bovine serum, glutamine, penicillin-streptomycin). If not indicated otherwise, FLS were routinely treated with the above NaHS solutions for 45 minutes, after that, the H₂S containing medium was removed and replaced with fresh, pre-warmed medium. At the concentrations used, H₂S did not interfere with any of the assay systems used; nonetheless, where possible, H₂S containing medium was routinely removed before adding additional reagents.

With regard to *in vivo* experiments, mice were randomly chosen (non-sex-, non-age-matched) and exposed either to ambient air (controls) or to the H₂S-containing atmosphere (~5 ppm H₂S) of a sulfur spa (Oberlaa, Vienna, Austria) by placing animals in containers close to a basin normally used for sulfur spa treatment. The ethics committee of the institute approved the *in vivo* experiments. Parts of tissue (lung, liver, heart) were placed in RNeasy lysis and Western blot sample buffer, minced immediately with a tissue grinder (Ultra-Turrax T8, IKA-Werke, Staufen, Germany) and subsequently stored at –20° C.

Western Blot Experiments. SDS-PAGE and Western blotting were carried out essentially as described (37). In short, for *in vitro* experiments, FLS were washed twice in ice-cold PBS and subsequently dissolved in SDS sample buffer without bromophenol blue. Proteins were quantitated, separated, blotted and stained with indicated antibodies. Proteins were made visible using RenaissancePlus (PerkinElmer Life Science, Boston, USA) and Kodak BioMax MR films or the chemiluminescence detection device GeneGnome (Syngene, Cambridge, UK). In Western blot experiments, lower concentrations of proteins were loaded on separate gels that served as controls for loading and protein transfer (LC). Such blots were stained with an antibody recognizing tubulin, and/or were stained with Ponceau red.

Cytokine Measurements. Quantikine ELISA kits from R&D Systems (Wiesbaden, Germany) were used to monitor release of TNF- α , IL-8 and IL-1 β by FLS exposed to H₂S. FLS, grown to high density, were cultured in

DMEM with or without H₂S for up to 32 hours. Medium (1 to 3 ml per 3 cm tissue culture dish) was removed, centrifuged, aliquoted and stored at -20°C for subsequent analyses. ELISA was carried out as described in the instruction booklet provided by the supplier. The detection limits of the ELISAs used are as follows: IL-1 β , 0.125 pg/ml; TNF- α , 0.5 pg/ml; and IL-8, 31.8 pg/ml.

Real Time RT-PCR, Data Analysis and Quality

Controls. Gene expression in FLS was measured by real-time RT-PCR on a Mx3000P (Stratagene, Amsterdam, The Netherlands), using SYBR green as reporter fluorophore for quantitating mRNA levels (37). To normalize the amount of total RNA present in each reaction, mRNA levels of HPRT and/or actin were used. Results are expressed as relative threshold cycle (ΔC_t -values) (C_t values of mRNA levels in stimulated, minus C_t values of a given gene in resting cells). Standard curves were generated for each gene using serial dilutions of RNA isolated from stimulated or unstimulated FLS as controls for amplification efficiency. Basal mRNA expression levels in unstimulated FLS were chosen to represent 1X expression of a given gene. Primer sequences, amplification curves and equations for calculations have been reported elsewhere (39). Primer sequences not previously published are as follows (orientation 5' to 3'): TNF- α (forward) GCT TTC CGA ATT CAC TGG AG, TNF- α (reverse) GCA CCT CAG GGA AGA GTC TG, IL-1 β (forward) GAG CCC ATC CTC TGT GAC TC, IL-1 β (reverse) TCC ATT GAG GTG GAG AGC TT, MIP-2 (forward) CAG ACT CCA GCC ACA CTT CA, MIP-2 (Cxcl2) (reverse) GGG TCT TCA GGC ATT GAC AG, IL-6 (forward) CAC AAG TCC GGA GAG GAG AC, IL-6 (reverse) CAG AAT TGC CAT TGC ACA AC, COX-1 (forward) TGC CCT CTG TAC CCA AAG AC, COX-1 (reverse) TGT GCA AAG AAG GCA AAC AG, COX-2 (forward) GTG GAA AAA CCT CGT CCA GA, COX-2 (reverse) GCT CGG CTT CCA GTA TTG AG, HSP-27 (forward) TCT GTC GCA CCT ATG TCC TG, HSP-27 (reverse) GCA GGA AGC AGG GAG ATG TA, HSP-70 (forward) AAC GTG CTC ATC TTC GAC CT, HSP-70 (reverse) CCT CTT GAA CTC CTC CAC GA, HO-1 (forward) GCT ACC TGG GTG ACC TCT CA, HO-1 (reverse) CCA GAG TGT TCA TTC GAG CA.

Assessing H₂S Effects on Mitochondrial Activity. The ability of cells to convert tetrazolium salt to formazan derivatives is frequently used to monitor cell proliferation and cytotoxicity. Conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) requires and assesses mitochondrial activity. FLS were treated with NaHS for 45 minutes after which cells were washed with pre-warmed medium. After that, FLS were incubated for indicated times in a fresh solution of medium containing MTT. Formazan formation was monitored at the indicated time points. With the exception that cells were grown in 3 cm tissue culture dishes, the MTT assay was carried out as described by the supplier of the assay (EZ4U Biomedica, Vienna, Austria).

Electrophoretic Mobility Shift Assay (EMSA). Preparation of nuclear extract, execution of EMSA as well as EMSA specificity control experiments have been described in detail elsewhere (37, 40). In short, the double-stranded oligonucleotides used in all experiments were end-labeled using T4 polynucleotide kinase and γ [³²P]ATP. After labeling and purification by chromatography, 5 μ g of nuclear extract was incubated with approximately 100,000 cpm of labeled probe in the presence of 1.5 μ g poly (dI-dC). The resulting mixture was separated on a native 6% polyacrylamide gel. For specific competition, 7 pmol of unlabeled NF- κ B oligonucleotides was included. For non-specific competition, 7 pmol of the double-stranded nucleotides was used. For supershift assays, 1 μ l of specific supershift antibodies (Santa Cruz, CA) was added to the nuclear extract 15 minutes prior to the addition of the labeled probe.

Quality Controls and Statistical Analysis. Basal mRNA expression levels in unstimulated FLS or control animals were chosen to represent 1X expression of a given gene. Time-course experiments (ELISA, I κ B α degradation and EMSA) were done twice; all other experiments were done at least three times. At selected time points, EMSA as well as ELISA time-course data were confirmed by additional experiments. Control experiments for EMSA such as supershift and competition experiments were performed as published (39). A number of quality controls, routinely performed to ensure RT-PCR specificity and linearity, have been reported elsewhere (37, 39, 41). Statistical analysis was done using the unpaired *t* test; *P* values $\leq$ 0.05 are considered significant.

Results

H₂S Induced a Profound Stress Response in FLS. FLS were treated with freshly prepared NaHS solution (0.05 to 5 mM). The H₂S-containing medium was exchanged for regular medium after 45 minutes. Cells were harvested 2, 4, 6, 8, 14 and 24 hours after the initial addition of NaHS. Shown in Figure 1A are data from three independent experiments, in which cells were left untreated (label "0") or were exposed to 2 mM NaHS. Such real time RT-PCR data demonstrate significantly higher levels of HO-1 and HSP-70 mRNA for up to 14 hours in cells exposed to H₂S. Only when measured 24 hours later, the mRNA levels for these stress proteins showed no significant increase.

Whether the elevated steady state mRNA levels translate into higher levels of stress proteins, was assessed by Western blot experiments. Shown in Figure 1B are representative data demonstrating that both HO-1 and HSP-70 were elevated in a dose dependent manner. FLS were treated with the indicated concentrations of NaHS. After 45 minutes NaHS medium was replaced with fresh medium. In this experiment FLS were harvested 12 hours later. As shown here, compared to untreated cells (lane, MED) levels of HSP-70 protein were higher in cells treated

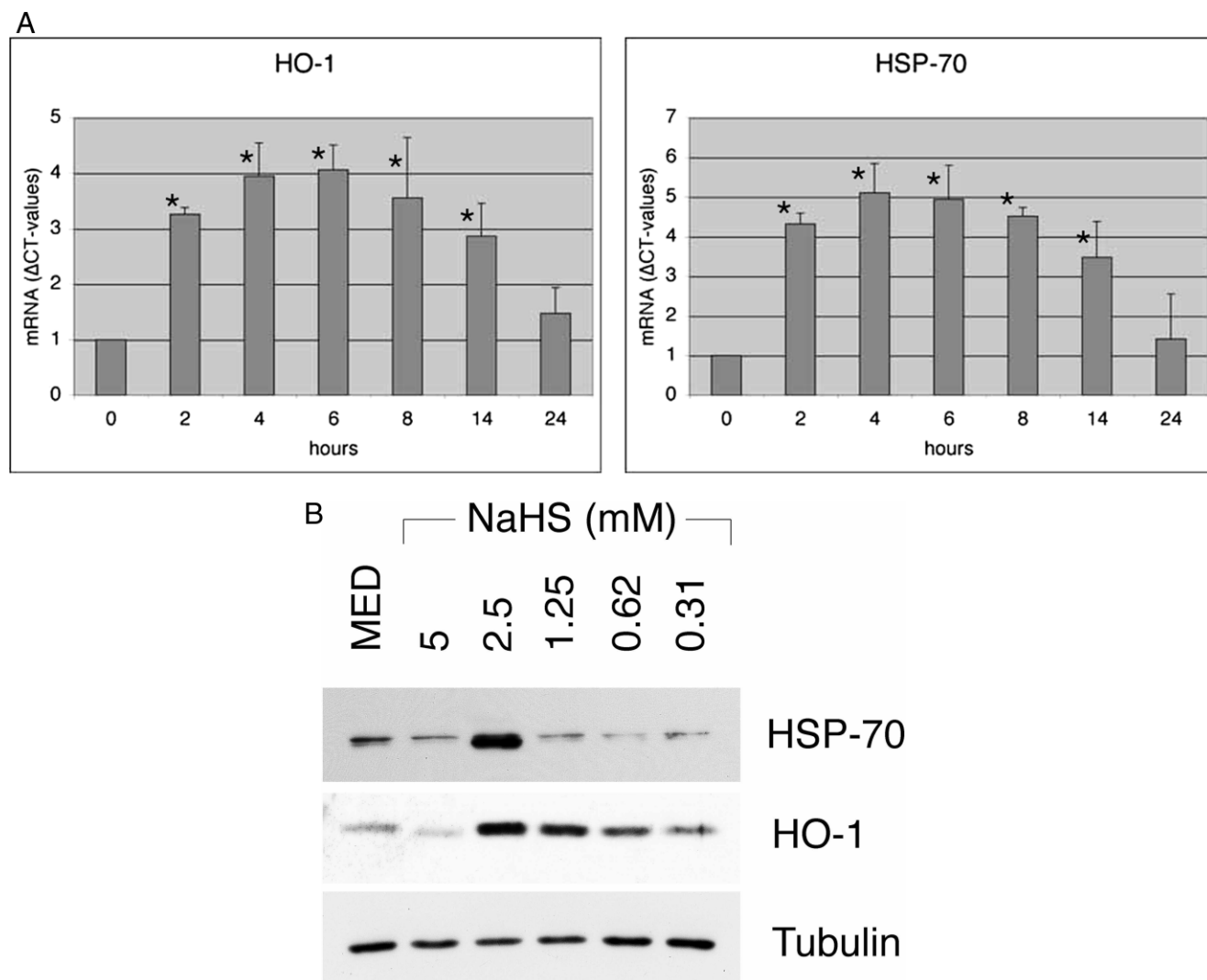


Figure 1. Short-term exposure of FLS to H_2S induces a profound stress response. As shown in the RT-PCR data in Figure 1A, after a 45 minute exposure to NaHS (2 mM), mRNA levels of HO-1 and HSP70 are significantly elevated for up to 14 hours ($n=3$, $P \leq 0.05$). Shown in Figure 1B are representative Western blot data demonstrating both increased HSP70 and HO-1 protein levels in FLS exposed to H_2S . Data in Figure 1B were generated from cells harvested 12 hours after the initial addition of NaHS. Asterisks indicate steady state mRNA levels that are significantly higher ($n \geq 3$, $P \leq 0.05$).

with 2.5 mM NaHS. Similarly, maximal levels of HO-1 protein were detectable in cells that were exposed to 2.5 mM NaHS. Also of interest, increasing NaHS to 5 mM did not result in higher levels of these two stress proteins, while less than 2 mM of NaHS did not result in elevated levels of HSP-70 protein, HO-1 protein levels were increased in FLS exposed to 1.25 and 0.625 mM NaHS. One blot, stained with anti-tubulin, is included as a control for proper protein adjustments and protein transfer.

H_2S Treatment Does Not Affect IL-1 β Induced Activation of NF- κ B. After establishing that H_2S does induce a stress response, we tested whether HSP-70 and/or HO-1 were able to prevent IL-1 β induced activation and translocation of NF- κ B. As demonstrated in prior work from this laboratory, IL-1 β treatment of FLS results in NF- κ B activation that peaks at around 45 minutes (41). Shown in Figure 2A are EMSA data resulting from experiments in

which FLS were left untreated (lane MED), treated with IL-1 β (2.5 ng/ml) for 45 minutes (lane IL-1 β (45 min.)), or exposed to NaHS (0.625 mM–5 mM) for 4 hours prior to exposure to IL-1 β for a further 45 minutes. As expected, IL-1 β treatment results in shifted NF- κ B DNA complexes; however, up to a concentration of 2.5 mM, NaHS did not affect such interactions. Higher concentrations of NaHS not only blocked NF- κ B-DNA binding but also the binding of a non-NF- κ B protein (NS) that also interacts with the consensus NF- κ B elements in this type of assay as shown before (39, 41).

Whether the presence of NaHS/ H_2S at the time of stimulation or the duration of time given to recover and express stress proteins influence the outcome of such experiments was next examined. Shown in Figure 2B are EMSA experiments in which FLS were treated with NaHS for 45 minutes, after which cells were washed and fresh,

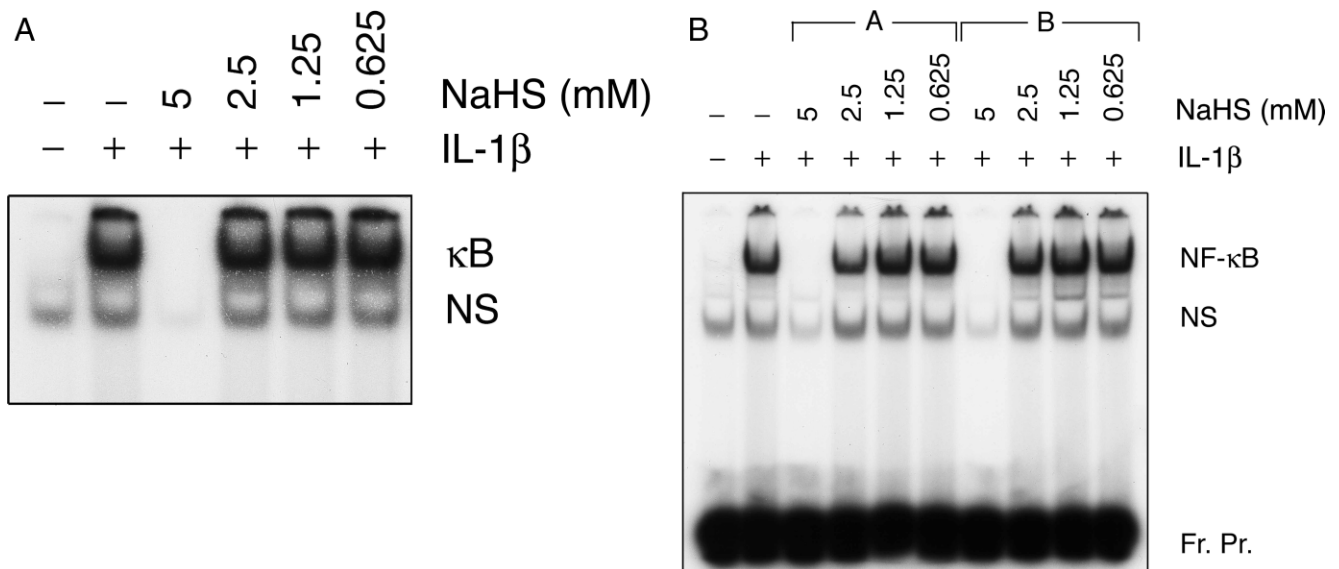


Figure 2. An H₂S induced stress response does not prevent IL-1 β induced NF- κ B-DNA interactions. Shown in Figure 2A are EMSA data in which FLS were left untreated (MED) or stimulated with IL-1 β for 45 minutes. Where indicated, NaHS was added 4 hours prior to the addition of IL-1 β . Shown in Figure 2B are data in which NaHS was removed prior to the addition of IL-1 β and cells were given a period of 1 (A) or 12 (B) hours to recover and to respond with the activation of stress proteins. The label "MED" refers to non-stimulated controls, "NS" to non-specific protein-DNA interactions and the label "Fr. Pr." indicates the position of the free probe.

pre-warmed regular medium (not containing H₂S) was added. One or 12 hours after the removal of NaHS-containing medium FLS were stimulated with IL-1 β (2.5 ng/ml) for 45 minutes. Such a procedure was opted for to (i) allow for optimal expression of the stress response genes and (ii) to prevent direct effects of NaHS/H₂S on IL-1 β . As shown in Figure 2B, no effect of NaHS/H₂S exposure could be observed at concentrations ≤ 2.5 mM NaHS. Similar to prior experiments 5 mM NaHS prevented both NF- κ B and non-NF- κ B-DNA interactions.

H₂S Treatment Resulted in Elevated mRNA Levels for a Number of Proinflammatory Genes. At high concentrations of NaHS, phase contrast microscopy revealed signs of activation in FLS. Therefore we decided to investigate the effect of H₂S on the activation of genes other than the classical stress genes. FLS were treated with 2 mM of NaHS for 45 minutes, after which medium containing H₂S was replaced with fresh, pre-warmed medium. Shown in Figure 3A are the results of 3 independent experiments in which steady state mRNA levels of a number of genes were analyzed at the indicated time points. As shown, levels of mRNA for TNF, IL-8, IL-1 α , IL-1 β and COX-2 were significantly elevated. With the exception of IL-8, mRNA levels of all the above genes were still significantly higher 24 hours after the initial exposure to NaHS. Among the genes that were not significantly affected were COX-1 and IL-6 (data not shown).

In order to gain insight into dose-effects of H₂S, mRNA levels of IL-8 and COX-2 were also monitored in cells treated with medium containing 0.125 to 2 mM of NaHS. FLS were treated as above and mRNA levels were determined 5 hours later. As shown in Figure 3B, IL-8 as

well as COX-2 mRNA levels were significantly elevated in FLS treated for 45 minutes with NaHS at concentrations as low as 0.25 mM ($n = 3$, $P \leq 0.05$).

Whether the observed increase in mRNA levels also translates into higher levels of proteins was determined in the next set of experiments. Shown in Figure 3C is one representative Western blot in which FLS were exposed to 2 mM of NaHS according to the standard protocol. Cells were harvested at time points ranging from 0 to 24 hours. As shown, COX-2 protein in non-stimulated cells is largely absent but readily detectable at each of the examined time points after exposure to NaHS.

Like COX-2, elevated IL-1 β , IL-8 and TNF- α proteins were also detected in supernatants of FLS exposed to NaHS (2 mM). As shown in Figure 3D, levels of IL-1 β in NaHS treated FLS were monitored for up to 32 hours and, at all time points measured, IL-1 β was significantly elevated. IL-8 and TNF- α , measured by ELISA at 5.5 and 14 hours after the initial treatment with NaHS, were also significantly increased ($n = 3$, $P \leq 0.05$).

Exposure to H₂S Does Not Result in Activation and Translocation of NF- κ B. Most of the above genes have been shown to depend on the activation of the transcription factor NF- κ B for their expression (35). Therefore, the question arose as to whether in FLS NaHS, instead of indirectly preventing NF- κ B activation, might actually result in the activation of this transcription factor. Shown in Figure 4A is a time-course EMSA experiment demonstrating that exposure of FLS to NaHS (2 mM for 45 minutes) does not result in significant activation of NF- κ B. While over time there is a noticeable increase in NF- κ B-DNA complexes, these complexes are not significantly activated

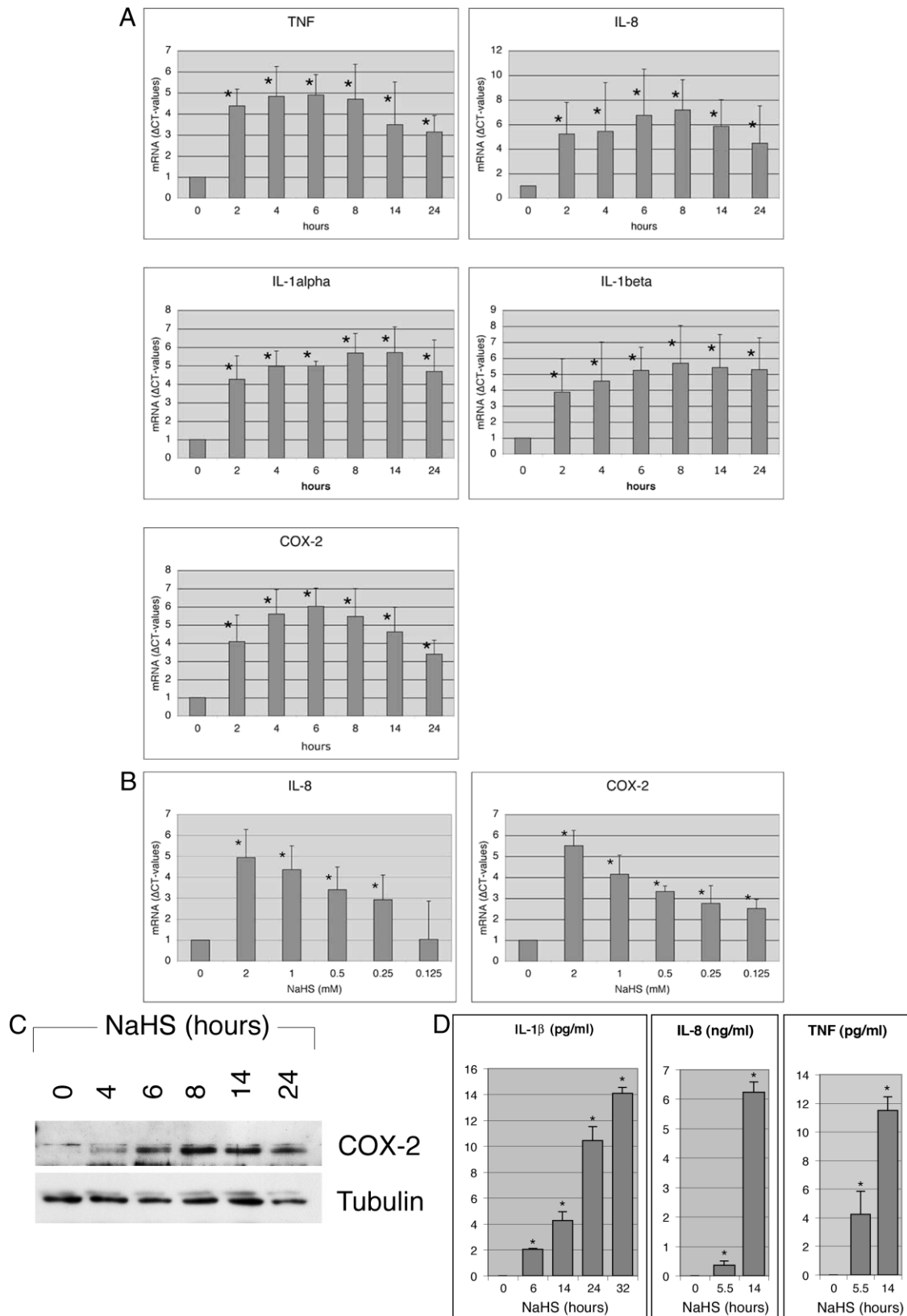


Figure 3. Short time exposure of FLS to H_2S activates proinflammatory genes. Genes relevant to inflammation were chosen to test the influence of H_2S . Figures 3A and 3B demonstrate that mRNA levels of a number of pro-inflammatory genes are elevated in a time and dose dependent manner in FLS that were exposed to a short course of NaHS (2 mM) treatment. Figure 3C demonstrates that higher levels of mRNA also translate into higher levels of COX-2 proteins, and Figure 3D demonstrates that 45 minutes of exposure to H_2S subsequently results in the release of significantly elevated levels of IL-1 β , IL-8 and TNF- α . Asterisks in Figures 3A, 3B and 3D indicate mRNA and protein levels that are, compared to controls, significantly changed ($n \geq 3$, $P \leq 0.05$).

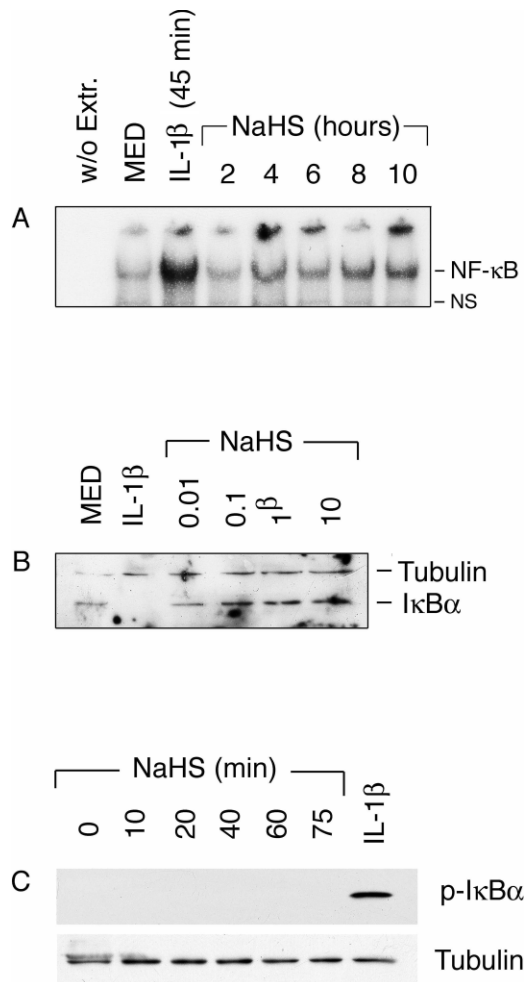


Figure 4. H₂S does not activate the NF-κB signaling pathway in FLS. IL-1β treatment results in IκBα phosphorylation (Fig. 4C), IκB degradation (Fig. 4B), in NF-κB translocation into the nucleus and in the formation of large NF-κB-DNA complexes (Fig. 4A)—all within 45 minutes. None of these events can be observed in FLS exposed to NaHS. Neither is there any sign of IκB phosphorylation, nor of IκB degradation. Slightly increased NF-κB-DNA complexes at later time points (8–10 hours) seen in Figure 4A are thought to be secondary effects e.g. release of cytokines, etc. The label “MED” indicates the lanes where proteins of unstimulated cells were separated and the label “IL-1 (45 min)” points to the positive control, a shifted complex induced by treatment of FLS with IL-1β for 45 minutes. The label “w/o Extr.” refers to the lane where protein extract was omitted from the reaction mixture.

within the first few hours when significantly increased levels of steady state mRNA for IL-1, TNF-α and COX-2 could be detected.

In further support of the notion that H₂S is able to activate the above genes without the involvement of NF-κB are data presented in Figure 4B and Figure 4C. Shown in Figure 4B is a Western blot experiment in which FLS were treated for 20 minutes either with IL-1β or with increasing concentrations (0.01 to 10 mM) of NaHS. As shown, in this blot stained simultaneously with anti-IκBα and anti-tubulin antibodies, IL-1β treatment resulted in complete degradation of IκBα, while NaHS treatment was without effect. In

subsequent experiments, possible IκBα degradation, induced by 2 mM NaHS, was monitored for up to 75 minutes. At none of the time-points examined were IκBα protein levels significantly altered (data not shown). Such data are in agreement with data presented in Figure 4C examining the phosphorylation of IκB by IL-1β and NaHS (2.5 ng/ml and 2 mM, respectively). Again, exposure to IL-1β for 10 minutes resulted in readily detectable phosphorylation of IκB; however, at none of the time points measured (10, 20, 40, 60 and 75 minutes) could we detect any effect of NaHS on the phosphorylation of IκB.

Activation of the Stress Response- and Proinflammatory Genes in Animals Exposed to H₂S. Despite the activation of “protective” genes by H₂S, the above *in vitro* experiments point at a number of potentially harmful effects as a consequence of exposure to H₂S, events that might well occur at low to moderate concentrations. To assess the extent that the above *in vitro* findings are mirrored *in vivo*, animal experiments were carried out. Groups of mice (3 each) were either kept in normal, ambient air, or were exposed to the H₂S (~5 ppm) containing atmosphere of a sulfur bath. Two groups of animals, exposed to H₂S for 1 and 6 hours, respectively, were sacrificed immediately; other animals, exposed to the H₂S atmosphere for 8 hours, were left to recover at ambient air for an additional 2 hours before being sacrificed. Shown in Figure 5 are data demonstrating that animals exposed to H₂S respond in an analogous way to the FLS. mRNA levels in lung and liver tissues of animals kept in ambient air (grey columns) were compared with mRNA levels in animals exposed to H₂S for 8 hours followed by 2 hours of recovery in ambient air (black columns). As shown in Figure 5A, H₂S induces a potent stress response as indicated by increased steady state HSP-70 and HO-1 mRNA levels in animals exposed to H₂S ($n = 3$, $P \leq 0.05$). However, similar to the *in vitro* data, steady state mRNA levels of genes associated with proinflammatory activities were also elevated. COX-2, the IL-8 equivalent MIP-2 and IL-1β are significantly elevated in lung tissue of mice exposed to H₂S ($n = 3$, $P \leq 0.05$).

The effect of H₂S exposure is not limited to lung tissue. Figure 5B shows real time RT-PCR data from liver tissue. As demonstrated, inhaling air containing H₂S results in significantly higher levels of HSP-70 and HO-1 in liver tissue. In addition, the average mRNA levels of proinflammatory genes in liver tissue of mice exposed to H₂S seem clearly higher than in mice kept in ambient air (Fig. 5B). However, such differences were not statistically significant. Among the tissues tested (lung, liver, heart), heart appeared to be the least affected as none of the genes whose mRNA levels were monitored were significantly elevated in mice exposed to a sulfur spa atmosphere (data not shown). Similarly, exposure of mice to 5 ppm H₂S for 1 hour only, was not sufficient to result in significantly elevated mRNA levels of stress proteins and/or proinflammatory genes (data not shown).

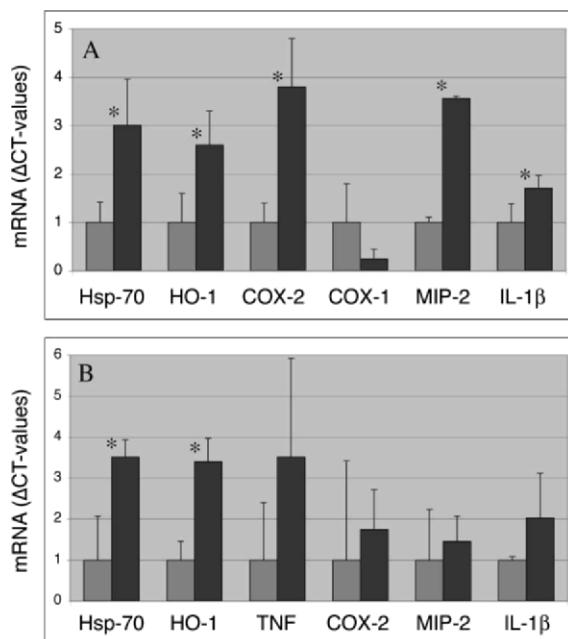


Figure 5. mRNA levels of a number of genes are higher in lung and liver tissue of mice exposed to a H₂S-containing atmosphere. Shown in Figure 5A are PCR data demonstrating that 8 hours of exposure to H₂S induces HSP70 and HO-1, but also a number of proinflammatory genes, in lung tissue. Asterisks indicate mRNA levels that are significantly higher ($n = 3$, $P \leq 0.05$). As shown in Figure 5B, liver tissue is affected as well. Steady state mRNA levels of HSP70 and HO-1 are significantly elevated. mRNA levels of the tested proinflammatory genes are also higher in animals exposed to 5 ppm H₂S; however, the observed increase in liver tissues is not statistically significant.

Exposure to H₂S Severely Affects Mitochondrial Activity in FLS. As mentioned above, cells exposed to ≥ 5 mM NaHS for an extended period of time showed clear signs of stress, resulting, among other things, in changes in cell-shape as well as effects such as the prevention of complex formation in EMSA experiments. One method well suited to assessing the potential toxicity of a given substance is to measure its impact on mitochondrial activity. Conversion of MTT to formazan was measured in cells left untreated and in FLS exposed to increasing concentrations of NaHS. FLS were exposed to NaHS for 45 minutes after which cells were washed with pre-warmed medium and incubated in fresh medium. MTT was added either immediately after replacing the H₂S-containing medium (grey columns) or 2 hours afterwards (black columns). Conversion of MTT was analyzed 1 and 6 hours after replacing the NaHS/H₂S-containing medium. The data obtained demonstrate that mitochondrial activity is not immediately impaired (Fig. 6). MTT conversion within the first hour occurs at a rate that is indistinguishable from untreated cells (Fig. 6, grey columns). However, between 2 and 6 hours effects of the short treatment with H₂S become obvious. As shown (Fig. 6, black columns), mitochondrial activity was already significantly reduced in cells that were exposed to concentrations of NaHS as low as 1.25 mM ($n = 3$,

$P = 0.05$). Adding IL-1β (5 ng/ml) or TNFα (5 ng/ml) alone had no significant effect on mitochondrial activity (Fig. 6).

Exposure of FLS to H₂S Results in the Activation of MAPK. As shown in Figure 4A, the transcription factor NF-κB does not seem to play a role in H₂S mediated cell activation. In a first attempt to identify and dissect other possible pathways utilized by H₂S, phosphorylation of the MAPK p38, JNK and ERK were investigated. As shown in the Western blot data presented in Figure 7, exposing FLS to 2 mM of NaHS results in the phosphorylation of ERK, p38 as well as JNK. In dishes that served as controls, FLS were also treated with IL-1β (5 ng/ml for 10 minutes). As a comparison of IL-1β with NaHS treated cells shows, H₂S is indeed a potent inducer of ERK and p-38, and to a lesser degree also of JNK phosphorylation.

A Multitude of Signaling Pathways Are Activated and to Different Degrees Essential for H₂S Induced Gene Activation. Using a number of specific inhibitors for MAPK, we next tried to gain insight into the relevance of MAPKs for the activation of proinflammatory genes by H₂S. FLS were left untreated or were treated with the following substances for 60 minutes: SB-203580; a p38 inhibitor, PD-98059; an ERK inhibitor, or with the JNK inhibitor II SP600125. After initial dose finding experiments, p38 and JNK inhibitors were used at a 10 μM concentration, while the ERK inhibitor was used at a final concentration of 30 μM. Subsequently, H₂S (2 mM) was added and cells were incubated for an additional hour, after which the H₂S-containing medium was replaced with fresh, pre-warmed medium. Experiments were terminated 5 hours after the initial addition of H₂S. Importantly, cells serving as negative and positive controls were mock-treated and, where appropriate, MAPK inhibitors were added again at each step of the experiments. Shown in Figure 8 are data demonstrating that genes activated by H₂S rely to varying degrees on the activation of MAPK.

As shown here, steady state mRNA levels for COX-2 were $38 \pm 16\%$ (SD) lower in cells that were treated with a p38 inhibitor, while blocking the activation of JNK was essentially without effect ($93 \pm 12\%$ (SD) of H₂S only controls). Interestingly, signaling pathways leading to IL-1α and IL-1β transcription seem to differ in that blocking p38 MAPK had little effect on mRNA levels of IL-1β ($79 \pm 9\%$ (SD) of H₂S only controls) while at the same time effects on IL-1α mRNA were much more pronounced ($42 \pm 13\%$ (SD) of H₂S only controls). These data show that activation of MAPK appears to play a role in the expression of COX-2, IL-1α and IL-1β, but not IL-8. As shown, none of the MAPK inhibitors affected H₂S induced IL-8 mRNA levels. In these experiments, mRNA levels of a given gene in untreated and H₂S treated cells represent 0% and 100%, respectively.

Discussion

The presented data are the results of experiments carried out with the working hypothesis that H₂S and/or H₂S

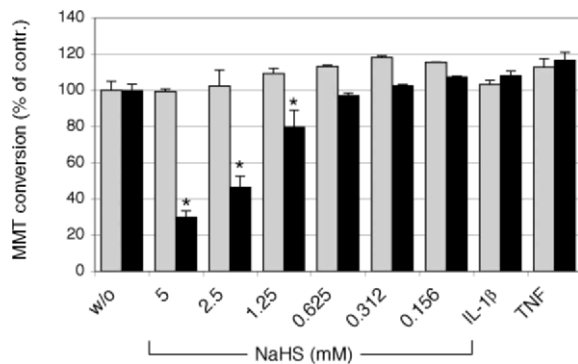


Figure 6. Effects of H₂S on mitochondrial activity. The effects of increasing concentrations of H₂S and high amounts of IL-1β and TNFα on mitochondrial activity were assessed 1 (grey columns) and 6 hours (black columns) after replacing NaHS/H₂S-containing medium. While neither TNFα nor IL-1β had any noticeable effects on mitochondrial activity, concentrations of NaHS as low as 1.25 mM for a duration of 45 minutes were sufficient to significantly impact enzyme activity in mitochondria. Asterisks indicate MTT conversion rates that are significantly lower than conversion rates in untreated cells ($n \geq 3$, $P \leq 0.05$).

dissociation products might be able to induce a protective stress response and that such a response might prevent or limit inflammation. Such an outcome would lend credence to a form of balneology, namely sulfur bath therapy, as a method to ameliorate inflammatory processes that are the hallmark of many ailments such as arthritis. While the first

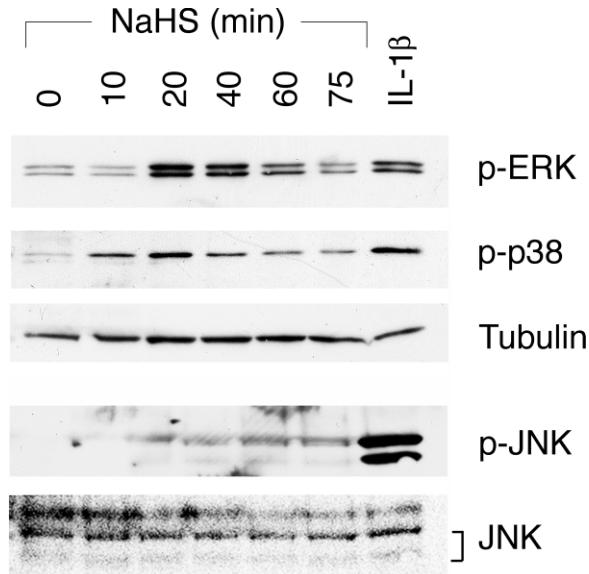


Figure 7. Phosphorylation of the MAPK ERK, p38 and JNK by H₂S. FLS were treated with NaHS (2 mM) for times ranging from 0 to 75 minutes; IL-1β (2.5 ng/ml) was included as an additional control. As shown here, FLS respond to H₂S exposure with the activation of all three MAPK in a time dependent manner. Two blots serving as loading and protein transfer controls are included as well. Data, labeled “JNK”, are the results of a blot (p-JNK) that has been stripped and re-stained with antibody specific for the non-phosphorylated form of JNK. On the control blot labeled “Tubulin” only a fraction of proteins is loaded in order to adjust for differences in the abundance of proteins and to prevent “overloading”.

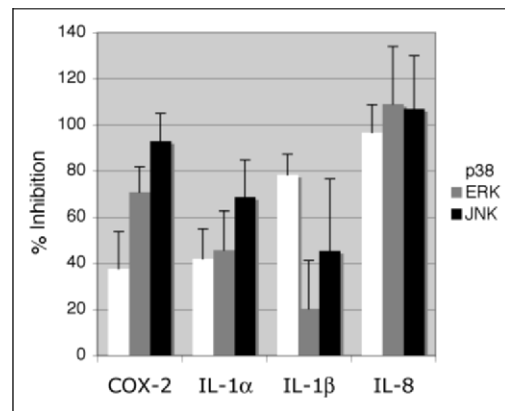


Figure 8. Differences in the necessity of MAPK activation for genes induced by H₂S. Specific MAPK inhibitors were used to probe the involvement of signaling pathways leading to the activation of a select group of genes. As shown here, H₂S activates IL-8 in FLS without the involvement of MAPK. However, for the activation of the genes COX-2, IL-1α and IL-1β, MAPK are, to varying degrees, essential. Inhibition (% of positive controls) of three independent experiments was calculated and indicated on the y-axis. The labels p38, ERK, and JNK refer to inhibitors of MAPK used.

part of our hypothesis turned out to be correct, the second part proved to be false. A potent H₂S induced stress response is able to overcome neither the cytokine-induced activation of proinflammatory genes nor the induction of these genes by H₂S itself. Activation of MAPK is generally seen as signs of cell activation. Moreover, MAPK also play a role in the induction of many proinflammatory genes (42). The demonstration that concentrations of H₂S lower than the 10 ppm maximal allowable concentration (MAC) currently implemented in many countries are able to induce transcription and translation of a series of proinflammatory genes has implications for other areas of health care and industrial activities. Such data seemingly also contradict the (scientifically unproven) conventional wisdom of sulfur bath therapy. As reflected in the ongoing discussion regarding exposure limits to H₂S, concerns regarding exposure to low levels of H₂S are not new. In light of the data presented here, it is also noteworthy that H₂S concentrations in tobacco-smoke reportedly far exceed concentrations that, in our experiments, resulted in the upregulation of proinflammatory genes (43). Aside from the significant activation of a series of proinflammatory genes, MAPK activation was also detected. Activation of MAPK is usually interpreted as a clear sign of ongoing inflammatory processes (42). The demonstration that a relatively short-term exposure to moderate levels of H₂S is sufficient to activate all three MAPK also points to detrimental effects of low concentrations of H₂S. The conclusions that lower than expected levels of H₂S might have undesired health effects are further supported by our data showing that mitochondrial activity is significantly affected at H₂S concentrations that are approximately 1/10 of the current MAC.

Sulfur bath therapy has a long tradition and supporting anecdotal evidence, going back centuries, abounds as to its

effectiveness as a remedy for a number of ailments. Patients suffering from several forms of rheumatic disorders, especially osteo- and rheumatoid arthritis (RA), are among those that are said to benefit from sulfur spa treatment. Arthritis is characterized by inflammatory processes that are driven in large part by the presence of increased levels of cytokines such as TNF, IL-1 β , IL-8, etc. Among other relevant genes is COX-2. For this reason it is of concern that this same panel of genes implicated in the progression of arthritis (TNF, COX-2, etc.) is highly activated by exposure to H₂S. Whether findings from our animal experiments can be applied to humans is currently unclear. However, by placing mice next to the basin normally used for therapy, the animals in our study were only exposed to the gaseous atmosphere of a typical sulfur spa. Since humans, in addition to being exposed to the H₂S-containing atmosphere are also immersed in sulfur water, it is reasonable to expect that they are typically exposed to even higher levels of H₂S. It is interesting that, contrary to our findings, investigations in the past reported mostly favorable effects of sulfur spa therapy. In a number of previous studies, skin and cells present in this tissue, as well as a series of other parameters, were the preferred objects of investigation. However, to our knowledge the effects of H₂S on the regulation of pro- and/or anti-inflammatory genes have never been directly investigated. Given the large surface area of lung tissue, as well as the ease of exchange of gases between atmosphere, lung tissue and the blood stream, we opted to investigate the effects of sulfur compounds on this organ rather than on skin tissue.

Next to NO and CO, hydrogen sulfide (H₂S) has recently been described as being the third physiologically relevant gaseous signaling molecule discovered (28, 29, 44). All the above gases share the characteristics of being poisonous at certain concentrations. To date, very little is known about short- and long-term effects of exposure to low and moderately elevated concentrations of H₂S. However, due to the presence of H₂S in many industries—for example in paper production, mining, oil- and gas-industry, farming, sewer- and water-treatment—H₂S has attracted the attention of regulatory bodies in the past. As a result of studies to define the health effects of H₂S it has been reported that guinea pigs but not rats die from exposure to 100 ppm of H₂S (36). Also of interest, at a concentration of 100 ppm, the mortality of canaries is 100 percent (36). In addition to such studies on mammals and birds, there are invertebrate studies that point toward toxic effects of H₂S (e.g. on the central nervous system) at concentrations that are well below those currently considered safe (45).

At present, in many countries, including Austria, the exposure limit (MAC) of humans to H₂S is set at 10 ppm (46–48). Accordingly, care is taken in spas not to exceed such levels. For example, H₂S concentrations in Vienna-Oberlaa are kept at around 5 ppm and patients as well as therapists are exposed to such concentrations either temporarily or for the duration of their working hours. It

is also noteworthy that H₂S exposure limits were initially established to protect individuals from eye damage, and that (for reasons laid out above) such criteria might not be sufficient to protect the pulmonary system from H₂S damage (36).

As shown herein, at least in mice and in *in vitro* experiments, H₂S activates MAPK and probably a series of other events as manifested in the MAPK independent activation of IL-8. H₂S also induces a strong stress response. The activation of stress proteins provides yet another indicator that concentrations considerably lower than 10 ppm can be highly bioactive. More important, however, is the question why we were unable to demonstrate any beneficial effects of the high levels of HSP-70 and/or HO-1 resulting from exposure to H₂S—especially in light of data from our laboratory demonstrating that stress genes induced by short-term hyperthermia can prevent cytokine induced activation of proinflammatory genes in FLS (37).

Next to HSP-70, HO-1 has repeatedly been shown to be beneficial and, like CO, H₂S is a very potent inducer of this gene. In the present studies a 45-minute exposure to this gas was sufficient to induce HO-1 transcription and translation for up to 24 hours. Unexpectedly, and contrary to our hypothesis, the induced activation of inflammatory processes was not altered despite the presence of the above stress proteins. The explanation for this phenomenon might, at least in part, lie in the demonstration that in FLS, H₂S induces the expression of proinflammatory genes without the involvement of the transcription factor NF- κ B. This transcription factor has been shown numerous times to play an essential role in the activation of most proinflammatory genes (35). However, Western blot, EMSA and inhibitor experiments using pyrrolidine dithiocarbamate (PDTC) illustrate that exposure to H₂S does not result in the activation of NF- κ B (the appearance of low levels of NF- κ B-DNA complexes at later time-points is thought to be due to secondary effects such as the release of H₂S induced cytokines). Since HSP-70 and HO-1 reportedly exert their effects mainly by blocking NF- κ B activation, it is not surprising that these proteins are largely ineffective in preventing H₂S induced gene activation. As shown here, higher concentrations of NaHS did appear to block NF- κ B-DNA binding. However, as indicated by dose dependent changes of the bands labeled “NS” in EMSA experiments shown in Figure 2, NaHS also affected the binding of, as of yet undefined non-NF- κ B protein(s) that also interact with the consensus NF- κ B elements in this type of assay (39). This points to toxic “non-specific” effects of NaHS at concentrations higher than 2.5 mM. This view is supported by the observation that at 5 mM NaHS the high basal AP-1-DNA complex-formation too is blocked (data not shown). The presented data, namely the absence of an effect by H₂S on phosphorylation and degradation of I κ B, as well as the demonstration that H₂S is unable to induce significant NF- κ B-DNA interactions by EMSA, supports the above interpretation and points to a rather unique mode of

activation of proinflammatory genes by H₂S. Such conclusions are further supported by the outcome of experiments using PDTC. This substance has been shown to be a potent inhibitor of the transcription factor NF- κ B; however, PDTC, like stress proteins, failed to prevent H₂S induced gene activation in FLS (data not shown). The existence of NF- κ B dependent as well as NF- κ B independent pathways leading to the activation of one and the same gene has been noted before. We demonstrated for example that HAS1 in FLS is a gene that depends on NF- κ B in cases where IL-1 β is used as a stimulus. However, the same gene is activated without the participation of NF- κ B if TGF- β is used to activate this gene (39). Likewise, the pro-inflammatory dextran sulfate sodium salt induced inflammation in mice, without the activation of NF- κ B (49). Similarly, lipopolysaccharides induce κ light chain synthesis by activating NF- κ B, whereas IFN- γ has been shown to induce surface IgM expression without the need for NF- κ B activation (50).

Taken together, despite increasing efforts by the scientific community, there is still little known about the effects of H₂S, either with regard to potential biological function(s) of H₂S at physiological concentrations, or about the possible detrimental or beneficial effects of exposure to elevated concentrations of this gaseous mediator. The presented data show that H₂S induces a potent stress response but also that H₂S activates an array of genes with proinflammatory properties. Both stress- and proinflammatory genes are activated by concentrations significantly lower than the current MAC. Furthermore, H₂S induces proinflammatory genes in FLS by an, as yet, undefined mechanism but clearly without the involvement of NF- κ B. Of further importance to individuals exposed to H₂S as well as to lawmakers is the *in vitro* observation that mitochondrial activity is severely affected at concentrations that until now have been considered safe.

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