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Original Article

Nitrosopersulfide (SSNO⁻) targets the Keap-1/Nrf2 redox system

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Abstract

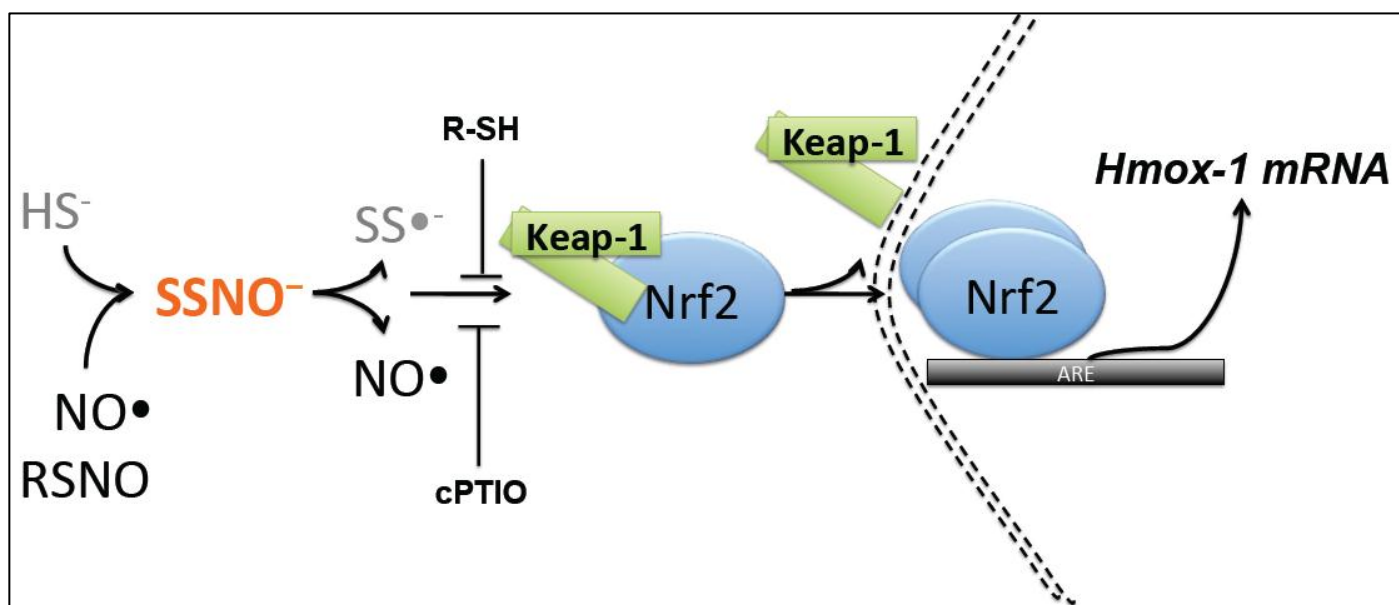
Nitric oxide (NO), hydrogen sulfide and polysulfides have been proposed to contribute to redox signaling by activating the Keap-1/Nrf2 stress response system. Nitrosopersulfide (SSNO⁻) recently emerged as a bioactive product of the chemical interaction of NO or nitrosothiols with sulfide; upon decomposition it generates polysulfides and free NO, triggering the activation of soluble guanylate cyclase, inducing blood vessel relaxation *in vitro* and lowering blood pressure *in vivo*. Whether SSNO⁻ itself interacts with the Keap-1/Nrf2 system is unknown. We therefore sought to investigate the ability of SSNO⁻ to activate Nrf2-dependent processes in human vascular endothelial cells, and to compare the pharmacological effects of SSNO⁻ with those of its precursors NO and sulfide at multiple levels of target engagement. We here demonstrate that SSNO⁻ strongly increases Nrf2 nuclear levels, Nrf2-binding activity and transactivation activity, thereby increasing mRNA expression of Hmox-1, the gene encoding for heme oxygenase 1, without adversely affecting cell viability. Under all conditions, SSNO⁻ appeared to be more potent than its parent compounds, NO and sulfide. SSNO⁻-induced Nrf2 transactivation activity was abrogated by either NO scavenging with cPTIO or inhibition of thiol sulfuration by high concentrations of cysteine, implying a role for both persulfides/polysulfides and NO in SSNO⁻ mediated Nrf2 activation. Taken together, our studies demonstrate that the Keap-1/Nrf2 redox system is a biological target of SSNO⁻, enriching the portfolio of bioactivity of this vasoactive molecule to also engage in the regulation of redox signaling processes. The latter suggests a possible role as messenger and/or mediator in cellular sensing and adaptations processes.

Keywords: NO and H₂S cross talk, nitroxyl, nitrosopersulfide, redox sensing, polysulfide, persulfide.

Abbreviations

Heme oxygenase-1 (Hmox1), Kelch-like ECH-associated protein (Keap)-1, nitrosopersulfide (SSNO⁻), nuclear factor (erythroid-derived 2)-like 2 (Nrf2), phosphodiesterase (PDE), soluble guanylate cyclase (sGC).

Graphical Abstract



Highlights

- SSNO⁻ increases nuclear levels of Nrf2
- SSNO⁻ increases Nrf2 binding activity to ARE
- SSNO⁻ increases the expression of heme oxygenase-1
- Activation of Nrf2 by SSNO⁻ is blocked by thiols (L-Cys) and NO scavenging

Introduction

Hydrogen sulfide (H_2S), a malodorous gas known to be toxic when inhaled, has recently been shown to exert a variety of interesting pharmacological actions including smooth muscle relaxant effects(1-4). It is also produced endogenously in the course of sulfur-containing amino acid metabolism by enzymes of the trans-sulfuration pathway including cystathionine- β -synthase (CBS), cystathionine- γ -lyase (CSE) and 3-mercaptopyruvate sulfotransferase (3-MST), along with cysteine aminotransferase (CAT), and presumed to serve a function as signaling molecule(5). Following nitric oxide (NO) and carbon monoxide (CO), H_2S was therefore proposed to represent the third endogenous gasotransmitter (6).

On dissolution in aqueous media of physiological pH, as a dibasic acid ($\text{pK}_{a1} \sim 7$, $\text{pK}_{a2} \sim 12$) about two thirds of the H_2S undergoes rapid dissociation to generate hydrosulfide anions (HS^-) and minor amounts of the fully deprotonated dianion, S^{2-} . Thus, a considerable part of the biological effects and pharmacological actions of both, endogenous and exogenous H_2S may be mediated by HS^- rather than the uncharged (fully protonated) solute. For simplicity we will here use the term 'sulfide', to define all solutes. Several groups independently proposed that H_2S signaling may be mediated by sulfide metabolites containing a highly reactive sulfane sulfur (7, 8) such as persulfides (9) and polysulfides (10), and that at least part of the biological signals are mediated by electrophilic sulfhydration (persulfidation) of reactive cysteines (forming a persulfide (-SSH))(9-15) rather than via interaction of H_2S with metals in e.g. heme-containing targets.

A further line of complexity is added by the notion that sulfide was shown to exert similar and, in some cases, interdependent effects with the nitric oxide (NO) signaling pathway, in particular with regard to smooth muscle relaxation(1, 4, 16-18). This 'NO/ H_2S cross-talk' has been shown to occur at different levels, from a coordinated control of proteins belonging to the same signaling cascade (e.g. soluble guanylate cyclase and phosphodiesterases) (16-18), over reciprocal biochemical control of activity of NO or sulfide producing enzymes (19, 20), to chemical interaction between the two (21-24).

Nitrosopersulfide (SSNO⁻) is an S/N hybrid molecule, which has been shown to be formed by the chemical interaction between NO-related species (including NO itself, NO donors and S-nitrosothiols) with H₂S or polysulfides (22-24). Chemical and pharmacological studies revealed that SSNO⁻ releases both NO and sulfane sulfur equivalents. At low μ M concentrations SSNO⁻ is a potent activator of the canonical NO-receptor, soluble guanylate cyclase (sGC) (23, 24), thereby increasing intracellular cGMP levels (24) and inducing vasorelaxation of pre-constricted aortic rings in vitro (25); in vivo, administration of SSNO⁻ leads to a dose-dependent decrease in mean arterial pressure (24). Considering its rather complex biological chemistry, SSNO⁻ may interact with biological targets other than sGC, and this may contribute to its overall pharmacological effects.

The transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is a key master redox switch ubiquitously controlling the expression of antioxidant and protective enzymes (26-28) carrying an antioxidant response element (ARE, 5'-TGACTnnnGC-3') in its promoter (26). The activity and stability of Nrf2, as well as the sensitivity for its inducers is mainly regulated by the ubiquitin ligase adaptor Kelch-like ECH-associated protein-(Keap)-1 (29). It was shown that, due to the presence of highly conserved redox-sensitive cysteines, Keap-1 is able to sense different chemical classes of inducers including NO, electrophiles, heavy metals and alkylating agents (30-32). These redox responsive domains include the highly-reactive Cys-151, which is responsible for sensing of NO and nitrosative species (30, 31), as well as exogenous xenobiotics like the canonical Nrf2 inducer tert-butylhydroquinone (tBHQ)(30); other cysteines include Cys-288, which is the alkenal sensor, as well as Cys-226 and Cys-613, which - together with histidine residues - form the Zn²⁺ and transition metal sensing elements (30). Formation of a long-range disulfide bridge between Cys-226 and Cys-613 was shown to be responsible for inactivation of Keap-1 by H₂O₂ and thereby activation of Nrf2-dependent transcription (31). Specific substitution of these three cysteines with other amino acids effectively abrogates the sensing properties of the Nrf2/Keap-1 system (30, 31).

Sulfide has also been shown to activate Nrf2-dependent responses in vivo and contribute to cardioprotection against I/R injury (20, 33). The molecular mechanism of sulfide and/or polysulfide

activation of Nrf2 is unclear at present, but seems to involve direct inactivation of Keap-1 by targeting its redox sensitive cysteines. Lower concentrations of sulfide (30 μM NaHS) were described to induce persulfidation of redox-sensing cysteines including Cys-151 (34), while higher concentrations of sodium hydrosulfide (400 μM NaHS)(35) or addition of sodium tetrasulfide (Na_2S_4 ; 25 μM) (14) inhibit Keap-1 by formation of a disulfide bond between Cys-226 and Cys-613, possibly via intermediate sulfhydration (35). Taken together, these results show that the Keap-1/Nrf2 redox system is a target for sulfide/persulfide signaling.

Although SSNO^- is formally considered a “nitrosated persulfide”, it shows some peculiar characteristics: differently from classical persulfides and polysulfides it is resistant to high concentrations of thiols (including cysteine, glutathione and DTT)(21, 23, 24). SSNO^- was shown to release NO^\bullet and sulfane sulfur on decomposition (21-24, 36-38), possibly via homolysis of the S-N bond yielding NO^\bullet and the persulfide radical anion, $\text{SS}^{\bullet-}$. We therefore hypothesized that SSNO^- is a strong activator of the Keap-1/Nrf2 redox system.

The aim of the present study was to investigate whether SSNO^- activates Nrf2 in cultured vascular endothelial cells. We found that SSNO^- indeed potently activates Nrf2 binding activity and increases the expression of the Nrf2 target gene heme oxygenase (Hmox)-1 in a concentration-dependent fashion. These results identify the Nrf2-mediated signaling pathway as a target for nitrosopersulfide in cells.

Materials and Methods

Materials

Ultrapure water (Milli-Q, Millipore), S-nitroso-N-acetyl-DL-penicillamine (SNAP), p-methoxyphenyl-morpholino-phosphinodithioic acid (GY 4137), 3'-methoxy-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthen]-6'-yl 2-(pyridin-2-yl)disulfanylbenzoate (Washington State Probe-1, WSP-1) were from Cayman Chemicals (Biomol, Hamburg, Germany). Unless otherwise specified, all other chemicals were of the highest purity available and purchased from Sigma-Aldrich (Schnelldorf, Germany), cell culture plastics from Greiner (Frickenhausen, Germany), endothelial cell culture medium and supplements from Promocell (Heidelberg, Germany) and other cell culture material from PAA (Pasing, Austria).

Preparation of stock solutions

Stock solutions of diethylamine NONOate (DEA/NO), spermine NONOate (Sper/NO) and Angeli's salt (sodium oxyhyponitrite, $\text{Na}_2\text{N}_2\text{O}_3$; 50 mM in NaOH 0.01 M) were prepared fresh and used within 1-2 hours. tert-Butylhydroquinone (tBHQ, 50 mM in DMSO), SNAP (200 mM, DMSO; supersaturated), GYY4137 stocks (40 mM, DMSO), and WSP-1 (5 mM, DMSO) and cPTIO (50 mM, DMSO) were kept aliquoted at -20°C until use. Sulfide stock solutions (500 mM) were prepared fresh before each experiment by dissolving anhydrous Na_2S in Milli-Q water and further dilution in Tris buffer (100 mM pH 7.4) immediately before use, as required. Control measurements confirmed that the pH of the cell culture medium remained at pH 7.4 even at the highest final target concentration. To obtain a 1 mM stock solution of SSNO^- , the reaction mixture was prepared by adding to 795 μl of 100 mM Tris buffer pH 7.4 a volume of 5 μl of 200 μM SNAP (final concentration 1 mM) and 200 μl of 50 mM Na_2S (final concentration 10 mM). The solutions for the control treatments with SNAP (1 mM) or sulfide (10 mM) were prepared in exactly the same manner, except that sulfide or SNAP were omitted. All SSNO^- incubations were carried out for 10 min under exclusion of light. To remove excess sulfide the stock solution was gassed with N_2 for 10 min, and removal of sulfide was confirmed by UV-visible

spectrometry. (see also Fig. 1A). After gassing, the absorption band below 220 nm (corresponding to HS⁻) disappeared, while the peak at λ_{\max} 412 nm (corresponding to SSNO⁻) was not affected by gassing. As observed previously (23, 24), addition of 1 mM DTT removed polysulfide ($\lambda = 290\text{--}350$ nm) but did not affect the peak at λ_{\max} 412 nm (corresponding to SSNO⁻).

Reaction monitoring and assessment product stability by UV-visible spectroscopy

UV-visible spectroscopy was used to monitor the formation and decomposition of SSNO⁻ from SNAP and Na₂S and to confirm removal of excess sulfide employing a FLUOstar Omega microplate reader (BMG Labtech, Ortenberg, Germany). Stock solutions (100 mM) were diluted 1:100 in 1 M TRIS pH 7.4 transferred to a UV-transparent 96-well plate (200 μ l/well), and spectra were analyzed using Omega data analysis software (BMG Labtech).

Culture of vascular endothelial cells.

For all cell culture experiments c-pooled human umbilical vein endothelial cells (HUVECs; Cat No. C-12203, lot number 1110701, PromoCell) were used. HUVECs were cultured for a maximum of two passages in 100 mm diameter Petri dishes pre-coated with fibronectin (10 μ g/ml fibronectin in PBS) containing 8 ml of complete endothelial growth medium with 2% fetal bovine serum (PromoCell) prepared following the manufacturer's instruction, and splitted every 3 days by using trypsin when they reached about 90% confluence. All experiments were performed with cells of passage 2.

Assessment of cell survival by neutral red assay.

Cell viability was assessed by neutral red staining as previously described (39, 40) and expressed as the percentage of viable treated cells vs. viable controls.

Assessment of Nrf2-binding activity from nuclear extract by DNA-binding ELISA

Cells (10^6 cells/well) were cultured in 8 ml complete endothelial cell medium for 24h. On the day of the experiment cells were washed with PBS once and treated in fresh medium. Treatments were added directly to the cells at the final concentrations indicated, and cells were incubated for 1 hour at standard culture conditions. cPTIO and Cys were added to the cell supernatant 1-3 min before addition of SSNO⁻. Nuclear proteins were extracted using a Nuclear Extraction Kit (Active Motif, Carlsbad, CA) following the manufacturer's protocol. Briefly, cells were washed, and cellular membranes were lysed in 500 μ l hypotonic solution containing a detergent, nuclei collected by centrifugation and lysed at 4°C by incubation for 30 min in 50 μ l nuclear complete lysis buffer on a rocking platform at 150 rpm, followed by 30 s vortexing at highest speed, and cleared by centrifugation at 10,000 g, 4°C for 10 min. Samples were rapidly aliquoted for Nrf2-binding assay (40 μ l) and protein assay (5 μ l), frozen in liquid nitrogen and stored at -80°C. Protein concentrations of nuclear extracts were determined according to Lowry using a commercial kit (DC BioRad Protein Assay, Bio-Rad Laboratories GmbH, Munich, Germany). The Nrf2-binding activity of nuclear extracts was quantified using a commercial DNA-binding ELISA (TransAM® Nrf2, ActiveMotif, La Hulpe, Belgium) following the manufacturer's instructions. Briefly, nuclear proteins (10 μ g/well) were loaded in duplicate onto a plate coated with ARE-oligonucleotides, and incubated for 1h at room temperature; after washing, Nrf2 bound to ARE was assessed by a colorimetric ELISA analyzing the adsorption at 450 nm. Complete nuclear lysis buffer was used as blank, and nuclear extracts from activated COS-7 cells transfected with Nrf2 were used as positive controls. Percent increase in ARE binding as compared to control was calculated from blank subtracted absorbance as $\Delta\% = (\text{treatment-untreated control})/\text{control}$.

Assessment of the Nrf2 and Nrf1 localization and nuclear levels by Western blot analysis

Nuclear levels of Nrf2 and Nrf1 were determined by Western blot analysis of nuclear extracts, as previously described(41, 42), and compared to the nuclear protein Lamin A. Briefly, 15 μ g nuclear

protein extracts were loaded on a pre-cast 3–8% NuPAGE Tris-Acetate pre-cast gels under reducing conditions, transferred onto a PVDF membrane (GE Healthcare, Little Chalfont, UK), stained with Ponceau S (SERVA Electrophoresis GmbH, Heidelberg, Germany) and blocked with 5% skimmed milk in T-TBS (0.1% Tween-20 100 mM NaCl in 10 mM Tris pH 7.4) for 2h. Membranes were incubated overnight with monoclonal mouse anti-human Nrf2 (1:200; ab62352, Abcam), monoclonal mouse anti-human Nrf1 (1:1000, ab90524, Abcam), or anti-Lamin A (1:500, ab8980, Abcam) antibodies. After washing in T-TBS bands were detected by using a rabbit-antimouse-HRP conjugated antibody (1:5000 BD Bioscience, New Jersey, USA) and bands were detected by chemiluminescence (SuperSignal West Pico Chemiluminescent substrate, Thermo Fischer Scientific, Oberhausen, Germany) in an ImageQuant LAS 4000 (GE Healthcare). Bands were quantified using Image J (NIH, Bethesda, Maryland, USA).

Assessment of SSNO⁻ mediated increases in Hmox-1 expression by real time RT-PCR

Endothelial cells (2×10^5 cells/well) were grown in a 6-well plate for 48 h, treated for 24 h and then lysed with 350 μ l RLT-lysis buffer (RNeasy, Qiagen, Hilden, Germany). RNA concentration was quantified using a NanoDrop ND 2000 spectrophotometer (NanoDrop Products, Wilmington, DE, USA) and RNA quality and integrity using an Agilent Bioanalyser (Agilent Technology, Santa Clara, CA, USA). After removal of genomic DNA, the RNA (1 μ g) was reversely transcribed using a MasterCycler (Eppendorf, Hamburg, Germany) using a QuantiTect Reverse Transcription Kit (Qiagen) containing gDNAse wipe-out buffer following manufacturer's instructions. The cDNA (5 ng) or control RNA was used as a template for real-time PCR performed in triplicate, using TaqMan Universal PCR Master Mix in ABI PRISM 7900 (Applied Biosystem) and Rplo as a housekeeping gene. Primers Hmox-1 (Hs01110250_m1) were purchased from Applied Biosystem, and results analyzed as described (39, 40).

Measurement of reduced and total glutathione (GSH)

Endothelial cells (2×10^5) were cultured in 6-well plates for 24 h, washed and treated for 24 hours as described. Cells were lysed in 200 μ l of ice-cold 100 mM HCl and sonicated for 30 s at 4 °C. After centrifugation at 14,000 x g for 10 minutes (min) at 4 °C, the supernatant was mixed with 5% sulfosalicylic acid (2.5% final concentration) to precipitate proteins, and centrifuged again. The clear supernatant was used for GSH measurements using the DetectX Fluorescent Detection Kit (Arbor Assays, Ann Arbor, MI, USA) following the manufacturer's instructions, and normalized for protein content assessed after buffering with 100 mM Tris pH 7.4 using the NanoQuant kit (Carl Roth, Karlsruhe, Germany).

Statistical analysis

Experiments were repeated at least three times, and all measurements (except for western blot analysis) were performed in double. Data are reported as means \pm SEM. ANOVA followed by an appropriate *post hoc* multiple comparison test (Dunnett's, Fisher's TSD or Student's T test) was used to test for statistical significance. $P < 0.05$ was considered as significant.

Results

1. NO• potently activates Nrf2 in a concentration-dependent manner, while nitrosating species (NO⁺ donors) and nitroxyl (HNO) are rather weak inducers of Nrf2 in vascular endothelial cells.

In this work we sought to compare the effects of nitrosopersulfide with those of NO and sulfide in terms of activation of Nrf2 binding and transactivation activity in endothelial cells.

While the Nrf2 system is ubiquitously expressed, the magnitude of the response to a stress signal and the outcome of Nrf2 activation (i.e. expression of its target genes) are strictly dependent on tissue/cell

type, cellular redox status and culture conditions. To properly adjust our experimental conditions for HUVECs, we tested the responsiveness of the cellular Nrf2/Keap-1 system to tert-butylhydroquinone (t-BHQ), a control stimulus known to inactivate Keap-1 by reacting with Cys-151 of the BTB domain (30, 43). We found that responsiveness and magnitude of the Nrf2 response was strictly dependent on the source of endothelial cells (i.e. the human donor) and strongly decreased by culturing HUVECs for more than three passages or maintaining cells in culture longer than 1-2 days after reaching confluence. To confirm system responsiveness and reduce batch-to-batch variability in magnitude of response, we worked with a defined lot of HUVECs obtained by pooling different donors and cultured the cells until passage 2 under standardized culture/growing conditions (seeding, grow intervals, culturing surface, and volume of supernatant). Under these conditions, a non-toxic concentration of tBHQ (10 μ M) increased the binding activity of Nrf2 to ARE by 100% as compared to baseline activation level in untreated cells (Fig. 1A).

Using this cellular system, we first investigated the activation of Nrf2 binding activity of nuclear extracts to ARE by a variety of chemically related compounds including donors of NO^{*} (100 μ M DEA/NO, 1-100 μ M Sper/NO), nitrosonium (NO⁺) equivalents (i.e. 1-100 μ M of the S-nitrosothiol SNAP), and nitroxyl (HNO; Angeli's salt (1-20 μ M AS) (Fig. 1A). We found that both NO^{*} donors, DEA/NO and Sper/NO strongly increased Nrf2 binding activity in a concentration-dependent fashion (Fig. 1A). Similar, albeit less potent effects were induced by the treatment of cells with SNAP (20 μ M increased ARE binding by 25%), while equimolar concentrations of AS enhanced ARE binding by only 19%. This result was unexpected, considering that S-nitrosation of Cys-151 on the BTB domain of Keap-1 has been proposed to account for Nrf2 activation by nitrosating agents and NO (30-32). Although mechanisms, stoichiometry and kinetics of NO, NO⁺ and HNO/NO⁻ release from the donors applied in this study are distinct (see Discussion), preventing a direct comparison of their profile of activation under standardized assay conditions, these results nevertheless show that Nrf2 activation can be triggered by NO and nitrosothiols, but less effectively by HNO.

2. Sulfide is a weak activator of Nrf2 in human vascular endothelial cells.

Next we aimed to compare the NO-induced enhancement of Nrf2 binding activity to ARE to effects of sulfide generated from two different sources, an inorganic sodium salt (Na_2S) and the slow sulfide releasing agent, GYY 4137. We found that concentration jumps induced by addition of buffered sulfide solutions containing dissolved H_2S (approx 25%) and hydrosulfide anion (HS^- ; approx 75%), obtained by dissolving Na_2S in aqueous buffers pH 7.4 (44), are comparatively weak inducers of Nrf2 binding activity, as only sulfide concentrations of 100 μM and higher activated Nrf2-binding activity by more than 20% (Fig. 1B – Na_2S). Similarly, the cell-permeable slow sulfide donor GYY4137 was also only a weak inducer of Nrf2 binding activity (Fig. 1B - GYY). Considering that the corresponding vehicle control, DMSO, causes a significant increase in Nrf2 binding itself (Fig. 1B – 1:5 and 1:50 DMSO) the potency of GYY 4137 to activate Nrf2 in this system is at least 10-fold lower when compared to Na_2S .

Taken together, these results demonstrate that NO is more potent than sulfide in inducing Nrf2-binding activity in human vascular endothelial cells.

3. Nitrosopersulfide increases nuclear Nrf2 levels and Nrf2 binding activity in human endothelial cells.

The chemical biology of SSNO^- (23, 24) suggests that SSNO^- should be a potent activator of the Keap-1/Nrf2 redox system. SSNO^- decomposition at physiological pH leads to formation of bioactive NO (23) (as assessed by gas-phase chemiluminescence and activation of sGC in the NO reporter cell line, RFL6) and polysulfides/sulfane sulfur (as assessed by UV/Vis spectrophotometry and high-resolution mass spectrometry (23, 24)). As described in detail elsewhere (21, 23), and in keeping with the chemical properties of other persulfides/polysulfides (45-47), for the purpose of carrying out biological experiments aqueous stock solutions of SSNO^- have to be prepared in situ. For the current study, SSNO^- was prepared by reacting the nitrosothiol SNAP with a 10-fold molar excess of sulfide in buffer at pH 7.4 (23). Formation of SSNO^- can be readily followed by UV-visible spectrophotometry

monitoring the formation of a new absorbance feature at $\lambda_{\max} = 412$ nm and disappearance of the nitrosothiol peak at $\lambda_{\max} = 340$ nm until the reaction runs to completion (Ref(23); Fig. 2A). In addition to the stable sulfur oxidation products thiosulfate and sulfate, the crude reaction mixture of SSNO⁻ (in the figures labelled as “crude SSNO⁻”) also contains unreacted sulfide, polysulfides and the weak nitroxyl donor SULFI/NO, as confirmed by high-resolution mass spectrometry (24).

We found that crude SSNO⁻ induces Nrf2 binding activity in a concentration-dependent fashion (Fig. 2B), with a considerably higher potency compared to equivalent concentrations of either SNAP or sulfide (compare Fig. 2B with Figs, 1A and 1B). The finding that under the same conditions 200 μ M and 400 μ M sulfide enhanced Nrf2 binding activity by only 50% and 100 %, respectively, suggested this effect was not due to unreacted sulfide in the reaction mixture. To unequivocally confirm this assumption, we removed excess sulfide from the crude SSNO⁻ preparations by gassing the fully reacted mixture with N₂ until complete disappearance of the sulfide absorption band below 220 nm (Fig. 2A; see Methods for details). We found that treating HUVECs with sulfide-free solutions of SSNO⁻ (2-200 μ M) increased the levels of Nrf2 as assessed by Western blot analysis of nuclear extracts (Fig. 3A,B – lanes 2-5) and Nrf2 binding activity (Fig. 3B – bars 2-5) in a concentration-dependent fashion. Addition of 1 mM cysteine (L-Cys) fully blocked SSNO⁻ mediated increases in nuclear Nrf2 levels (Fig. 3A,B – lane #6) and ARE binding activity (Fig. 3C, bar #6), consistent with the likely mechanistic involvement of persulfide formation. Addition of the NO scavenger cPTIO also blocked both, SSNO⁻ mediated increases in Nrf2 levels in the nucleus (Fig. 3A,B – lane #7), and binding activity (Fig. 3C, bar #7). Treatment of endothelial cells with fully decomposed SSNO⁻, obtained by incubating the gassed SSNO⁻ preparation for 24 hours in the dark (see (23)) before addition to endothelial cells, failed to increase nuclear protein levels of Nrf2 (Fig. 3A,B –lane #8) and Nrf2 binding activity (Fig. 3C – bar #8). There was no significant difference between the effects of 20 μ M crude SSNO⁻ (Fig 3A,B – lane #9 and Fig3C –bar #9) and that of N₂-gassed SSNO⁻ (Fig 3A,B – lane #3 and Fig3C –bar #), corroborating our findings that sulfide itself has only minor effects on the Keap-1/Nrf2 system in this particular cell type. Moreover, 20 μ M sulfide-free SSNO⁻, which results in 76% activation of ARE binding, was more

potent in activating Nrf2 binding activity than equimolar concentrations of 20 μ M SNAP; this is consistent with lower levels of stimulation of Nrf2 binding by 20 μ M of either Sper/NO or Angeli's salt, as shown in Fig. 1A. Importantly, SSNO⁻ did not affect nuclear Nrf1 levels as assessed by Western blot analysis of the same nuclear extracts (Fig. 3A), indicating that changes in ARE binding described here were dependent on increases in Nrf2 and not Nrf1 nuclear levels.

Taken together these results show that SSNO⁻ is a potent activator of Nrf2 in human endothelial cells the action of which appears to depend on both, the release of NO and sulfuration of a reactive cysteine on Keap1.

4. Nitrosopersulfide increases expressional levels of the Nrf2-target gene heme oxygenase 1 in human endothelial cells.

We finally aimed to verify whether SSNO⁻ also stimulated Nrf2 transactivation levels by quantifying the level of expression of the Nrf2 target gene, Hmox-1. The incubation time was chosen according to previous analyses of time-dependend regulation of Hmox-1 mRNA in response to NO^{*} (39) and other Nrf2 inducers like zinc (40). We found that incubation of vascular endothelial cells for 6 hours with SSNO⁻ strongly and concentration-dependently (2 – 200 μ M) increased the levels of Hmox-1 mRNA (Fig. 4 – treatments #2 to #5). As seen for nuclear Nrf2 expression and ARE binding, treatment with high concentrations of L-Cys (1 mM, Fig. 4 - treatment #6) or the NO scavenger cPTIO (200 μ M, Fig. 4 - treatment #7) fully blocked SSNO⁻ mediated activation of Hmox-1 gene expression, and treatment with 20 μ M decomposed SSNO⁻ failed to stimulate Hmox-1 expression (Fig. 4 – treatment #8). By contrast, treatment of endothelial cells with crude SSNO⁻ significantly increased Hmox-1 mRNA levels (20 μ M; Fig. 4 - treatment #9), and the extent of this stimulation did not differ from that using equimolar concentrations of sulfide-free SSNO⁻ (20 μ M; Fig. 4 - treatment #9). At variance with the results on Nrf2 binding activity, SSNO⁻ (20 μ M) was equipotent to Sper/NO (20 μ M) in inducing expression of Hmox-1, probably a result of the well-known stabilization of Hmox-1 mRNA by NO^{*} (48).

Taken together, our results demonstrate that SSNO⁻ activates Nrf2 translocation into the nucleus, Nrf2-binding activity and expression of Hmox-1 in endothelial cells, identifying Nrf2 as an additional target for SSNO⁻ bioactivity in cells.

Discussion

The Keap-1/Nrf2 redox system was previously shown to be targeted by NO and nitrosating species(30-32) as well as by sulfide and polysulfide(14, 34, 35, 49), and is thought to account for part of the pharmacological responses to donors of these species. The S/N-hybrid, nitrosopersulfide (SSNO⁻) recently emerged as a major reaction product of the interaction between NO/NO⁻ donors and sulfide (24). This compound has a rather peculiar chemistry, generating both free NO[•] and sulfane sulfur upon decomposition (24). In the present study we sought to investigate whether SSNO⁻ itself may also be an activator of the Keap-1/Nrf2 system by comparing its effects with those of its precursors NO and sulfide in terms of activation of Nrf2 binding and transactivation activity in human endothelial cells. We here demonstrate that 1. both, NO and sulfide activate Nrf2 binding activity in cultured endothelial cells, with donors of different redox forms of NO showing different activation profiles; 2. SSNO⁻ increases Nrf2 nuclear levels, Nrf2-binding and transactivation activity, and expression of the Nrf2 downstream target Hmox-1 more potently than its precursors, NO and sulfide; and 3. SSNO⁻ induced Nrf2 transactivation activity was blocked by both, NO scavenging (cPTIO) and inhibition of persulfidation reactions (high thiol concentrations), indicating that persulfide and/or polysulfide generation and NO[•] reactivity may be involved in Nrf2 activation by SSNO⁻. Taken together, our studies imply that the Keap-1/Nrf2 redox system is another biological target of SSNO⁻, thus adding 'stress signaling' to the bioactivity profile of this vasodilatory molecule.

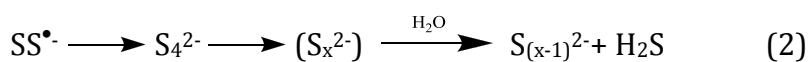
Nitrosopersulfide as activator of the Keap-1/Nrf2 redox system

SSNO⁻ was discovered and fully characterized many years ago by Seel et al. as a product of the interaction of either sulfur or polysulfides with nitrite in polar organic solvents, but also identified as a product of the reaction between gaseous NO[•] and sulfide (or polysulfide) in polar organic solvents and anaerobic alkaline aqueous solutions (21). Of note, Seel observed that decomposition of SSNO⁻ leads to release of NO[•] into the headspace and generation of either polysulfides in solution and/or sulfur

precipitation. Re-introducing NO[•] to these solutions again forms SSNO⁻ suggesting that SSNO⁻ was in equilibrium with NO[•] and S₃^{•-} and S₄^{•-} polysulfide radicals (21). Subsequent work by others revealed that SSNO⁻ is also generated in the course of the reaction between nitrosothiols and sulfide under both aqueous (50) (23, 24) and non-aqueous conditions (22).

If prepared in buffered aqueous solution, SSNO⁻ was found to be rather stable at physiological pH ($t_{1/2}$ >30 min at RT; (22-24) (21)); unexpectedly, its stability was neither affected by the presence of excess sulfide nor by the addition of other reduced thiols (tested for glutathione and cysteine, both at 1 mM; (23) or DTT (23, 24)). Its decomposition at physiological pH leads to formation of NO, as assessed by gas phase chemiluminescence (23, 24), and formation of polysulfides in solution (24). In contrast to SSNO⁻ itself the polysulfides generated are susceptible to decomposition by dithiothreitol (DTT, 1 mM)(23, 24). These properties are in line with those observed by Seel et al (see discussion in (21)) and by Victor (36) who studied the behavior of SSNO⁻ salts in organic polar solvents.

Assuming homolytic cleavage of SSNO⁻, the most likely initial product formed in addition to NO[•] is the persulfide radical, SS^{•-} (Eq 1). Dimerization and reaction with excess thiolate may give rise to formation of higher polysulfides (S_x²⁻; RSS_xH). In aqueous solution at pH 7.4, this is followed by hydrolysis and disproportionation reactions (51), yielding polysulfides of varying chain lengths in addition to free H₂S (eq. 2).



The decomposition products of SSNO⁻ are well-known activators of the Keap-1/Nrf2 redox system, including NO, sulfide, sulfane sulfur radicals and polysulfide. The sensitivity of this system for NO and sulfide/sulfane sulfur is due to the presence of highly conserved cysteines on Keap-1 (30, 31, 43), as demonstrated by site directed mutagenesis. However, how exactly these residues are modified by the action of reactive nitrogen and sulfur species is not fully understood. The highly-reactive Cys-151 on Keap-1 is responsible for sensing of NO and nitrosative species via S-nitrosation (30, 31). Of interest,

Cys-151 was also shown to be targeted by sulfide-mediated persulfidation in mesenchymal embryonic fibroblasts treated with moderate concentrations of sulfide (30 μM NaHS)(34). Exposing the same cell type to higher concentrations of sulfide (400 μM NaHS) others proposed that Cys-151 was not involved in sulfide-mediated Keap-1 inactivation, but instead induced formation of Keap-1 dimers via formation of a long-range disulfide bridge between Cys-226 and Cys-613, preceded by transient persulfidation of one of these cysteines (35). Formation of Keap-1 dimers is typically observed after treatments of cells with H_2O_2 (31); since addition of catalase inhibited the effects of high concentrations of sulfide (35), the authors of this study concluded that sulfide-induced H_2O_2 formation was involved in Keap-1 inhibition under these conditions. Both groups verified the involvement of Cys-151 or Cys-226/Cy-613 in the inhibition of Keap-1 by sulfide using site-directed mutagenesis (31, 35), suggesting that the concentration of sulfide rather than other experimental conditions were responsible for the discordance in results. Interestingly, treatment of neuroblastoma cells with polysulfides (25 μM Na_2S_4) also induced formation of Keap-1 dimers (14). It is therefore tempting to speculate that oxidation of Cys-226/Cy-613 in Keap-1 by high sulfide concentrations, as described in Ref (35), is secondary to the formation of polysulfides in the cells and/or polysulfide contamination of the NaHS solution. This mechanism shares similarity with the activation of transient receptor potential (TRP)A1 channels, another biological target of polysulfides involved in the transmission of pain signals, in which two cysteine residues of the amino terminus are targeted as shown by site-directed mutagenesis (10, 15). Kimura et al. observed that polysulfides are considerably more potent activators of the TRPA1 channel than sulfide itself and concluded that the former are the signaling entities responsible for the effects of sulfide (15); similar results were reported for another thiol-rich protein PTEN by another group (52).

An important observation of the present study is that SSNO^- is more potent than its parent/precursor compounds NO and sulfide in inducing Nrf2 accumulation into the nucleus, Nrf2 binding activity and Nrf2-mediated transactivation of the Hmox-1 gene, regardless of whether equimolar concentrations of NO donor, nitrosothiol or nitroxyl were compared. These effects were observed without causing overt

signs of toxicity or inducing changes in cellular GSH/GSSG redox status (see supplementary information Fig. S1). This may be simply due to a different biological chemistry of SSNO⁻ as compared to NO[•] and sulfide. Alternatively, the higher potency of SSNO⁻ in activating the Keap-1/Nrf2 redox system may be a result of concomitant release of both NO[•] and SS⁻ / S_x⁻ (according to Eq. 1 and 2), which are potent inhibitors of Keap-1 and activators of Nrf2-mediated response. As a result, SSNO⁻ may be considered a sulfane sulfur/NO donor hybrid with a dual pharmacological activity on systems able to sense both persulfides/polysulfides and NO[•]. In fact, we found that treatment with the NO scavenger cPTIO as well as inhibition of persulfidation by treatment with high concentrations of cysteine abrogates SSNO⁻-dependent activation of the Keap-1/Nrf2 redox system and consecutive Hmox-1 expression. As demonstrated earlier, addition of high concentrations of reduced biological thiols (cysteine and glutathione) or DTT did not affect the decomposition kinetics of SSNO⁻ (23, 24), excluding an effect of cysteine on stability of SSNO⁻. A clear limitation of the present study is that we cannot meaningfully compare the pharmacological effects of SSNO⁻ preparations with polysulfide mixtures obtained by dissolving a small-chain polysulfide (like Na₂S₃ or Na₂S₄) in water, buffer or cell culture medium. Preparations of polysulfides undergo rapid disproportionation reactions yielding different equilibrium proportions depending on the conditions, and on the presence of other reactants (as for example NO[•], sulfite or SULFI/NO in the SSNO⁻ preparation (24), as discussed below. The resulting fluxes and speciation of polysulfides in aqueous solution can neither be predicted with any degree of accuracy nor can it be reliably quantified at present.

Nevertheless, the effects of SSNO⁻ are consistent across all levels of the Keap-1/Nrf2 redox system, including Nrf2 accumulation, Nrf2 binding and Hmox-1 mRNA levels, with one exception: while the NO scavenger cPTIO potently inhibits SSNO⁻-mediated increases of mRNA levels, its effects on Nrf2-binding activity are weak. This may be due to stronger effects of SS⁻/polysulfides as compared to NO[•] on Nrf2 binding activity, or be related to the stabilization effects of NO[•] on mRNA levels of Hmox-1 (48). The latter is consistent with the observation that the effects of SSNO⁻ on Nrf2 binding activity are stronger than those of Sper/NO, but equipotent to Sper/NO in elevating Hmox-1 mRNA.

Since cPTIO efficiently converts NO to NO₂, this also indicates that NO₂ (a potential O₂/NO reaction product) is not responsible for Nrf2 activation under the conditions analyzed in this study. This may be significant since NO₂ is definitely a strong enough oxidant to modify thiols in Keap-1. Our observation that DMSO (which was used as a vehicle to prepare cPTIO stock solutions) increases Nrf2-binding activity on its own is a potential confounder. Future analysis of cysteine modifications on Keap-1 by biotin switch assay followed by mass spectrometric analysis, accompanied by the use of site-directed mutagenesis will reveal whether SSNO⁻ induces nitrosative or sulfurative modifications or leads to oxidation/disulfide formation of the redox-reactive cysteines of Keap-1.

Similar to the metastable nature of short-chain polysulfides and persulfides in aqueous solution (45-47), the chemistry and pharmacology of SSNO⁻ can only be studied if this species is generated *in situ* in this medium. In the present study we prepared SSNO⁻ by reacting the nitrosothiol SNAP with sulfide at a 1:10 molar ratio, followed by removal of excess sulfide by gassing with nitrogen (23, 24); the reaction was carefully characterized previously by applying independent analytical methods (24). As demonstrated by high-resolution mass spectrometry earlier, the reaction between NO or nitrosothiols with excess sulfide leads not only to the formation of SSNO⁻ and persulfide/polysulfides (Eq 1 and 2) but also to production of dinitrososulfite (24), a molecule belonging to the class of NONOates (also known as SULFI/NO in the literature). This molecule was found to be a weak NO donor and a weak nitroxyl donor, and its main decomposition products are N₂O and sulfate (24). Although we expected that nitroxyl (HNO) might be a potent activator of the Keap-1/Nrf2 system by targeting the redox-active cysteines of Keap-1, we found that the prototypical nitroxyl donor Angeli's salt only elicited a relatively weak increase in Nrf2-binding activity and Hmox-1 expression compared to NO. These findings imply that SULFI/NO-derived nitroxyl present in the SSNO⁻ reaction mixture employed in the current study is unlikely to have contributed significantly to the observed elevation of Nrf2 binding activity and Hmox-1 expression.

The NO sensing element of Keap-1 (Cys-151) is located in the vicinity of basic amino acids, reducing the pKa of its sulfhydryl moiety (30); under physiological conditions, Cys-151 is therefore expected to

exist largely in the thiolate form (30, 35) and be more prone to modification by electrophiles such as nitrosothiols. By contrast, NO^\bullet cannot react directly with thiolates. We were therefore expecting SNAP to be a more potent activator of the Keap-1/Nrf2 system than SSNO^- or the NO donors DEA/NO and Sper/NO. However, this was not what we found using cultured human endothelial cells. By way of reference, S-nitrosation of Keap-1 was previously achieved by treating pC12 cells with 500 μM SNAP (32) or HeLa cells with 500 μM CysNO (31), while treatment with 2 mM Sper/NO induced Keap-1 dimer formation but not nitrosation of Keap-1 (31); by contrast, another study found that Cys-151 was modified by treatment with (a cell-membrane permeable) DEA/NO in COS1 cells(30).

In this context, it is important to point out that the potency of activation of the Keap-1/Nrf2 redox system by SSNO^- , SNAP and NO donors cannot be readily compared on the basis of molar concentrations alone. In fact, mechanisms, stoichiometry and kinetics of NO^\bullet release and/or nitrosation by each of these donors differ markedly (53). At neutral pH, NONOates release NO^\bullet spontaneously, and the kinetics of NO release is strictly dependent on the structure of the amine backbone (54). Nitrosothiols may release NO by homolytic cleavage of the the S-N bond, a reaction which is favored in the presence of transition metals, or participate in transnitrosation reactions (formally, transfer of a NO^+ equivalent)(21). However, the stability of their S-N bond in biological systems is not easily predictable and depends not only on the chemical structure of the nitrosated thiol, but also on the presence of transition metals in culture media, cellular redox status and other factors. Which factors contribute to homolysis of the S-N bond in SSNO^- is poorly understood. Nevertheless, SSNO^- appears to be a powerful activator of the Keap-1/Nrf2 redox system.

Is the Keap-1/Nrf2 pathways a possible target of endogenous SSNO^- formation?

According to the results presented in this and earlier studies SSNO^- displays broad in vitro and in vivo pharmacological activity. An obvious question is whether SSNO^- can actually be formed in cells, and if so, by what mechanism. Similar discussions arose two decades ago when peroxyxynitrite (OONO^-) – the

oxygen analogue of SSNO⁻ – was shown for the first time to have pharmacological/toxicological activity and was proposed to be of biological relevance (55). Peroxynitrite is formed according to a radical-radical reaction between O₂^{•-} and NO[•] at a rate close to the diffusion-controlled limit (Eq. 3) (55).



At first, OONO⁻ was considered to be too unstable to be of much relevance to biology and unlikely to be formed in tissues, not least because of the presence of superoxide dismutase removing the precursor O₂^{•-} by catalyzing one of the fastest reactions known at that time (56-58). Following the discovery of several different enzymatic sources (including NADPH oxidases and uncoupled nitric oxide synthase itself) forming O₂^{•-} in close proximity to enzymatically generated NO from NO synthase, OONO⁻ is now an established cellular mediator contributing either to oxidant signaling or to “the dark side” of NO, in particular under inflammatory conditions (58). Interestingly, recent data also show that low concentrations of peroxynitrite induce Nrf2, potentially conferring cell protection (59). According to Eq. 1, SSNO⁻ is in equilibrium with its products of homolysis, S₂^{•-} and NO[•]. We therefore recently speculated that in biological environments SSNO⁻ may be formed by a radical-radical reaction between S₂^{•-} and NO[•] according to the following reaction(21):



Therefore, an important question to address is then whether the precursors of $SSNO^-$ can be found in the cellular environment. The disulfide radical ion $S_2^{\bullet-}$ is a well-known species that can be formed in aqueous environments by non-enzymatic and enzymatic reactions involving polysulfides and persulfides (60, 61). Possible precursors of $S_2^{\bullet-}$ are cysteine persulfide (CySSH) and glutathione persulfide (GSSH)(62), which have been found in micromolar concentrations in tissues (9), and proposed to be formed in cells from cystine or GSSG by enzymes involved in sulfur amino acid metabolism, including CBS, CSE (9). According to kinetic analyses and computer modeling, others predicted that the major products of CBS and CSE in the reducing environment of the cell should be sulfide and mixed polysulfides, while CySSH was proposed to be unstable under these conditions (63). These findings are at variance with the experimental data demonstrating the presence of rather high concentrations of CySSH and GSSH in cells (9). Interestingly, MST was reported to produce polysulfides (64), which may generate $S_2^{\bullet-}$ by homolytic cleavage (inverse of Eq. 2). Other sources of $S_2^{\bullet-}$ and persulfide may be the mitochondria, in particular the mitochondrial sulfide:quinone reductase (SQR) as well as red blood cells via the hemoglobin-mediated oxidation of sulfur-centered radicals (65). Since the kinetics of most radical-radical reactions are typically very fast, we propose that $SSNO^-$ might be formed in cells and tissues where sources for its precursors are found in close proximity to each other. A challenge in this field is finding detection methods capable of determining concentrations and flux rates of $S_2^{\bullet-}$ and $SSNO^-$ at low physiological levels in cells and tissues without perturbing natural equilibria.

Summary and conclusion

The Keap-1/Nrf2 redox system evolved to adapt and respond to environmental insults, including alkenals, reactive species, and heavy metals (30). During its evolution some of the stimuli, including

reactive species and Zn^{2+} became signaling molecules and “messengers”, and therefore the system itself became an integral part of eukaryotic cellular signaling as both the target (Keap-1) and effector (Nrf2) of these new messengers. Keap-1 is able to detect the activators via specific redox-sensing cysteines and regulate the effector Nrf2 by fine-tuning its localization, stability and activity to transcriptionally activate a battery of downstream targets involved in cellular defense. In addition to protecting against injury, a lesser-known function of the Nrf2 signaling system is to adjust the cellular metabolic machinery to nutritional stresses (66). Earlier work showed that NO, sulfide and polysulfides can all activate the Keap-1/Nrf2 redox system. Here we show that $SSNO^-$, a molecule emerging as a product of the chemical cross-talk between NO and sulfide, itself potently activates the Keap-1/Nrf2 redox system, leading to Nrf2 accumulation in the nucleus, increasing Nrf2 binding activity and transactivation capacity. These effects appear to be mediated by a dual effect of $SSNO^-$ -derived NO and per/polysulfide generation. These results imply that the Keap-1/Nrf2 redox system is a target of $SSNO^-$, adding redox signaling to the vasoactive properties of this interesting molecule.

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Author contributions.

M.M.C.-K designed the experiments and analyzed the data; D.P. performed research, collected data and drafted Materials and Methods; M.M.C.-K. and M.F. wrote the paper.

Conflict of interest statement.

The authors declare no competing financial interests.

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Figures and legends.

Figure 1.

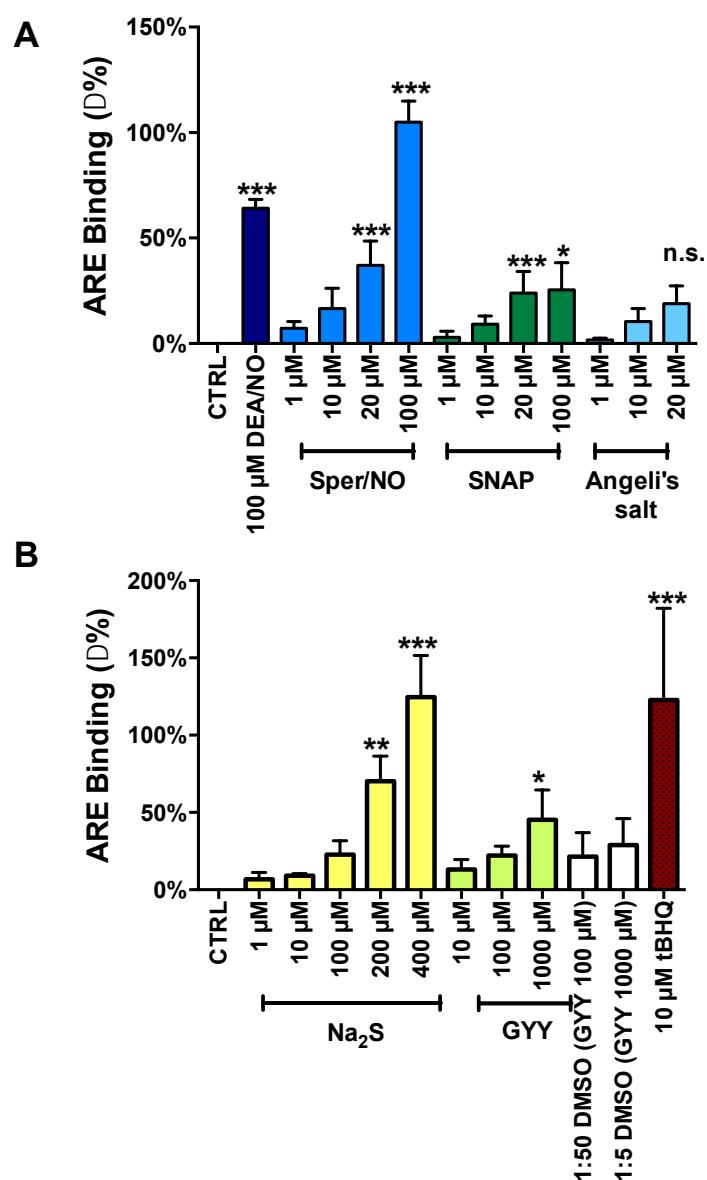


Figure 1: Activation of ARE binding by NO and sulfide in endothelial cells. A) Concentration-dependent activation of Nrf2 induced by 1 hour incubations of cultured human endothelial cells with the NO donors DEA/NO (100 μ M) or Sper/NO (1-100 μ M), the nitrosothiol SNAP (1-100 μ M) and the nitroxyl donor Angeli's salt (1-20 μ M). n=3-4; 1-way ANOVA $p < 0.0001$; Dunnett's * $p < 0.01$, *** $p < 0.0001$. n.s., non significant. B) Concentration-dependent activation of Nrf2 by incubation of human endothelial cells for 1 hour with aqueous solutions of a sulfide salt (Na_2S , 1-400 μ M) or a sulfide donor (GYG4137, 10-1000 μ M) dissolved in DMSO, and the corresponding vehicle control, as

well as the Nrf2 activator tert-butylhydroquinone (t-BHQ, 10 μ M), which was applied as a positive control. Binding of Nrf2 to oligonucleotides containing the ARE sequence was assessed by an ARE-oligonucleotide-based ELISA. n = 3-6. 1-way ANOVA $p < 0.001$; Dunnett's ** $p < 0.01$, *** $p < 0.001$

Figure 2

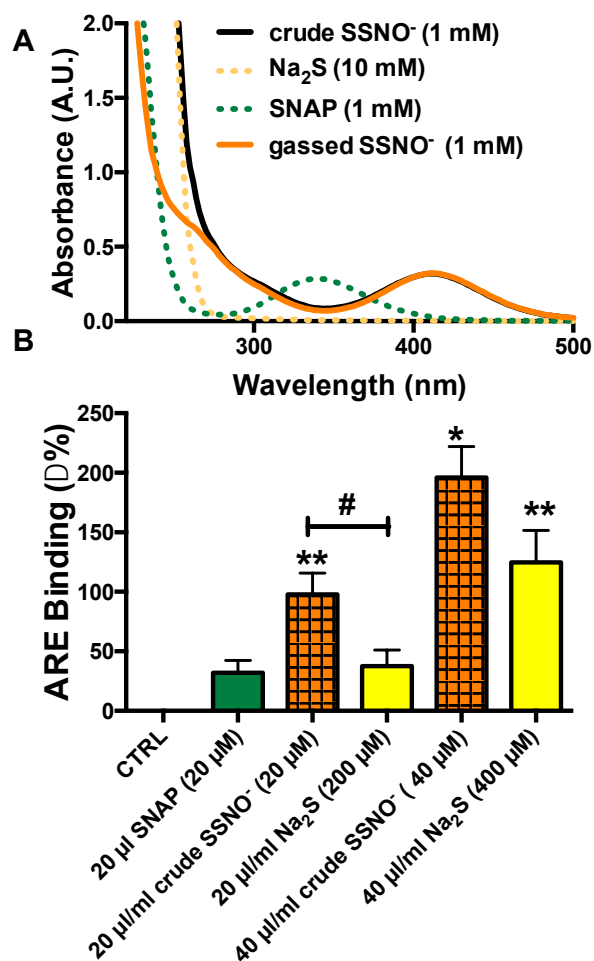


Figure 2 – A crude preparation of SSNO⁻ mix activates Nrf2 more potently than the parent compounds, SNAP and sulfide. A) Representative UV-visible spectra of a crude preparation of SSNO⁻ (1 mM ; λ_{\max} 412 nm), its starting compounds SNAP (λ_{\max} 340 nm, 1 mM) and Na₂S (λ_{\max} <250 nm; 10 mM), and a sulfide-free preparation of SSNO⁻ (1 mM ; λ_{\max} 412 nm) obtained by removing excess Na₂S by gassing the crude preparation with N₂. B) Concentration-dependent activation of Nrf2 in HUVECs by crude SSNO⁻ mix as compared to the equivalent concentration of the starting materials, SNAP and Na₂S (final concentrations of treatments added to the cells in brackets). n = 3-7, 1-way-ANOVA p<0.0001, Dunnett's **p<0.001; *** p< 0.0001.

Figure 3

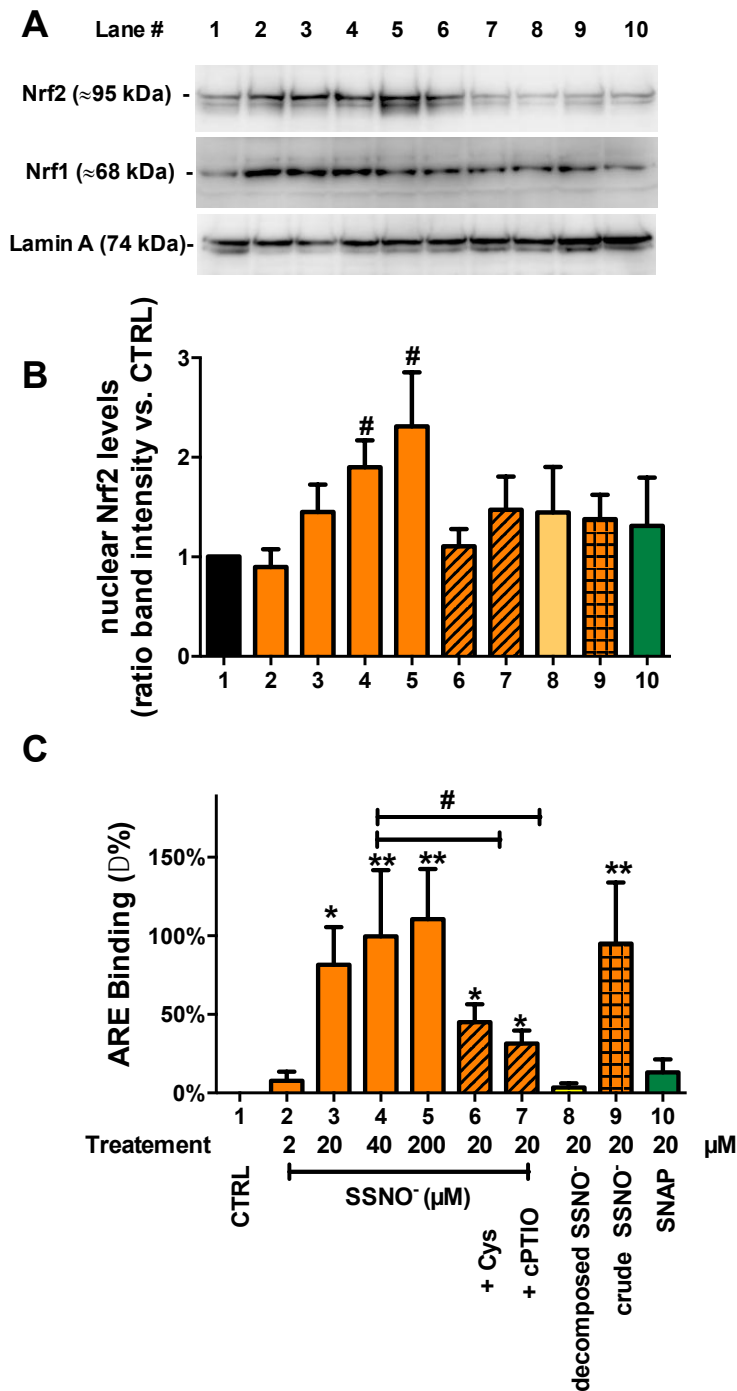


Figure 3 -Sulfide-free SSNO⁻ increases Nrf2 nuclear levels and ARE binding activity in a concentration, sulfuration and NO-dependent manner. Sulfide-free SSNO⁻ preparations increase the levels of Nrf2 in the nucleus of vascular endothelial cells in a concentration-dependent fashion. Increases are blocked by treatment with high concentrations of L-cysteine (Cys, 1mM) or the NO scavenger cPTIO (200μM). Decomposed SSNO⁻ does not increase nuclear Nrf2 levels. A)

Representative Western blot of nuclear extract stained for Nrf2 and Nrf1. Lamin A was used as loading control. B) Densitometric analysis of blots from three individual experiments. T-test vs. CTRL # $p < 0.05$ (1-tailed). C) Sulfide-free SSNO⁻ increases Nrf2 ARE-binding activity in a concentration-dependent fashion; this activation is blocked by treatment with high cysteine concentrations (Cys, 1mM) or the NO scavenger cPTIO (200 μ M). Decomposed SSNO⁻ does not increase Nrf2-binding activity. n = 3, 1 way-ANOVA, p = 0.0071, Fisher's LSD * $p < 0.05$, ** $p < 0.001$. T-test (2 tailed) # $p < 0.05$

Figure 4

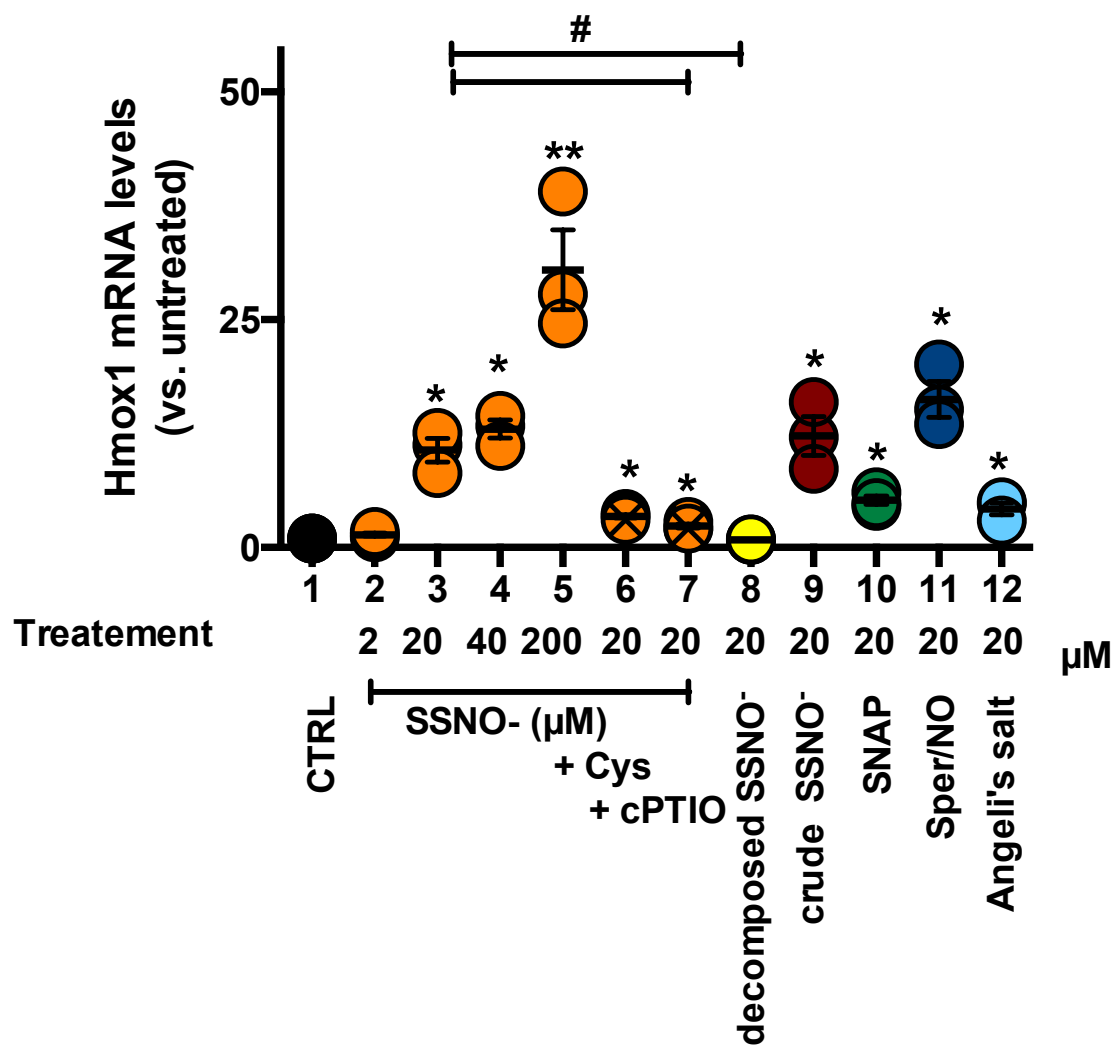


Figure 4. Sulfide-free SSNO⁻ activates Hmox-1 gene expression in a concentration and NO-dependent manner. A 6h incubation of HUVECs with SSNO⁻ increases Hmox 1 mRNA levels; addition of high concentrations of L-cysteine (Cys, 1 mM) to destroy persulfide/polysulfides and addition of the NO-scavenger cPTIO blocks SSNO⁻ mediated increases in Hmox-1 expression, while decomposed SSNO⁻ does not exert any effects. The NO donor Sper/NO increases Hmox-1 expression, while the nitrosothiol SNAP and the nitroxyl donor Angeli's salt are considerably weaker activators of Hmox-1-gene expression. The Hmox-1 mRNA levels were measured by real time RT-PCR and quantified vs. Rpl0, which was used as housekeeping gene (n = 3, RM 1-way-ANOVA, p = 0.0177, Fisher's LSD * p<0.05, ** p<0.01, T-test (2-tailed), # p<0.01).

Supplemental materials

Supplemental figures

Figure S1

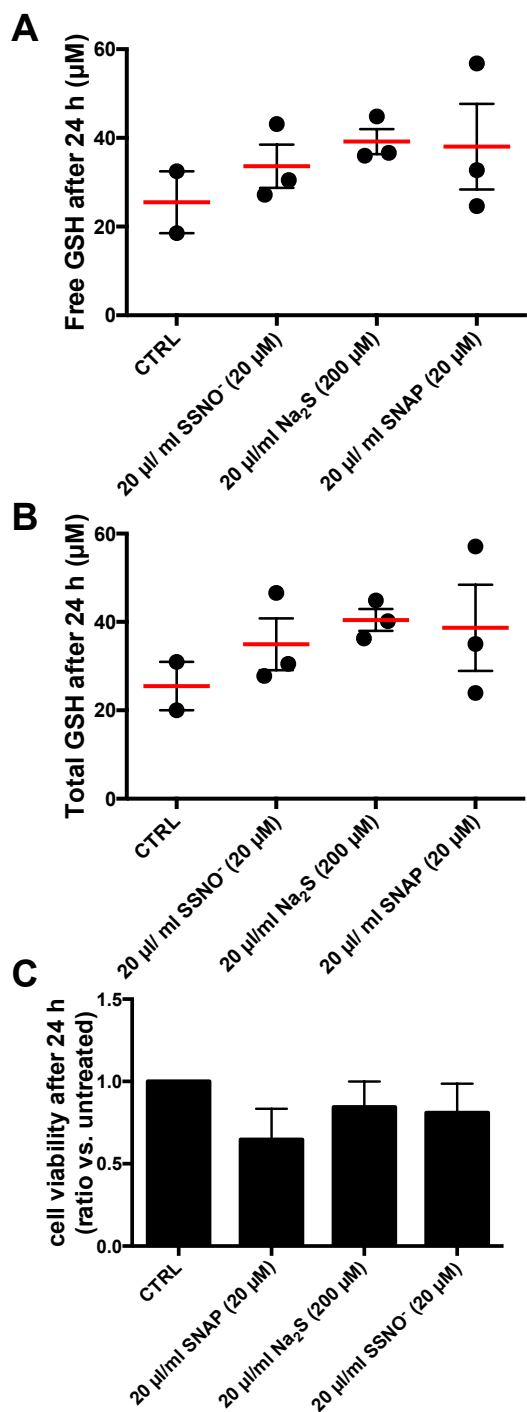


Figure S1. Nitrosopersulfide does not affect reduced (“free”) and total GSH in endothelial cells.

Acute incubation of HUVECs with crude preparations of SSNO⁻ or equivalent concentrations of sulfide

or SNAP did not affect the levels of intracellular (A) reduced glutathione (GSH), or (B) total glutathione (GSH+GSSG), nor (C) the viability of cells, assessed by neutral red staining, 24 after incubation.

Figure S2

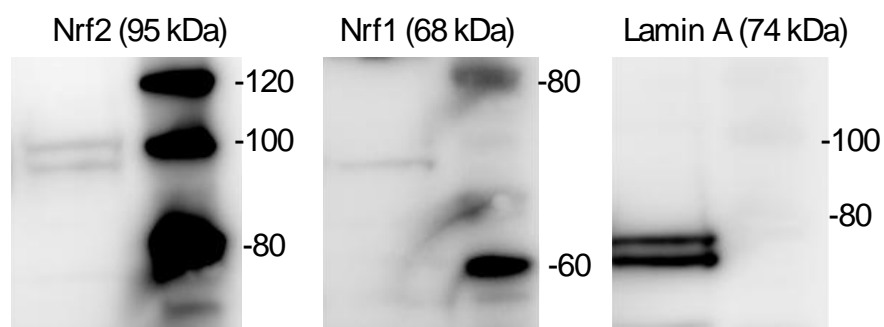


Figure S3 - Apparent molecular weight of Nrf2, Nrf2 and lamin A/C in Western blot analysis of nuclear extracts from HUVECs. Staining was carried out with a rabbit monoclonal antibody anti-Nrf2 (Ab62352, Abcam), anti-Nrf1 (ab90524) and anti-Lamin A/C (ab8984). Nrf2 in Western blots has an apparent molecular weight of approximately 95 kDa, and a predicted molecular weight of 68 kDa (see (1))

Figure S3.

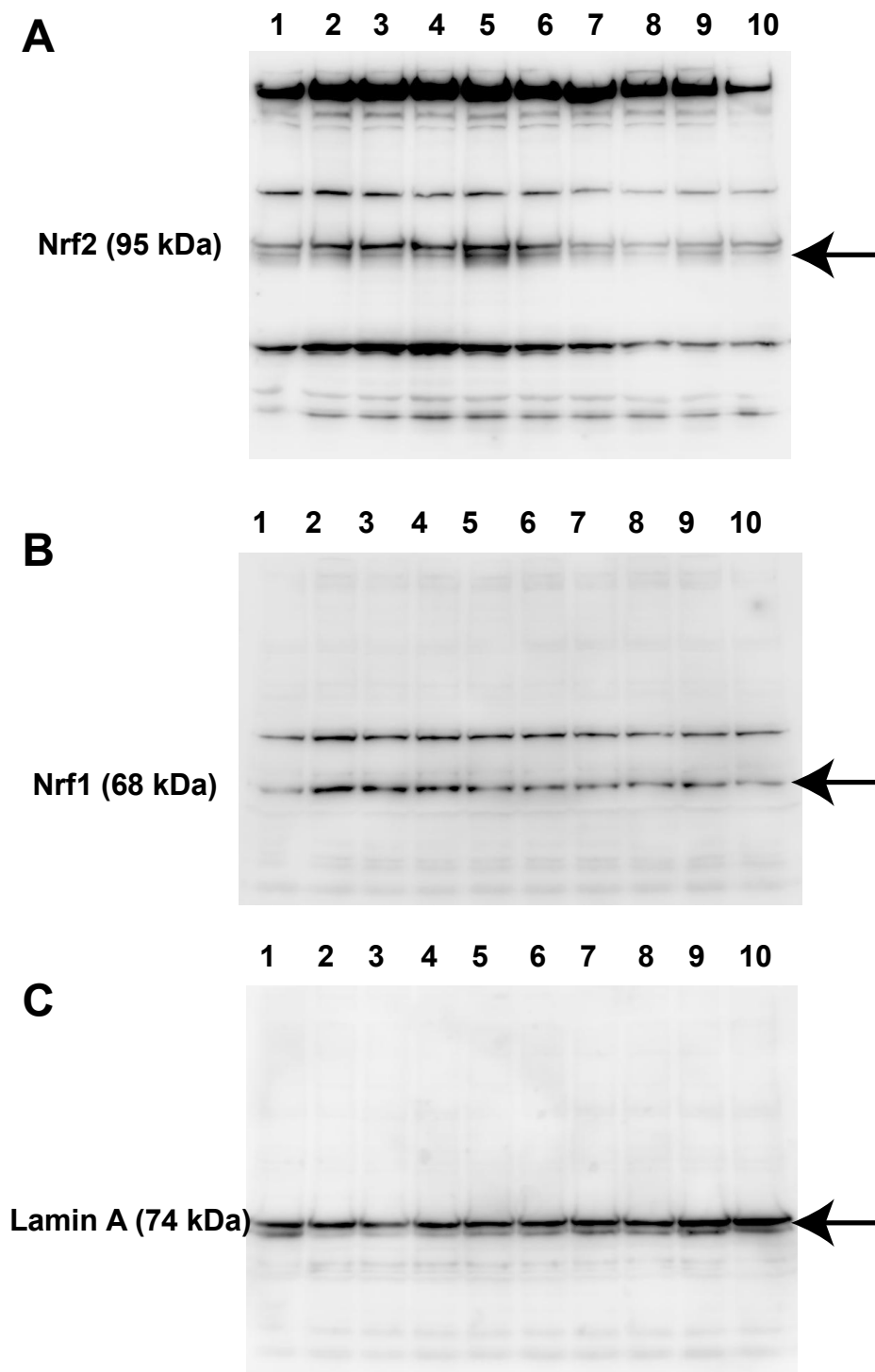


Figure S3. Whole un-cropped images of the original Western blots shown in Fig. 3.

Supplemental References

1. Lau A, Tian W, Whitman SA, Zhang DD. The Predicted Molecular Weight of Nrf2: It Is What It Is Not. *Antioxidants & redox signaling*. 2013;18(1):91-93.

