

Selective Irreversible Inhibition of Neuronal and Inducible Nitric-oxide Synthase in the Combined Presence of Hydrogen Sulfide and Nitric Oxide*

Received for publication, April 20, 2015, and in revised form, August 19, 2015. Published, JBC Papers in Press, August 20, 2015, DOI 10.1074/jbc.M115.660316

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Background: NO and H₂S signaling pathways may be interdependent.

Results: Citrulline formation and NADPH oxidation by neuronal and inducible but not endothelial nitric-oxide synthase were inhibited irreversibly by H₂S in the presence of NO.

Conclusion: NO synthase is isoform-specifically inactivated by a reaction product of NO and H₂S.

Significance: Inhibition by NO/H₂S may represent an autoregulatory feedback mechanism.

Citrulline formation by both human neuronal nitric-oxide synthase (nNOS) and mouse macrophage inducible NOS was inhibited by the hydrogen sulfide (H₂S) donor Na₂S with IC₅₀ values of $\sim 2.4 \cdot 10^{-5}$ and $\sim 7.9 \cdot 10^{-5}$ M, respectively, whereas human endothelial NOS was hardly affected at all. Inhibition of nNOS was not affected by the concentrations of L-arginine (Arg), NADPH, FAD, FMN, tetrahydrobiopterin (BH₄), and calmodulin, indicating that H₂S does not interfere with substrate or cofactor binding. The IC₅₀ decreased to $\sim 1.5 \cdot 10^{-5}$ M at pH 6.0 and increased to $\sim 8.3 \cdot 10^{-5}$ M at pH 8.0. Preincubation of concentrated nNOS with H₂S under turnover conditions decreased activity after dilution by $\sim 70\%$, suggesting irreversible inhibition. However, when calmodulin was omitted during preincubation, activity was not affected, suggesting that irreversible inhibition requires both H₂S and NO. Likewise, NADPH oxidation was inhibited with an IC₅₀ of $\sim 1.9 \cdot 10^{-5}$ M in the presence of Arg and BH₄ but exhibited much higher IC₅₀ values ($\sim 1.0 - 6.1 \cdot 10^{-4}$ M) when Arg and/or BH₄ was omitted. Moreover, the relatively weak inhibition of nNOS by Na₂S in the absence of Arg and/or BH₄ was markedly potentiated by the NO donor 1-(hydroxy-NNO-azoxy)-L-proline, disodium salt (IC₅₀ $\sim 1.3 - 2.0 \cdot 10^{-5}$ M). These results suggest that nNOS and inducible NOS but not endothelial NOS are irreversibly inhibited by H₂S/NO at modest concentrations of H₂S in a reaction that may allow feedback inhibition of NO production under conditions of excessive NO/H₂S formation.

Nitric oxide (NO) and hydrogen sulfide (H₂S) are two endogenously generated molecules that perform important functions in signal transduction (1–3). Nitric oxide is formed from L-ar-

ginine (Arg), molecular oxygen (O₂), and NADPH-derived electrons in a reaction catalyzed by nitric-oxide synthase (NOS; EC 1.14.13.39). NOS is only active as a dimer and exists in three isoforms, neuronal, endothelial, and inducible NOS (nNOS,² eNOS, and iNOS, respectively), that differ in tissue distribution and physiological function (4–6). The constitutive isozymes nNOS and eNOS are activated by Ca²⁺/calmodulin (CaM), whereas the much higher affinity of iNOS for CaM renders its activity [Ca²⁺]-independent under physiological conditions. Formation of NO requires the cofactor tetrahydrobiopterin (BH₄), which couples NADPH oxidation to NO synthesis. In the absence of BH₄, oxidation of NADPH results in O₂^{•-} formation (5, 7, 8).

In mammals, generation of H₂S is catalyzed by cystathionine β-synthase (EC 4.2.1.22), cystathionine γ-lyase (EC 4.4.1.1), and 3-mercaptopyruvate sulfurtransferase (EC 2.8.1.2) (3, 9, 10). There is growing evidence that the NO and H₂S signaling pathways are interdependent with both stimulatory and inhibitory effects being reported (2, 11–14). Although many of these effects appear to be indirect, there are some reports of direct effects of NO on the H₂S-generating enzymes and of inhibition of NOS by H₂S (15, 16). Furthermore, recent data suggest that reactions among NO, H₂S, and their derivatives may be (patho)physiologically relevant. H₂S as a reducing agent and nucleophile is predicted to react with a variety of NO-derived species, possibly yielding nitroxyl (HNO), thionitrous acid (HSNO), and nitrosopersulfide (SSNO⁻) as reaction products (9, 11–13, 17, 18).

In the present study, we investigated whether H₂S is able to directly affect NOS activity. We found that recombinant human nNOS and murine iNOS but not human eNOS were

* This work was supported by Austrian Science Fund (FWF) Grants P23135 (to A. C. F. G.) and P24946 (to B. M.). The authors declare that they have no conflicts of interest with the contents of this article.

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² The abbreviations used are: nNOS, neuronal NOS; iNOS, inducible NOS; eNOS, endothelial NOS; CaM, calmodulin; BH₄, tetrahydrobiopterin ((6R)-2-amino-6-[[1R,2S)-1,2-dihydroxypropyl]-5,6,7,8-tetrahydropteridin-4(1H)-one); [³H]Arg, L-[2,3,4,5-³H]arginine hydrochloride; [³H]citrulline, L-[2,3,4,5-³H]citrulline; PROLI/NO, proline NONOate (1-(hydroxy-NNO-azoxy)-L-proline, disodium salt); SPER/NO, spermine NONOate ((Z)-1-[N-[3-aminopropyl]-N-[4-(3-aminopropylammonio)butyl]amino]diazene-1-ium-1,2-diolate; Angeli's salt, disodium diazen-1-ium-1,2,2-triolate; 2-ME, 2-mercaptoethanol; TEA, triethanolamine HCl; Bis-tris propane, 1,3-bis(tris(hydroxymethyl)methylamino)propane.

irreversibly inhibited by modest ($\sim 10^{-5}$ M) concentrations of H₂S under conditions that allowed NO formation (*i.e.* +Arg/+BH4). In the absence of NO formation, inhibition required much higher H₂S concentrations ($\sim 10^{-4}$ M) and was reversed by dilution. The results suggest that a product of the reaction between NO and H₂S, possibly SSNO⁻, irreversibly inhibits nNOS and iNOS. The potential physiological relevance of these observations is discussed.

Experimental Procedures

Materials—L-[2,3,4,5-³H]Arginine hydrochloride (³H]Arg; 57 Ci/mmol) was from American Radiolabeled Chemicals Inc. purchased through Humos Diagnostic GmbH (Maria Enzersdorf, Austria). BH4 was from Dr. B. Schircks Laboratories (Jona, Switzerland). Stock solutions of BH4 were prepared in 10 mM HCl. Stock solutions of Na₂S (Sigma-Aldrich, catalog number 407410) were prepared in Milli-Q water (Millipore; resistance, >18 megaohms·cm⁻¹) and stored in dark vessels. General materials for molecular biology were from New England Biolabs; Life Technologies, Inc.; and Qiagen. The EasySelect™ *Pichia* expression kit was from Invitrogen (Life Technologies, Inc.). Human nNOS cDNA was from Dr. John Parkinson (Berlex Biosciences, Richmond, CA). Purified yeast thioredoxin 1 and thioredoxin reductase were from Biomol (Sanova, Vienna, Austria). 1-(Hydroxy-NNO-azoxy)-L-proline, disodium salt (PROLI/NO) and spermine NONOate (SPER/NO) were from Enzo Life Sciences (Lausen, Switzerland). Disodium diazen-1-ium-1,2,2-triolate (Angeli's salt) was from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany). NADPH was purchased from Pharma Waldhof GmbH (Düsseldorf, Germany). Glutathione persulfide was prepared as published (19) and used immediately. Other chemicals were from Sigma-Aldrich.

Enzyme Expression and Purification—Mouse macrophage iNOS was expressed in *Escherichia coli* and purified as described (20). Human eNOS was expressed in and purified from *Pichia pastoris* as described elsewhere (21). To subclone cDNA of human nNOS, the *P. pastoris* expression vector pPICZA was used (EasySelect *Pichia* expression kit). The plasmid pBBS230 containing cDNA for human nNOS was double digested with XbaI and NotI. The recessed 3' termini from the XbaI digest were filled by the Klenow fragment of *E. coli* DNA polymerase I in the presence of appropriate deoxynucleoside triphosphates. The vector was subsequently double digested with EcoRI and after filling the recessed 3' termini with NotI. The 4.3-kb insert was ligated to the restricted pPICZA. *E. coli* TOP10F' cells were transformed with the resulting ligation products and plated on LB/Zeocin medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, and 25 μg/ml Zeocin at pH 7.5). The resulting transformants were tested by restriction analysis, and positive clones were amplified. The final DNA construct was linearized with PmeI, the DNA was transformed into *P. pastoris* GS115 (Mut⁺), and the cells were plated on YPDS/Zeocin medium (1% yeast extract, 2% peptone, 2% glucose, 1 M sorbitol, and 100 μg/ml Zeocin) to select recombinants. A single colony of the best clone was grown for 36 h at 30 °C in 50 ml of buffered minimal glycerol (BMGH) medium consisting of 100 mM potassium phosphate (pH 6.0), 13.4 g/liter yeast nitrogen base without amino acids, 400 μg/liter biotin, 40 mg/liter L-histidine, and

1% (v/v) glycerol. The overnight culture was diluted in BMGH medium (1:200) and grown overnight at 30 °C to an A₆₀₀ of 5–6. To induce nNOS expression, cells were harvested and resuspended in the presence of 4 mg/liter hemin chloride in buffered minimal methanol medium consisting of 100 mM potassium phosphate (pH 6.0), 13.4 g/liter yeast nitrogen base without amino acids, 400 μg/liter biotin, 40 mg/liter L-histidine, and 0.5% methanol at an A₆₀₀ of ~ 1 .

After 24 h of growth at 30 °C, cells were harvested by centrifugation at 2000 × g for 5 min at room temperature and resuspended at a concentration equivalent to an A₆₀₀ of 125 (based on the A₆₀₀ of the culture) in 50 mM Tris (pH 7.4) containing 1 mM EDTA, 5% glycerol, 12 mM 2-mercaptoethanol (2-ME), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM CHAPS. An equal volume of glass beads (0.5 mm) was added to the suspension, and the cells were broken by vigorous vortexing at 4 °C for a total of 10 min in bursts of 30 s alternating with cooling on ice. The glass beads were separated by centrifugation at 800 × g for 5 min. After a further clearing step at 1600 × g for 5 min, the supernatant was centrifuged at 30,000 × g for 15 min. The enzyme was purified from the resulting supernatant by affinity chromatography as described previously (22). Final elution was achieved with 20 mM Tris (pH 7.4), 150 mM NaCl, and 4 mM EGTA. After determination of the protein concentration according to Bradford (23) using bovine serum albumin as a standard, the enzyme was stored at -70 °C in the presence of 1 mM CHAPS. Enzyme concentrations are expressed as the concentration of the monomer, assuming molecular masses of 160 (nNOS), 130 (iNOS), and 135 kDa (eNOS).

Determination of Enzyme Activity—NOS activity was determined as the formation of L-[³H]citrulline from [³H]Arg (24). Unless indicated otherwise, purified nNOS (5 μg/ml; 31.3 nM), iNOS (2 μg/ml; 15.4 nM), or eNOS (5 μg/ml; 37 nM) was incubated for 10 min in 0.1 ml of 50 mM triethanolamine HCl (TEA) (pH 7.4) containing 0.1 mM [³H]Arg ($\sim 60,000$ cpm), 0.2 mM NADPH, 5 μM FAD, 5 μM FMN, 10 μM BH4, 0.5 mM CaCl₂, 10 μg/ml CaM, 0.2 mM CHAPS, 0.1 mM EDTA, and sodium sulfide (Na₂S) as indicated at 37 °C followed by separation and detection of [³H]citrulline. Blank values were determined in the absence of enzyme. For activity measurements at varying pH values, 50 mM Bis-tris propane (pH 6.3–9.5) was used instead of TEA.

To test for irreversibility of inhibition, nNOS (250 μg/ml; 1.6 μM) was preincubated for 3 min in 0.1 ml of 50 mM TEA (pH 7.4) containing 0.1 mM Arg, 0.2 mM NADPH, 5 μM FAD, 5 μM FMN, 10 μM BH4, 0.5 mM CaCl₂, 0.2 mM CHAPS, 0.1 mM EDTA, 300 μg/ml CaM, and 0.5 mM Na₂S as indicated at 37 °C. After preincubation, samples were 50-fold diluted in prechilled buffer containing 50 mM TEA (pH 7.4), 0.2 mM CHAPS, and 0.1 mM EDTA in the absence or presence of thiol (2 mM DTT, 2 mM GSH, or 2.9 mM 2-ME). These mixtures were diluted 3-fold in 0.1 ml of 50 mM TEA (pH 7.4) containing 0.1 mM [³H]Arg ($\sim 60,000$ cpm), 0.2 mM NADPH, 5 μM FAD, 5 μM FMN, 10 μM BH4, 0.5 mM CaCl₂, 10 μg/ml CaM, 0.2 mM CHAPS, and 0.1 mM EDTA in the absence or presence of 5 μM thioredoxin and 6 μM thioredoxin reductase followed by determination of [³H]citrulline formation at 37 °C for 10 min.

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NADPH oxidation was determined spectrophotometrically at 340 nm and 37 °C as described elsewhere (25). Unless indicated otherwise, samples containing 10 μg/ml nNOS (62.5 nM), 0.2 mM NADPH, 0.5 mM CaCl₂, 0.2 mM CHAPS, 0.1 mM EDTA, 0.1 mM Arg, 10 μM BH₄, 30 μM PROLI/NO, and Na₂S as indicated in 50 mM TEA (pH 7.4) were incubated at 37 °C. The reaction was initiated by the addition of 20 μg/ml CaM and monitored for 5 min. Rates were corrected by subtraction of blank rates obtained in the absence of CaM.

Concentration-effect curves (Figs. 1; 3; 4A; 7, B and C; 8, A and B; and 9A) were fitted to the Hill equation $Act = Act_{\infty} + (Act_0 - Act_{\infty}) / (1 + ([I]/IC_{50})^h)$ in which Act is the observed activity, [I] is the variable concentration of inhibitor (Na₂S in Figs. 1, 3, 4, and 7; PROLI/NO in Fig. 8; and Angeli's salt in Fig. 9), Act₀ and Act_∞ are the respective activities at zero and infinite inhibitor concentration, IC₅₀ is the half-maximal inhibitory concentration, and *h* is the Hill coefficient. Values for IC₅₀, *h*, and Act₀ and in Fig. 3 for Act_∞ were determined from the fits; Act_∞ was set to 0 in Figs. 1, 4, 7, 8, and 9. In Fig. 8B, *h* was set to 2.

The pH dependence of Fig. 4B was fitted to the equation $IC_{50} = K_i / (1 + 10^{pH - pK_a})$ where *K_i* and *pK_a* are the apparent inhibition constant of the protonated inhibitor and the corresponding acidity constant, respectively. This equation describes the dependence of the observed IC₅₀ on pH when inhibition involves the protonated form only. The time traces in the presence of CaM of Fig. 7A were fitted to single exponential functions.

UV/Visible Absorbance Spectroscopy—Spectra were measured with a Hewlett-Packard 8452A diode array spectrophotometer. For absorbance measurements, nNOS or eNOS samples were diluted to a final concentration of approximately 4 μM in 50 mM TEA (pH 7.4) in the absence or presence of 5 mM NaHS.

Gel Filtration—NOS dimerization was analyzed by gel filtration with a Superose 6 HR 10/30 column under the control of an ÄKTA chromatography system at 8 °C. The flow rate was set to 0.3 ml·min⁻¹, and the elution buffer consisted of 20 mM TEA (pH 7.4), 150 mM NaCl, 5% (v/v) glycerol, and 0.5 mM diethylene triamine pentaacetic acid. Purified nNOS (250 μg/ml; 1.6 μM) was incubated for 10 min in 0.4 ml of 50 mM TEA (pH 7.4) containing 0.1 mM Arg, 0.2 mM NADPH, 5 μM FAD, 5 μM FMN, 10 μM BH₄, 0.5 mM CaCl₂, 0.2 mM CHAPS, 0.1 mM EDTA, and 300 μg/ml CaM as indicated in the absence or presence of 0.1 mM Na₂S at 37 °C. Subsequently, 80 μl of ice-cold EGTA (30 mM) was added, and samples were immediately frozen in liquid nitrogen. After thawing, 250-μl aliquots (50 μg of protein) were injected and monitored by UV/visible absorption at 280 nm.

Low Temperature Polyacrylamide Gel Electrophoresis-Western Blotting Analysis—Dimerization was also analyzed by low temperature PAGE (26) followed by Western blotting. Purified nNOS (5 μg/ml; 31.3 nM) or eNOS (5 μg/ml; 37 nM) was incubated for 10 min in 0.1 ml of 50 mM TEA (pH 7.4) containing 0.1 mM Arg, 0.2 mM NADPH, 5 μM FAD, 5 μM FMN, 10 μM BH₄, 0.5 mM CaCl₂, 0.2 mM CHAPS, and 0.1 mM EDTA in the absence or presence of 10 μg/ml CaM and 0.3 mM Na₂S at 37 °C. Reactions were terminated by the addition of 0.1 ml of chilled 0.125 M Tris (pH 6.8) containing 4% (w/v) SDS, 10% (v/v) 2-ME, 20% (w/v) glycerol, and 0.02% (w/v) bromphenol blue.

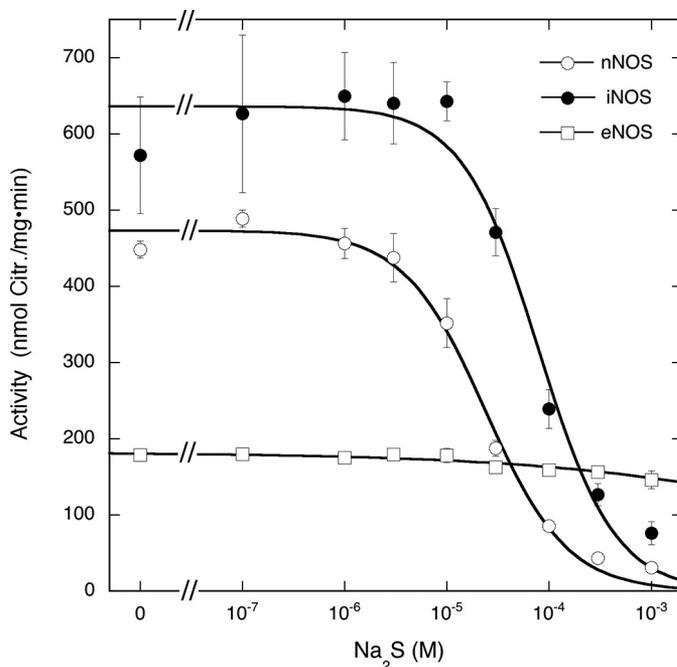


FIGURE 1. Effect of Na₂S on NOS-catalyzed citrulline formation. The figure shows the effect of Na₂S on citrulline (Citr.) formation by nNOS (open circles), iNOS (closed circles), and eNOS (open squares). Samples containing 0.2–0.5 μg of NOS, 0.1 mM [³H]Arg (~60,000 cpm), 0.2 mM NADPH, 5 μM FAD, 5 μM FMN, 10 μM BH₄, 0.5 mM CaCl₂, 10 μg/ml CaM, 0.2 mM CHAPS, 0.1 mM EDTA, and Na₂S as indicated in 0.1 ml of 50 mM TEA (pH 7.4) were incubated for 10 min at 37 °C. Data points (*n* = 3) are presented as mean values ± S.E. (error bars).

Samples containing 50 ng of nNOS or eNOS were subjected to SDS-PAGE for 100 min at 100 V on discontinuous 4% SDS gels (1.5 mm) using the Mini-Protean II system from Bio-Rad. Gels and buffers were equilibrated at 4 °C, and the buffer tank was cooled during electrophoresis in an ice bath. Separated proteins were transferred to nitrocellulose membranes (0.45 μm) by electroblotting at 240 mA for 110 min followed by immunodetection with anti-nNOS or anti-eNOS antibodies (1:1000 or 1:2000 dilution, respectively; BD Transduction Laboratories) using horseradish peroxidase-conjugated anti-mouse IgG (1:5000; BD Transduction Laboratories) and ECL detection reagent (Biozym, Hessisch Oldendorf, Germany). Immunoreactive bands were quantified densitometrically using E.A.S.Y. 1.3 Win 32 (Herolab, Vienna, Austria) and ImageJ 1.46r software (Wayne Rasband, National Institutes of Health).

Results

Effect of Na₂S on Citrulline Formation by nNOS, iNOS, and eNOS—To determine the effect of H₂S on NOS activity, we measured citrulline formation by the NOS isoforms in the presence of varying concentrations of Na₂S. As illustrated in Fig. 1, Na₂S inhibited nNOS and iNOS with IC₅₀ values of $(2.4 \pm 0.3) \cdot 10^{-5}$ and $(7.9 \pm 1.6) \cdot 10^{-5}$ M, respectively, whereas eNOS was only marginally affected.

Effect of Na₂S on the Optical Absorbance Spectra of nNOS—Because it has been demonstrated that DTT and other thiols inhibit NOS by binding to the heme (27), we measured the effect of Na₂S on the UV/visible absorbance of

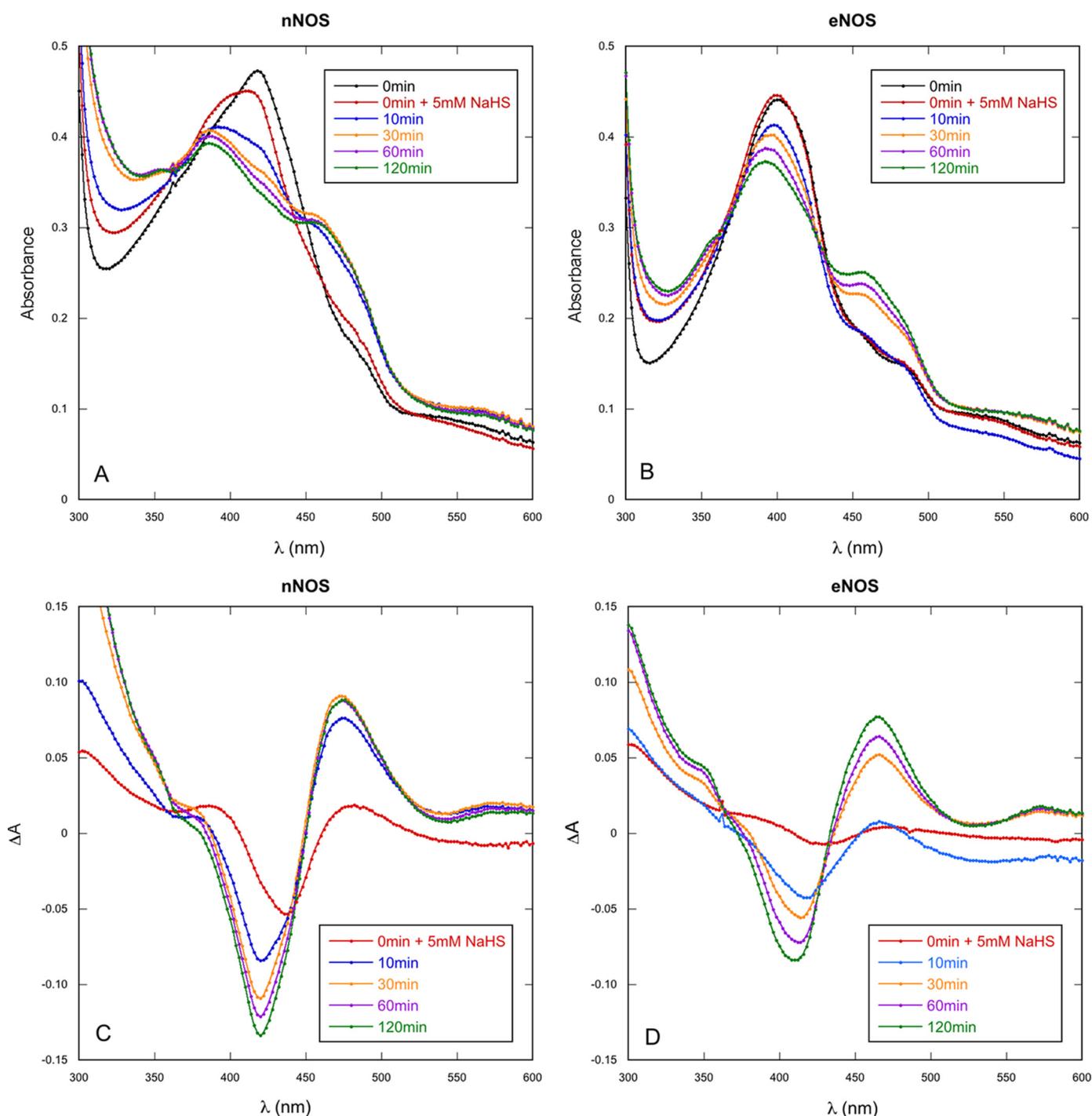


FIGURE 2. Effect of Na₂S on the UV/visible absorbance spectra of nNOS and eNOS. The enzyme (nNOS or eNOS) was diluted to a final concentration of $\sim 4 \mu\text{M}$ in 50 mM TEA (pH 7.4). At time 0, NaHS (5 mM) was added, and spectra were measured at the indicated times. *A* and *B* show the absolute absorbance spectra of nNOS and eNOS, respectively. *C* and *D* show the corresponding difference spectra with the spectrum before Na₂S addition subtracted from all other spectra.

nNOS and eNOS. We observed spectral changes typical of the conversion to a thiol complex (Fig. 2). However, the transition was slow ($t_{1/2} = 3.5 \pm 1.6$ min for nNOS), incomplete (approximately 50%), and required high concentrations (5 mM) of the H₂S donor, suggesting that binding of the thiol to the heme is not involved in NOS inhibition.

Effect of Substrate and Cofactor Concentration on Inhibition of Citrulline Formation by Na₂S—To investigate whether H₂S inhibition is competitive with substrates or cofactors, citrulline

formation by nNOS was examined in the presence of 10-fold higher concentrations of Arg, NADPH, FAD/FMN, BH₄, or CaM. IC₅₀ values were not affected by higher concentrations of these compounds (IC₅₀ values: with 1 mM Arg, $(2.8 \pm 0.4) \cdot 10^{-5}$ M; with 2 mM NADPH, $(2.6 \pm 0.4) \cdot 10^{-5}$ M; with 50 μM FAD and 50 μM FMN, $(2.3 \pm 0.4) \cdot 10^{-5}$ M; with 100 μM BH₄, $(2.3 \pm 0.3) \cdot 10^{-5}$ M; with 100 $\mu\text{g/ml}$ CaM, $(1.7 \pm 0.2) \cdot 10^{-5}$ M; Fig. 3), which indicates that H₂S does not interfere with substrate or cofactor binding.

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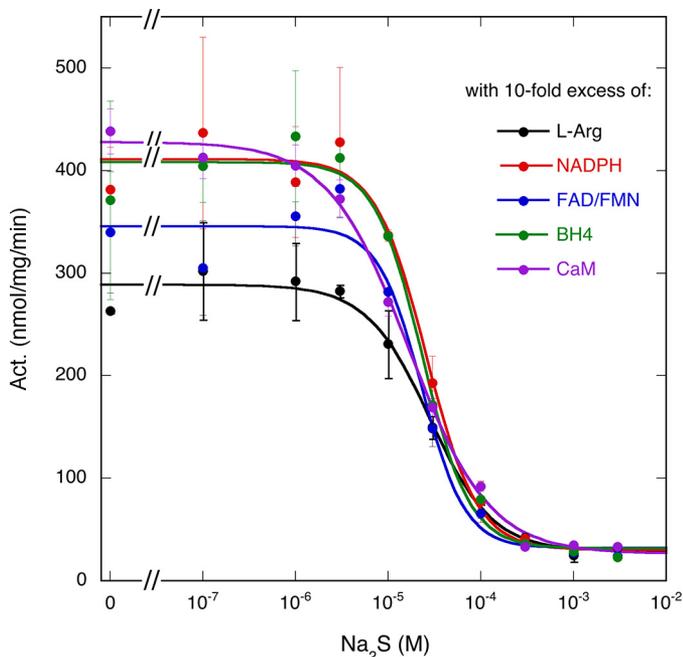


FIGURE 3. Effect of excess Arg, NADPH, FAD/FMN, BH₄, or CaM on Na₂S-induced inhibition of nNOS-catalyzed citrulline formation. The figure shows the effect of 10-fold excess Arg, NADPH, FAD/FMN, BH₄, or CaM on inhibition by Na₂S. Activity (Act.) was measured under the same experimental conditions as in Fig. 1 except that 1 mM Arg (black circles), 2 mM NADPH (red circles), 50 μM FAD/FMN (blue circles), 100 μM BH₄ (green circles), or 100 μg/ml CaM (violet circles) was present. Data points ($n = 2$) are presented as mean values \pm S.E. (error bars).

Effect of Thiols on Na₂S-induced Inhibition of Citrulline Formation by nNOS—Because H₂S can modulate enzyme function by sulphydration of cysteine residues (9–11, 13, 17, 28), it is conceivable that inhibition might be relieved in the presence of excess thiols. Therefore, we measured inhibition by Na₂S in the presence of 2 mM DTT, 2 mM GSH, or 2.9 mM 2-ME. However, none of these thiols had any impact on IC₅₀ values (results not shown).

Effect of Glutathione Persulfide on Citrulline Formation by nNOS—A potential complication is the facile formation of persulfides (RSS⁻) from H₂S in the presence of thiols (2, 9–11, 17, 18). To study the possible involvement of persulfides in H₂S-mediated inhibition of nNOS, we determined the effect of glutathione persulfide (GSSH) on nNOS activity. GSSH, synthesized from Na₂S and GSSG according to a published procedure (19), inhibited nNOS with lower affinity than Na₂S (IC₅₀ = $(1.16 \pm 0.11) \cdot 10^{-4}$ M, $n = 2$; not shown). Because the conversion of GSSG and Na₂S to GSSH by the applied method amounts to about 30–40% (19), the observed inhibition was most likely due to the remaining H₂S. This suggests that persulfides do not significantly contribute to nNOS inhibition.

Effect of pH on Na₂S-induced Inhibition of Citrulline Formation by nNOS—To study the effect of pH on Na₂S-induced inhibition of citrulline formation, we determined the activity of nNOS at pH 6.0, 7.4, and 8.0 (Fig. 4A). The IC₅₀ increased when the pH was raised from $(1.5 \pm 0.2) \cdot 10^{-5}$ M at pH 6.0 via $(3.1 \pm 0.5) \cdot 10^{-5}$ M at pH 7.4 to $(8.3 \pm 1.2) \cdot 10^{-5}$ M at pH 8.0. At first sight, these results suggest that Na₂S-induced inhibition involves interaction of nNOS with H₂S rather than with hydrogen sulfide anion (HS⁻). However, from a plot of IC₅₀ against

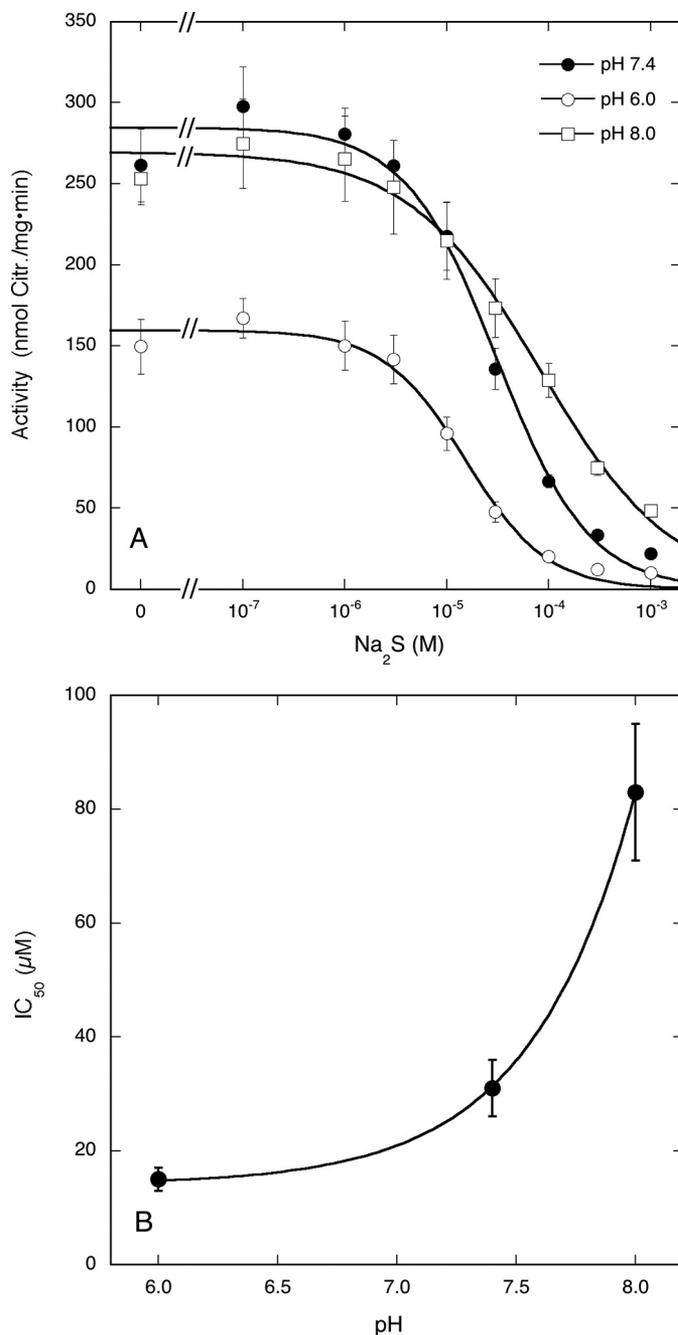


FIGURE 4. Effect of pH on Na₂S-induced inhibition of citrulline formation by nNOS. A shows the inhibition of nNOS-catalyzed citrulline (Citr.) formation by Na₂S at pH 7.4 (closed circles), 6.0 (open circles), and 8.0 (open squares). Samples containing 0.5 μg of NOS, 0.1 mM [³H]Arg (~60,000 cpm), 0.2 mM NADPH, 5 μM FAD, 5 μM FMN, 10 μM BH₄, 0.5 mM CaCl₂, 10 μg/ml CaM, 0.2 mM CHAPS, 0.1 mM EDTA, and Na₂S as indicated in 0.1 ml of 50 mM Bis-tris propane (pH as indicated) were incubated for 10 min at 37 °C. Data points ($n = 3-4$) are presented as mean values \pm S.E. (error bars). B shows the IC₅₀ values as a function of pH.

pH assuming inhibition by the low pH species only, we obtained a pK_a value of 7.310 ± 0.014 (Fig. 4B), which is considerably higher than the published pK_a (6.76 at 37 °C) of the H₂S/HS⁻ equilibrium (29). The pH profile of inhibition therefore appears to reflect the protonation state of another compound.

Irreversible Inhibition by Na₂S under Turnover Conditions—As illustrated in Fig. 5, preincubation of nNOS with Na₂S under turnover conditions decreased the activity after dilution (with

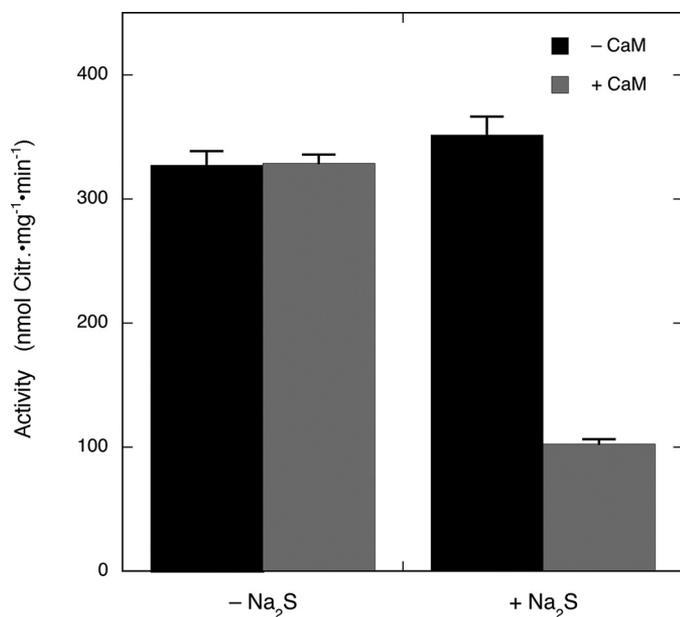


FIGURE 5. Reversibility of nNOS inhibition after Na₂S preincubation. Neuronal NOS was preincubated with or without Na₂S in the absence or presence of CaM. The figure shows citrulline (*Citr.*) formation by nNOS after 150-fold dilution of samples that were preincubated in the absence or presence of CaM and Na₂S. See "Experimental Procedures" for details. Samples containing nNOS (250 μg/ml; 1.6 μM), 0.1 mM Arg, 0.2 mM NADPH, 5 μM FAD, 5 μM FMN, 10 μM BH₄, 0.5 mM CaCl₂, 0.2 mM CHAPS, 0.1 mM EDTA, 300 μg/ml CaM, and 0.5 mM Na₂S as indicated in 0.1 ml of 50 mM TEA (pH 7.4) were preincubated for 3 min at 37 °C. After dilution, samples containing nNOS (1.7 μg/ml; 10.6 nM), 0.1 mM [³H]Arg (~60,000 cpm), 0.2 mM NADPH, 5 μM FAD, 5 μM FMN, 10 μM BH₄, 0.5 mM CaCl₂, 10 μg/ml CaM, 0.2 mM CHAPS, and 0.1 mM EDTA in 0.1 ml of 50 mM TEA (pH 7.4) were incubated for 10 min at 37 °C. Assays also contained ~3 μM Na₂S carried over from the preincubation mixture. Data (*n* = 3–4) are presented as mean values ± S.E. (error bars).

~3.3 μM Na₂S remaining) by approximately 70%, suggesting that inhibition by H₂S is irreversible. However, the activity of the diluted enzyme was not affected when CaM was omitted during preincubation, which suggests that irreversible inhibition requires the presence of both H₂S and NO. To elucidate whether thiols could reverse inhibition, we added 2 mM DTT, 2 mM GSH, or 2.9 mM 2-ME to the activity assay. As shown in Fig. 6A, none of these thiols restored the activity. Similarly, neither bovine serum albumin (2 mg/ml; data not shown) nor thioredoxin/thioredoxin reductase (Fig. 6B) reversed inhibition.

Similar observations were made with iNOS (not shown): preincubation in the absence and presence of 0.5 mM Na₂S yielded activities after dilution of 528 ± 75 and 257 ± 45 nmol of citrulline/mg/min when CaM was present during preincubation, whereas the corresponding activities were 569 ± 65 and 617 ± 90 nmol/mg/min when CaM was omitted. As with nNOS, virtually identical results were obtained when 2 mM GSH was added to the assay mixture.

Effect of the Enzyme Concentration on Inhibition of nNOS and eNOS by H₂S under Turnover Conditions—Because eNOS has lower turnover than nNOS and iNOS, the lack of inhibition of eNOS might be caused by the lower NO formation rate of that isoform. To explore that possibility, we determined the effect of the concentration of nNOS and eNOS (between 0.5 and 15.0 μg/ml and between 2.0 and 30.0 μg/ml, respectively) on the inhibition by 0.5 mM Na₂S (Table 1). Both isoforms exhibited constant specific activities over the studied concentration

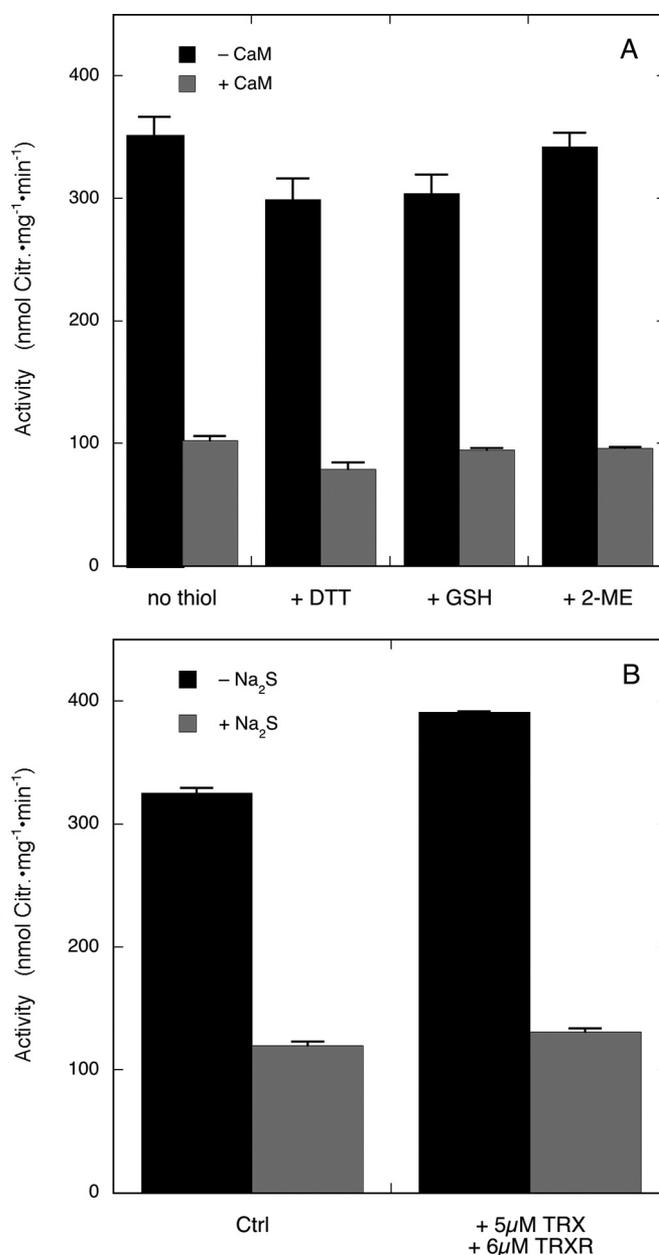


FIGURE 6. Effect of thiols and thioredoxin/thioredoxin reductase on inhibition of nNOS by Na₂S. A shows the effect of various thiols on the reversibility of inhibition of nNOS by Na₂S. Citrulline (*Citr.*) formation by nNOS was determined after preincubation in the absence or presence of Na₂S and CaM. Experimental conditions were the same as for Fig. 5 except that thiol (2 mM DTT, 2 mM GSH, or 2.9 mM 2-ME) was added to the 50-fold diluted reaction mixture (see "Experimental Procedures"). Note that the final mixtures contained ~3 μM Na₂S carried over from the preincubation mixture and ~0.7 mM DTT, ~0.7 mM GSH, or ~1 mM 2-ME carried over from the 50-fold diluted samples. Data (*n* = 2) are presented as mean values ± S.E. (error bars). B shows the effect of inclusion of thioredoxin/thioredoxin reductase on the reversibility of inhibition of nNOS by Na₂S. Citrulline formation by nNOS was determined after preincubation in the absence or presence of Na₂S. Experimental conditions were as for Fig. 5 except for the presence of thioredoxin reductase (TRXR) (6 μM) and thioredoxin-1 (TRX) (5 μM) during the final assay. Note that the final assay samples also contained ~3 μM Na₂S carried over from preincubation mixture. Data (*n* = 2) are presented as mean values ± S.E. (error bars). Ctrl, control.

range. However, whereas nNOS activity was almost completely (~90%) blocked at all concentrations, eNOS activity was hardly affected even though the estimated NO formation rate (in the absence of Na₂S) for the highest concentration of eNOS was

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TABLE 1

Effect of the enzyme concentration on H₂S-induced inhibition of nNOS and eNOS

Conc is the enzyme concentration; Act₀ and Act_{H₂S} are the specific activities measured as citrulline formation in the absence and presence of 0.5 mM H₂S, respectively; v_{NO}⁰ and v_{NO}^{H₂S} are the corresponding NO formation rates in μM/min. See the legend to Fig. 1 for other experimental conditions.

Isoform	Conc	Act ₀	v _{NO} ⁰	Act _{H₂S}	v _{NO} ^{H₂S}	Inhibition
	μg/ml	nmol/mg/min	μM/min	nmol/mg/min	μM/min	%
nNOS	0.5	383 ± 36	0.19 ± 0.02	53 ± 26	0.026 ± 0.013	86 ± 7
	2.0	405 ± 63	0.81 ± 0.13	31 ± 8	0.062 ± 0.016	92 ± 2
	10.0	306 ± 10	3.06 ± 0.10	24 ± 2	0.24 ± 0.02	92 ± 1
	15.0	309 ± 25	4.6 ± 0.4	29 ± 2	0.44 ± 0.03	91 ± 1
eNOS	2.0	129 ± 22	0.26 ± 0.04	98 ± 12	0.20 ± 0.02	24 ± 16
	10.0	160 ± 14	1.60 ± 0.14	134 ± 16	1.34 ± 0.16	16 ± 12
	15.0	153 ± 11	2.30 ± 0.17	146 ± 19	2.2 ± 0.3	5 ± 14
	30.0	128 ± 23	3.8 ± 0.7	136 ± 38	4.1 ± 1.1	-6 ± 35

20× as high as that for the lowest concentration of nNOS. In the presence of Na₂S, 30 μg/ml eNOS produced ~160× as much NO as 0.5 μg/ml nNOS did. These results clearly demonstrate that the lack of inhibition of eNOS is not due to its lower intrinsic activity.

Effect of Na₂S on NADPH Oxidation by nNOS—To examine whether inhibition of citrulline formation was accompanied by NOS uncoupling, we determined the effect of Na₂S on the rate of NADPH oxidation in the absence or presence of Arg and/or BH₄ (Fig. 7, A and B). The NADPH oxidation rate under control conditions in the presence of Arg and BH₄ (714 ± 23 nmol·mg⁻¹·min⁻¹) corresponds to a NADP⁺/citrulline stoichiometry of 1.51 ± 0.06, indicative of strong coupling (7). Na₂S completely blocked NADPH oxidation with an IC₅₀ of (1.9 ± 0.3)·10⁻⁵ M in good accordance with the value observed for citrulline formation (Fig. 1). This indicates that inhibition targets NADPH oxidation without any sign of uncoupling. Interestingly, when Arg and/or BH₄ were omitted, conditions under which no irreversible inhibition of citrulline formation occurs (see above), NADPH oxidation was still blocked but at considerably higher concentrations of Na₂S (IC₅₀ values: +Arg/-BH₄, (3.3 ± 0.4)·10⁻⁴ M; -Arg/+BH₄, (6.1 ± 1.1)·10⁻⁴ M; -Arg/-BH₄, (9.5 ± 1.4)·10⁻⁵ M). These observations suggest that H₂S alone inhibits nNOS reversibly with an IC₅₀ of ~0.1–0.6 mM but that inhibition becomes more pronounced and irreversible in the presence of NO.

To confirm this, we repeated the experiments in which Arg and/or BH₄ was omitted in the presence of the NO donor PROLI/NO (Fig. 7C). Under these conditions, 30 μM PROLI/NO lowered the IC₅₀ to values similar to those observed in the combined presence of Arg and BH₄ (IC₅₀ values: +Arg/-BH₄, (1.5 ± 0.5)·10⁻⁵ M; -Arg/+BH₄, (2.0 ± 0.9)·10⁻⁵ M; -Arg/-BH₄, (1.34 ± 0.19)·10⁻⁵ M). These results confirm that inhibition by H₂S is potentiated by NO.

Effect of the NO Concentration on Inhibition of nNOS and eNOS by Na₂S—To study the effect of the NO concentration, we measured the rate of NADPH formation at varying PROLI/NO concentrations in the presence of Arg but in the absence of BH₄ under which conditions the enzyme does not produce NO. Determination of the effect of the NO concentration is complicated by the fact that Na₂S alone already inhibits the enzyme (see Fig. 7B). Moreover, NO alone will also inhibit NOS activity by binding to the heme (30–32). Therefore we determined the effect of the NO concentration in the absence and presence of 10 μM Na₂S, a concentration that does not by

itself inhibit NADPH oxidation but that becomes inhibitory in the presence of PROLI/NO (Fig. 7, B and C, blue traces). As illustrated by Fig. 8A, PROLI/NO inhibited NADPH activity with an IC₅₀ of ~ (8.0 ± 0.5)·10⁻⁵ M in the absence of Na₂S, which is most likely caused by binding of NO to the heme. In the presence of Na₂S, the IC₅₀ shifted leftward to (1.1 ± 0.2)·10⁻⁵ M, probably reflecting the (irreversible) effect of NO on H₂S-induced inhibition.

For comparison, we also looked into the effect of the PROLI/NO concentration in the presence of Na₂S on eNOS activity. Unlike nNOS, eNOS exhibits greatly reduced NADPH oxidation when either BH₄ or Arg is omitted (33). Therefore we decided to include Arg and BH₄ in the reaction mixture, and as a consequence, the enzyme already produces NO in the absence of PROLI/NO. We also applied a much higher Na₂S concentration (1 mM) because at that concentration we earlier observed moderate inhibition of eNOS activity (see Fig. 1). Fig. 8B shows that in this case too NADPH oxidation was inhibited by high concentrations of PROLI/NO (IC₅₀ = (1.0 ± 0.2)·10⁻⁴ M). Fig. 8B also confirms the moderate effect of 1 mM Na₂S on NADPH oxidation. However, this weak inhibitory effect was not potentiated by PROLI/NO (IC₅₀ = (1.17 ± 0.16)·10⁻⁴). Taken together, these results demonstrate that NO concentration-dependently potentiates the inhibition by Na₂S of nNOS but not of eNOS.

In the experiments described above, the effect of NO on H₂S-induced inhibition of nNOS was clearly concentration-dependent. By contrast, in the experiments of Table 1, similar inhibition was observed at all nNOS concentrations and therefore at all NO concentrations. This suggests that in the studied enzyme concentration range (which corresponded to NO concentrations after 10 min between 2 and 50 μM in the absence of Na₂S and approximately 10-fold lower concentrations in the presence of Na₂S) the potentiation by NO of H₂S-induced inhibition is not affected by its concentration. To corroborate this finding, we determined the effect of the nNOS concentration on the IC₅₀ value of Na₂S. Variation of the nNOS concentration did not affect the IC₅₀ values (22 ± 5, 25 ± 6, and 37 ± 3 μM at 1, 5, and 15 μg/ml, respectively), confirming that in this concentration range, which corresponds to uninhibited NO formation rates between 0.37 and 4.3 μM/min, inhibition does not depend on the NO concentration.

Effect of Slow H₂S and NO Donors on nNOS Activity—To study the effect of the rate of H₂S generation on the inhibition of nNOS, we replaced Na₂S, which releases H₂S almost instantaneously, by the slow H₂S-releasing agent GYY4137 (t_{1/2} ~ 415

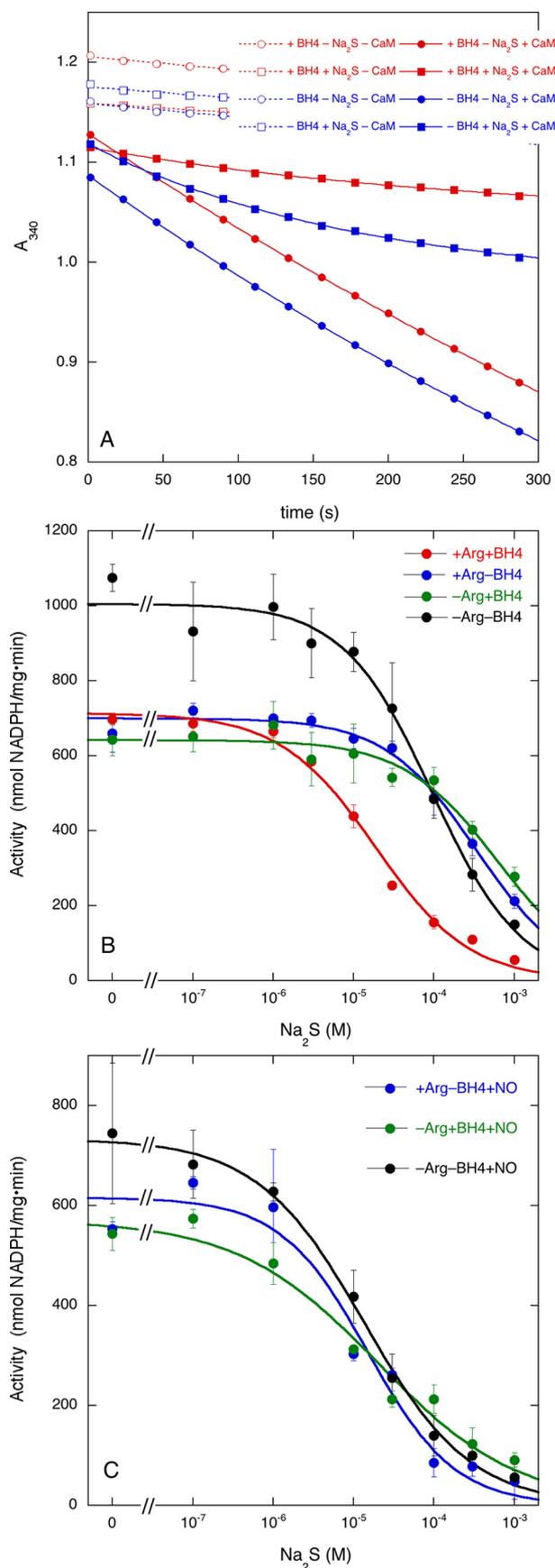


FIGURE 7. Effect of Na₂S on NADPH oxidation by nNOS in the absence or presence of NO. A shows representative NADPH oxidation traces as measured by the absorbance decrease at 340 nm. Activity was determined in the

min; Ref. 34). Under full-turnover conditions, *i.e.* in the presence of BH₄ and Arg, citrulline formation over a time interval of 25 min was inhibited by GYY4137 with an apparent IC₅₀ value of $(2.2 \pm 0.3) \cdot 10^{-3}$ M (not shown). At that concentration, GYY4137 will release $\sim 9 \cdot 10^{-5}$ M H₂S in fair agreement with the IC₅₀ value obtained with Na₂S under similar conditions (see Fig. 1).

To study the effect of the NO release rate, we replaced PROLI/NO ($t_{1/2} \sim 1$ –2 s) by SPER/NO ($t_{1/2} \sim 1800$ s) (35). In the absence of BH₄, *i.e.* when the enzyme does not produce NO, SPER/NO potentiated the inhibitory effect of Na₂S on NADPH formation after 10 min with an apparent IC₅₀ value of $(1.06 \pm 0.17) \cdot 10^{-4}$ M (not shown). At this concentration, SPER/NO will release approximately 24 μ M NO in 10 min in good agreement with the values obtained with PROLI/NO (see Fig. 7C).

Inhibition of nNOS by HNO—According to a recent report, the combination of NO and H₂S regulates vascular tone by the intermediate formation of HNO (36), and it has been reported in the past that HNO is a stronger inhibitor of nNOS than NO (37). To investigate the potential involvement of HNO in the inhibition observed here, we determined the effect of the HNO donor Angeli's salt on citrulline formation by nNOS. As illustrated by Fig. 9A, Angeli's salt inhibited nNOS, but the IC₅₀ value of $(1.9 \pm 0.4) \cdot 10^{-4}$ M was considerably higher than that of Na₂S (see Fig. 1). More importantly, unlike the effect of Na₂S, inhibition by HNO was completely reversed in the presence of thiols (Fig. 9B).

Effect of Na₂S on Dimeric Structure of nNOS and eNOS in the Absence or Presence of NO Synthesis—To investigate the effect of H₂S on the dimer content of nNOS, we performed gel filtration chromatography after preincubation under various conditions. Because dimer stability is affected by Arg and BH₄ but not by CaM (26), both Arg and BH₄ were included in all preincubations, and the effect of NO formation was instead determined by omitting or including CaM. As shown in Fig. 10, after preincubation in the absence of CaM and Na₂S, the enzyme was mostly ($\sim 70\%$) dimeric. Preincubation in the presence of CaM or Na₂S appeared to cause a slight decrease in dimer content, whereas a somewhat larger decrease was observed when CaM and Na₂S were both present. However, $\sim 55\%$ of the enzyme was still dimeric even after preincubation under full-turnover conditions. Similar observations were made with low temperature PAGE followed by Western blotting analysis. As shown in Fig. 11, A and B, Na₂S alone did not affect dimer stability (33.2 ± 2.6 versus $34.0 \pm 1.8\%$), whereas the combination of

presence (closed symbols, continuous lines) or absence (open symbols, dotted lines; "blanks") of 20 μ g/ml CaM in the presence (red symbols and lines; NO-producing conditions) or absence (blue symbols and lines; non-NO-producing conditions) of 10 μ M BH₄ and in the absence (circles; uninhibited traces) or presence (squares; inhibited traces) of 1 mM Na₂S. In the experiments shown in A, Arg was always present, but similar experiments were performed in the absence of Arg (not shown). B shows the effect of Na₂S on nNOS-catalyzed NADPH oxidation in the absence or presence of Arg and/or BH₄. C shows the effect of inclusion of the NO donor PROLI/NO in the assay mixture. Samples (0.25 ml) contained 2.5 μ g of NOS, 0.2 mM NADPH, 0.5 mM CaCl₂, 20 μ g/ml CaM (except for A, open symbols, dotted lines), 0.2 mM CHAPS, and 0.1 mM EDTA in 50 mM TEA (pH 7.4) and were incubated for 5 min at 37 °C; 0.1 mM Arg, 10 μ M BH₄, 30 μ M PROLI/NO, and Na₂S at varying concentrations (1 mM in A) were present as indicated. The reaction was initiated by the addition of CaM. Data ($n \geq 2$) are presented as mean values \pm S.E. (error bars).

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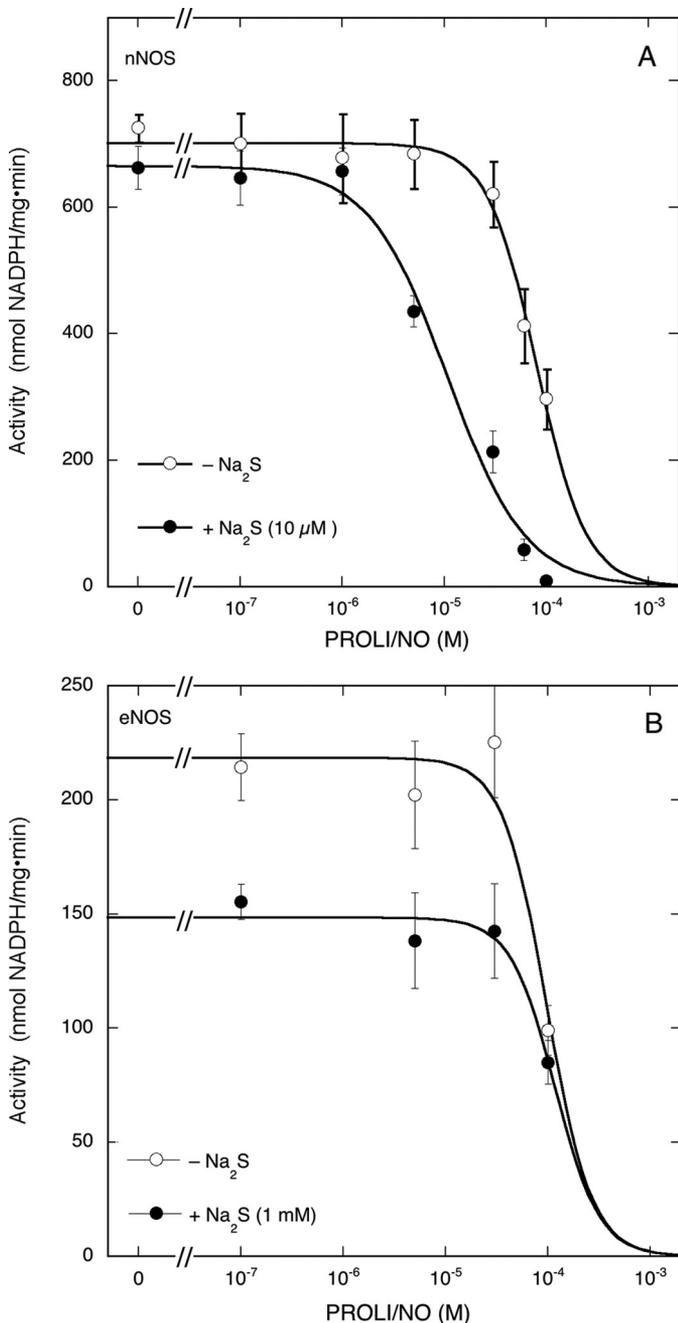


FIGURE 8. Effect of PROLI/NO on NADPH oxidation by nNOS and eNOS in the absence or presence of Na₂S. *A* shows the effect of PROLI/NO on nNOS-catalyzed NADPH oxidation in the presence of Arg and the absence of BH₄ with and without Na₂S. *B* shows the effect of PROLI/NO on eNOS-catalyzed NADPH oxidation in the presence of Arg and BH₄ with and without Na₂S. Samples (0.25 ml) containing 2.5 μg of nNOS (*A*) or 4 μg of eNOS (*B*), 0.1 mM Arg, 0.2 mM NADPH, 10 μM BH₄ (*B* only), 0.5 mM CaCl₂, 20 μg/ml CaM, 0.2 mM CHAPS, 0.1 mM EDTA, Na₂S (10 μM in *A*; 1 mM in *B*), and PROLI/NO as indicated in 50 mM TEA (pH 7.4) were incubated for 5 min at 37 °C. Reactions were initiated by the addition of CaM. Data points ($n \geq 3$) are presented as mean values \pm S.E. (error bars).

CaM and Na₂S reduced the amount of SDS-resistant dimers by more than half (11.0 ± 1.6 versus $25.6 \pm 1.5\%$). The dimer/monomer ratio of eNOS was not affected by Na₂S at all (Fig. 11C). Although these results suggest some correlation between dimer strength and NO/H₂S-induced inhibition, nNOS remained mainly dimeric under conditions that resulted in

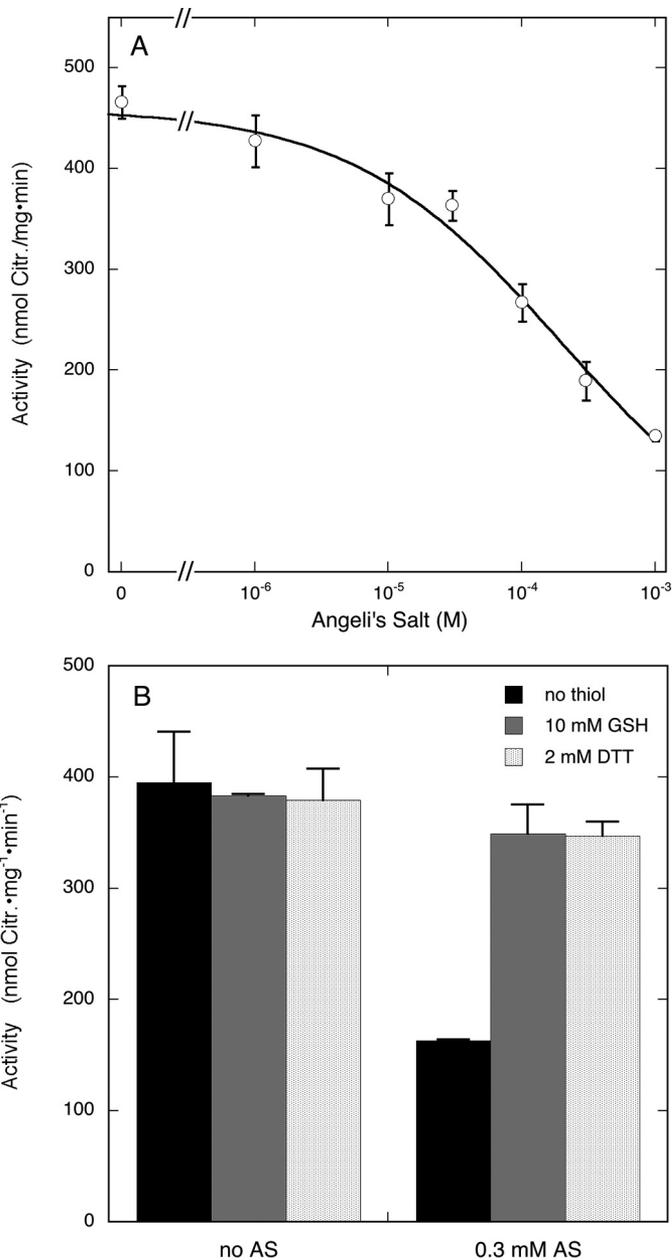


FIGURE 9. Effect of Angeli's salt on citrulline formation by nNOS. *A* shows the concentration dependence of the inhibition by Angeli's salt of nNOS-catalyzed citrulline (*Citr.*) formation. Samples (0.1 ml) containing 0.5 μg of NOS, 0.1 mM [³H]Arg (~60,000 cpm), 0.2 mM NADPH, 5 μM FAD, 5 μM FMN, 10 μM BH₄, 0.5 mM CaCl₂, 10 μg/ml CaM, 0.2 mM CHAPS, 0.1 mM EDTA, and Angeli's salt as indicated in 50 mM TEA (pH 7.4) were incubated for 10 min at 37 °C. Reactions were initiated by the addition of CaM. Data points ($n = 2$) are presented as mean values \pm S.E. (error bars). *B* shows the effect of thiols on the inhibition by Angeli's salt (AS). Experimental conditions were the same as for *A* except for the presence or absence of 10 mM GSH or 2 mM DTT as indicated ($n = 2$).

complete loss of activity, indicating that the main mechanism for inhibition does not involve monomerization.

Discussion

Inhibition of NOS by H₂S has been reported previously by Kubo *et al.* (15, 16). In those studies, all three isoforms were inhibited with comparably low potencies (IC₅₀ values between 0.13 and 0.21 mM). Furthermore (contrary to what was stated in Ref. 16), inhibition was partly or completely countered by

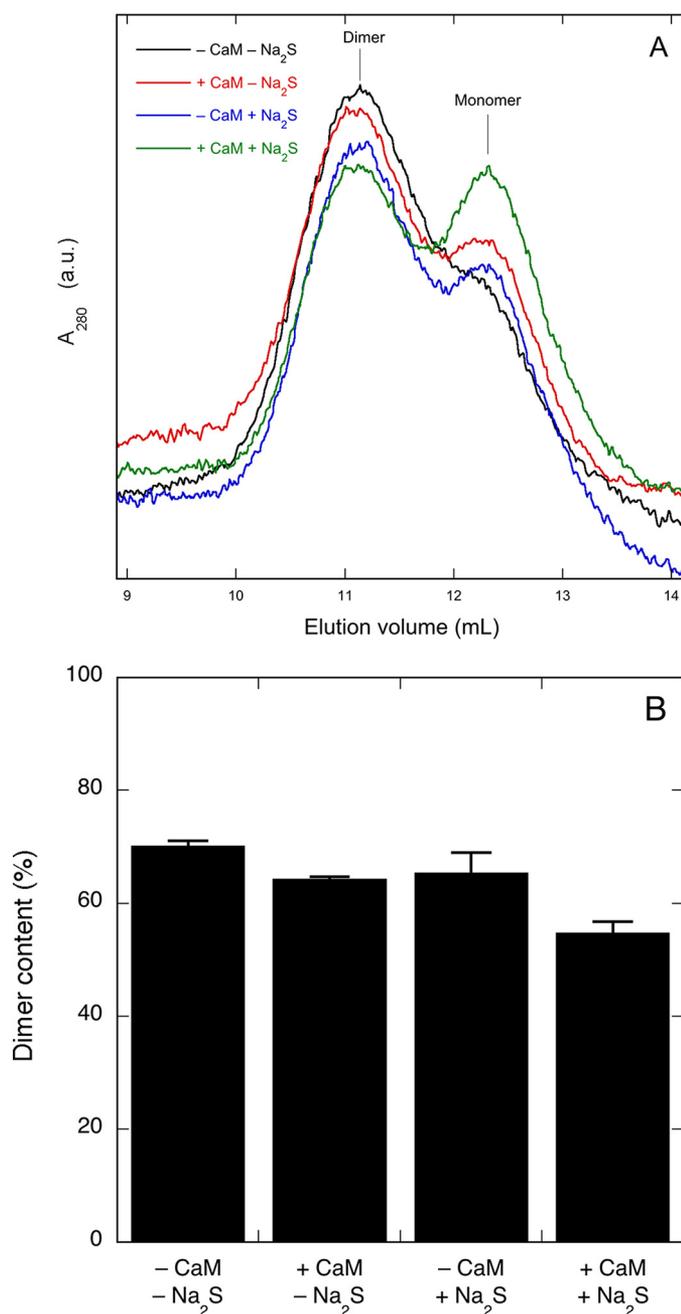


FIGURE 10. Effect of NO/H₂S on the dimer content of nNOS. A shows representative elution profiles of nNOS after preincubation in the presence or absence of CaM and Na₂S (black curve, -CaM -Na₂S; red curve, +CaM -Na₂S; blue curve, -CaM +Na₂S; green curve, +CaM +Na₂S). B shows the corresponding quantification (*n* = 2–3). Preincubation conditions were as follows: 250 μg/ml (1.6 μM) nNOS, 0.1 mM Arg, 0.2 mM NADPH, 5 μM FAD, 5 μM FMN, 10 μM BH₄, 0.5 mM CaCl₂, 0.2 mM CHAPS, 0.1 mM EDTA, 300 μg/ml CaM, and 0.1 mM Na₂S as indicated in 0.4 ml of 50 mM TEA (pH 7.4) at 37 °C for 10 min. Data are presented as mean values ± S.E. (error bars). a.u., absorbance units.

increasing the NADPH concentration and except for iNOS by increasing the BH₄ concentration. The reason for these and other discrepancies, the study also reported an IC₅₀ for inhibition of nNOS by DTT of 13.2 mM where we previously found 0.16 mM (27), cannot be resolved here but may be related to the use of commercial enzyme preparations with low activity and different experimental conditions (for instance, extremely low

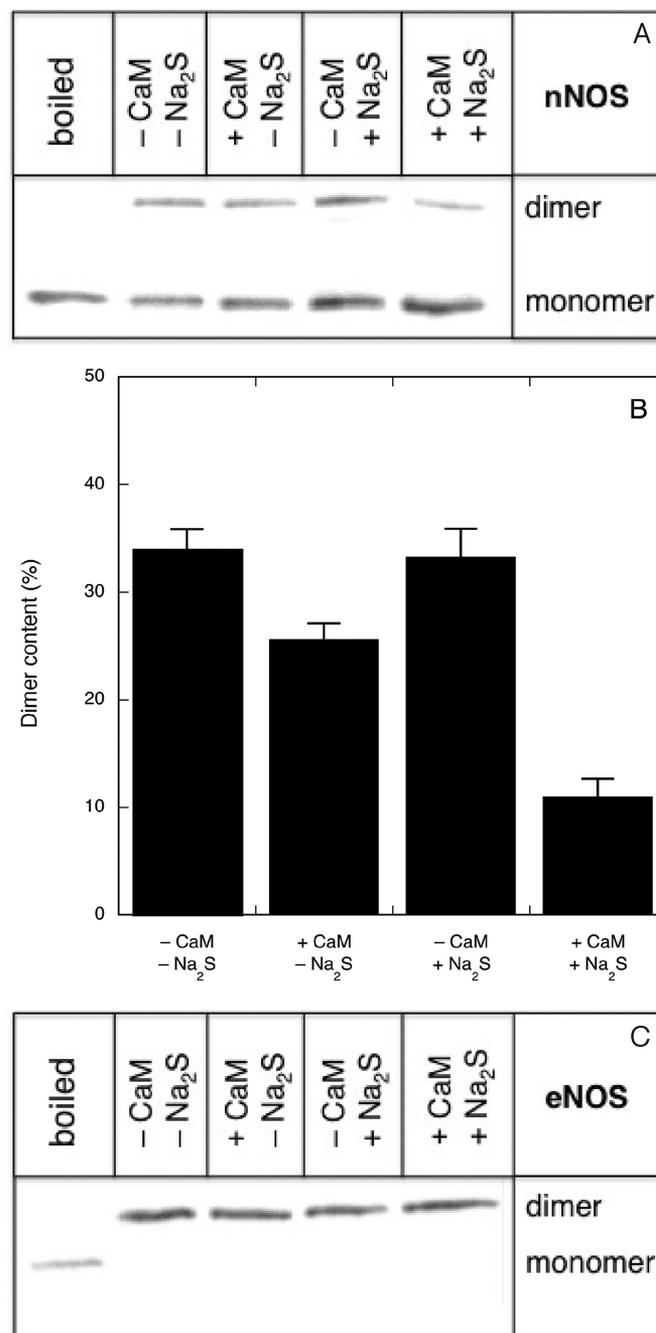


FIGURE 11. Effect of Na₂S on the strength of the dimeric structure of nNOS and eNOS in the absence or presence of NO synthesis. A shows a representative Western blot illustrating the SDS-resistant nNOS dimer/monomer ratio. B shows the corresponding densitometric quantification. Data are presented as mean values ± S.E. (error bars). C shows a representative blot of the effect of Na₂S on the strength of the dimeric structure of eNOS in the absence or presence of NO synthesis. nNOS (5 μg/ml; 31.3 nM) or eNOS (5 μg/ml; 37 nM) was incubated for 10 min in 0.1 ml of 50 mM TEA (pH 7.4) containing 0.1 mM Arg, 0.2 mM NADPH, 5 μM FAD, 5 μM FMN, 10 μM BH₄, 0.5 mM CaCl₂, 0.2 mM CHAPS, and 0.1 mM EDTA in the absence or presence of 10 μg/ml CaM and/or 0.3 mM Na₂S at 37 °C. Reactions were terminated by the addition 0.1 ml of chilled 0.125 M Tris (pH 6.8) containing 4% (w/v) SDS, 10% (v/v) 2-ME, 20% (w/v) glycerol, and 0.02% (w/v) bromphenol blue. See “Experimental Procedures” for further details (*n* = 2–4).

concentrations of Arg and CaM and very long incubation times).

The respective mechanisms of inhibition by H₂S and by NO/H₂S are unclear. Inhibition is not competitive with any

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of the substrates or cofactors. Furthermore, inhibition by NO/H₂S but not by H₂S alone is irreversible under the present conditions. A puzzling aspect of the present study is the apparent difference in the inhibitory efficiency of nNOS- and PROLI/NO-generated NO. No [NO] dependence was observable down to concentrations as low as 0.5 μg/ml nNOS, which corresponds to a production of NO of ~0.3 μM after 10 min in the presence of Na₂S. In contrast, PROLI/NO exhibited an apparent IC₅₀ of ~10⁻⁵ M. Tentatively, one may ascribe this remarkable difference to the close proximity of the sites of NO formation and H₂S inhibition in the case of endogenously produced NO.

Cysteiny side chains are the most likely targets for inhibition by H₂S. In the presence of an electron acceptor, H₂S may cause protein S-sulfhydration (17, 28). Indeed, S-sulfhydration of NOS has been reported recently (38). However, in that study, eNOS was stimulated rather than inhibited by sulfhydration. There have also been several reports on eNOS glutathionylation, which blocked NO synthesis but not NADPH oxidation, resulting in uncoupled catalysis (39–41). By contrast, we are not aware of any study on the glutathionylation or sulfhydration of the neuronal and inducible isoforms. All three NOS isoforms are also inhibited by S-nitrosation (42–45), which targets the cysteinyl side chains coordinating the zinc cation that stabilizes the NOS dimeric structure (46, 47). In addition to modification of cysteinyl side chains, it is conceivable that H₂S directly interferes with NOS zinc binding as has been proposed as a potential inhibitory mechanism in the case of angiotensin-converting enzyme and phosphodiesterase (48, 49). If the inter-domain zinc cation or its cysteinyl ligands are indeed the target for inhibition by H₂S, this would offer a tentative explanation for the remarkable resistance of eNOS to inhibition. Of the three isoforms, eNOS has by far the greatest dimer stability (50). Although the present results indicate that inhibition is not caused by NOS monomerization, it is conceivable that the same forces that stabilize the eNOS dimer also protect the zinc site against inhibition by H₂S.

Whereas sulfhydration of specific cysteinyl residues might be causing the low affinity reversible inhibition, irreversible inhibition in the presence of NO may involve a product of the reaction between H₂S and NO. We recently demonstrated efficient nitrosation of GSH and other thiols by NO at submicromolar concentrations (35, 51). A similar reaction with H₂S would yield HSNO, which in principal might inhibit NOS by transnitrosation of one of the cysteinyl zinc ligands. However, the observation that inhibition was not reversed by thiols or thioredoxin/thioredoxin reductase argues against that possibility. For the same reason, the involvement of HNO can be ruled out as well. In the presence of excess H₂S, the highly unstable HSNO is rapidly transformed to nitrosopersulfide (SSNO⁻) (17, 18, 52). Conceivably, it is this compound that is responsible for irreversible inhibition of nNOS and iNOS. Although as far as we are aware the pK_a of nitrosopersulfide has not been reported, it is tempting to ascribe the value of 7.3 that we observed for nNOS inhibition to the HSSNO/SSNO⁻ equilibrium. Alternatively, NO or an NO-derived compound may react with the sulfhydrated protein formed by H₂S in the absence of NO, which would possibly explain the absence of an effect of the NO con-

centration on inhibition (although not the higher potency of NO/H₂S compared with H₂S alone). Clearly, elucidation of the inhibitory mechanism must await identification and characterization of the inhibitory site. To this end, we are currently performing mass spectrometric analysis of the modification of nNOS by NO/H₂S. Preliminary results suggest that a specific cysteine residue in the reductase domain (Cys¹²³¹) becomes sulfinated in the presence of H₂S under turnover conditions.³ However, additional studies are required to confirm or refute these observations.

The present results demonstrate that H₂S completely blocks nNOS activity (coupled and uncoupled) at moderately high concentrations. Importantly, inhibition gets stronger and becomes irreversible under conditions of coupled turnover or when NO is co-administered. Similar effects were observed for iNOS but not for eNOS, demonstrating that inhibition by NO/H₂S is isoform-specific. There is controversy in the literature on the physiological levels of H₂S with earlier reports suggesting unrealistically high values (for a review, see Ref. 53), whereas more recent estimates seem to converge on values in the submicromolar or even low nanomolar range (2, 10, 54, 55). Whereas the higher estimates would render the effects observed here physiologically relevant, inhibition by H₂S alone would be too weak to play a significant role if the lower estimates apply. However, because of its apparent irreversible nature, inhibition by NO/H₂S might still be relevant. One may speculate that such inhibition could serve a protective role as a negative feedback mechanism in the case of excessive NO/H₂S production. It will therefore be important to establish whether inhibition by NO/H₂S remains irreversible in an *in vivo* setting.

In summary, we have observed inhibition of nNOS but not of eNOS that may be physiologically relevant provided that the irreversible character observed here persists under (patho)physiological conditions. If so, these observations may help resolve some of the controversies concerning the impact of H₂S on NO signaling where both stimulatory and inhibitory effects have been reported.

Author Contributions—The study was conceived by C. L. H., K. S., B. M., and A. C. F. G. and designed by C. L. H. and A. C. F. G. Data were acquired and analyzed by C. L. H., R. S., K. G., and B. G. and interpreted by C. L. H., A. S., and B. G. The paper was written by C. L. H. and A. C. F. G. and revised by A. S., K. S., and B. M. All authors reviewed the results and approved the final version.

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