

Activation of Soluble Guanylyl Cyclase by the Nitrovasodilator 3-Morpholinosydnonimine Involves Formation of S-Nitrosogluthathione

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ABSTRACT

Soluble guanylyl cyclase (sGC) is the major physiological target of sydnonimine-based vasodilators such as molsidomine. Decomposition of sydnonimines results in the stoichiometric formation of nitric oxide (NO) and superoxide ($O_2^{\cdot-}$), which rapidly react to form peroxynitrite. Inasmuch as sGC is activated by NO but not by peroxynitrite, we investigated the mechanisms underlying sGC activation by 3-morpholinosydnonimine (SIN-1). Stimulation of purified bovine lung sGC by SIN-1 was found to be strongly dependent on glutathione (GSH). By contrast, GSH did not affect sGC activation by NO released from 2,2-diethyl-1-nitroso-oxyhydrazine, indicating that NO/ $O_2^{\cdot-}$ released from SIN-1 converted GSH to an activator of sGC. High performance

liquid chromatography identified this product as the thionitrite S-nitrosogluthathione. Further, the reaction product decomposed to release NO upon addition of $Cu(NO_3)_2$ in the presence of GSH. Activation of sGC was antagonized by the Cu(I)-specific chelator neocuproine, whereas the Cu(II)-selective drug cuprizone was less potent. Carbon dioxide (delivered as $NaHCO_3$) antagonized S-nitrosation by peroxynitrite but not by SIN-1. Thus, NO/ $O_2^{\cdot-}$ released from SIN-1 mediates a CO_2 -insensitive conversion of GSH to S-nitrosogluthathione, a thionitrite that activates sGC via trace metal-catalyzed release of NO. These results may provide novel insights into the molecular mechanism underlying the nitrovasodilator action of SIN-1.

Nitrovasodilation is a prominent therapeutic strategy in the treatment of coronary artery disease. Because of their vasodilatory and antithrombotic properties, sydnonimine-based prodrugs such as molsidomine or pirsidomine are used clinically to improve the hemodynamics in distinct ischemic pathologies (Darius *et al.*, 1984). The pharmacological profile of sydnonimines is similar to that of organic nitrates, although the onset of action is slower and the preload-reducing effect is more pronounced with sydnonimines. The development of tolerance during long-term administration is disputed (Kaiser *et al.*, 1983; Thulesius, 1984).

Molsidomine is bioactivated in the liver by hydrolytic removal of the side chain in position 5 of the heterocycle (Fig. 1). This reaction is catalyzed by hepatic esterases and results in the formation of SIN-1, which decomposes nonenzymatically in a two-step reaction. In the first step, SIN-1 undergoes base-catalyzed ring opening to form SIN-1A. The second step requires an electron acceptor, usually oxygen, and yields NO and $O_2^{\cdot-}$ together with the stable metabolite SIN-1C (Bohn

and Schönafinger, 1989; Feelisch *et al.*, 1989). NO and $O_2^{\cdot-}$ combine rapidly to form peroxynitrite (Huie and Padmaja, 1993). The reported peroxynitrite-like effects of SIN-1 include oxidation of low-density lipoproteins (Darley-Usmar *et al.*, 1992), degradation of deoxyribose (Hogg *et al.*, 1992), and inhibition of glyceraldehyde-3-phosphate-dehydrogenase (Dimmeler *et al.*, 1992). Likewise, cytotoxic effects of SIN-1 comparable with those of authentic peroxynitrite have been demonstrated in various cell types (Lipton *et al.*, 1993; Brunelli *et al.*, 1995).

sGC [GTP pyrophosphate-lyase (cyclizing), E.C. 4.6.1.2.] is the most important physiological target of SIN-1 (Böhme *et al.*, 1982), a cytosolic enzyme that catalyzes the formation of the second messenger cGMP from Mg^{2+} GTP. The enzyme is an α/β heterodimer with an overall molecular mass of 150 kDa (Koesling *et al.*, 1991), containing stoichiometric amounts of ferroprotoporphyrin-IX bound to His105 of the β subunit (Wedel *et al.*, 1994). NO binds with high affinity to this heme iron, which leads to a change in heme geometry that confers enzyme stimulation (Ignarro, 1992). By contrast, peroxynitrite does not itself activate sGC, but leads to a slight enzyme activation in the presence of GSH because of

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ABBREVIATIONS: SIN-1, 3-morpholino-sydnonimine; DEA/NO, 2,2-diethyl-1-nitroso-oxyhydrazine; DTT, dithiothreitol; GSH, glutathione; GSNO, S-nitrosogluthathione; HPLC, high performance liquid chromatography; NO, nitric oxide; $O_2^{\cdot-}$, superoxide; sGC, soluble guanylyl cyclase; SIN-1A, N-morpholino-N-nitrosoaminoacetonitrile; SIN-1C, N-morpholinoiminoacetonitrile; SOD, superoxide dismutase.

formation of the thionitrite GSNO (Mayer *et al.*, 1995), which releases NO in a trace metal-catalyzed reaction (Dicks *et al.*, 1996; Gorren *et al.*, 1996).

It was the aim of the present study to investigate the molecular mechanism of sGC activation by SIN-1 *in vitro*. Based on very recent data obtained with purified neuronal nitric oxide synthase, which was shown to form NO and superoxide at the same time and thus might be regarded as endogenous SIN-1 (Mayer *et al.*, 1998), we gave special emphasis to the potential role of GSH and other thiols and the interplay between GSH and SOD.

Experimental Procedures

Materials. sGC was purified from bovine lung as described previously (Humbert *et al.*, 1990). Alkaline stock solutions of peroxy-nitrite (~100 mM) were synthesized and quantified as described (Mayer *et al.*, 1995). [α - 32 P]GTP (400 Ci/mmol) was purchased from MedPro (Amersham, Vienna, Austria). SIN-1 was a generous gift from Dr. K. Schönafinger (Höchst Marion Roussel, Frankfurt, Germany). DEA/NO and GSNO were obtained from Alexis Corporation (Läufelfingen, Switzerland). Cu, Zn-SOD (from bovine erythrocytes, 4200 units/mg), neocuproine (2,4-dimethyl-1, 10-phenanthroline), cuprizone (biscyclo-hexano-oxaldihydrazone), and all other chemicals were purchased from Sigma (Vienna, Austria). Solutions were prepared with Nanopure water (Barnstead ultrafiltered type I, resistance > 18 M Ω /cm). Stock solutions of peroxy-nitrite were first diluted 10-fold in water; further dilutions were prepared in 10 mM NaOH. Cuprizone was dissolved and diluted in dimethylsulfoxide, which was added as vehicle in the respective controls.

Light absorbance spectroscopy. Decomposition of SIN-1 was measured with a Hewlett Packard 8452A diode array spectrophotometer. Results were fitted to a simple model describing two consecutive first-order reactions, SIN-1 \rightarrow SIN-1A \rightarrow SIN-1C (Grossmann, 1984). The second step is a second order reaction (Feelisch *et al.*, 1989), but was treated as a pseudo-first order reaction to account for our experimental conditions (excess O₂). Samples containing 50 μ M SIN-1 in a 50 mM K₂HPO₄/KH₂PO₄ buffer, pH 7.4, were incubated at 37° in the absence and presence of 1 mM GSH. The rate constants of SIN-1 decomposition [i.e., SIN-1A formation (k_1)] were determined at 300 nm. Absorbance decreases were measured for 20 min and fitted to single exponentials (eq. 1). The rate constants for SIN-1A decay [i.e., SIN-1C formation (k_2)] were determined at 270

nm. Absorbance increases were monitored for 8 hr and fitted to eq. 2, substituting for k_1 the values obtained at 300 nm. The rate constants (k_1 , k_2) represent mean values \pm standard error of three experiments. Concentrations of SIN-1, SIN-1A, and SIN-1C as a function of time (t) were simulated by substituting k_1 and k_2 in the appropriate rate equation (eqs. 3–5).

$$A(t) = A_{\infty} - \Delta A * e^{-k_1 t} \quad (1)$$

$$A(t) = A_0 + \Delta A * \left(1 + \frac{k_2}{k_1 - k_2} * e^{-k_1 t} - \frac{k_1}{k_1 - k_2} * e^{-k_2 t} \right) + \alpha t \quad (2)$$

$$[\text{SIN} - 1]_t = [\text{SIN} - 1]_0 * e^{-k_1 t} \quad (3)$$

$$[\text{SIN} - 1\text{A}]_t = [\text{SIN} - 1]_0 * \frac{k_1}{k_1 - k_2} * (e^{-k_2 t} - e^{-k_1 t}) \quad (4)$$

$$[\text{SIN} - 1\text{C}]_t = [\text{SIN} - 1]_0 * \left(1 + \frac{k_2}{k_1 - k_2} * e^{-k_1 t} - \frac{k_1}{k_1 - k_2} * e^{-k_2 t} \right) \quad (5)$$

where A_0 is initial absorbance, A_{∞} is final absorbance, ΔA is absorbance change, k_1 is the first order rate constant, and k_2 is the apparent first order rate constant. The term αt in eq. 2 was introduced to account for a small linear increase in absorbance that became apparent at very long time scales. In this phase, the absorbance of the difference spectrum gradually increased toward shorter wavelengths. This increase in absorbance corresponded neither to the breakdown of SIN-1A nor to the formation of SIN-1C. After 100 min, when SIN-1C formation was more than 90% complete, this linear phase was 0–8% of the overall absorbance change. The origin of the effect was not further investigated.

Determination of sGC activity. Purified sGC (50 ng, $V_{\max} \sim 16$ μ mol/mg/min in the presence of 1 μ M DEA/NO) was incubated at 37° for 10 min in a total volume of 0.1 ml of a 50 mM K₂HPO₄/KH₂PO₄ buffer, pH 7.4, containing 0.5 mM [α - 32 P]GTP (200,000–300,000 cpm), 3 mM MgCl₂, 1 mM cGMP, and 0.05 mg/ml bovine serum albumin. Thiols, SOD, NaHCO₃, and chelators were present as indicated. Reactions were started by adding 10-fold concentrated stock solutions of SIN-1, peroxy-nitrite, DEA/NO, or vehicle to the assay mixtures and consequent transfer of the samples from 4 to 37°. Incubations were stopped by ZnCO₃ precipitation, and [32 P]cGMP was isolated by column chromatography as described previously (Schultz and Böhme, 1984). Results were corrected for enzyme-deficient blanks and recovery of cGMP. Data represent mean values \pm standard error of three experiments performed in duplicate. Parameters of the concentration-response curves were estimated using the Hill equation. The data shown in Fig. 3 were analyzed by one-way analysis of variance using the Scheffé F-test to compare single mean values.

HPLC analysis of GSNO. Samples (1 ml) containing SIN-1 (100 μ M) and GSH (1 mM) were incubated at 37° for 10 min in 50 mM K₂HPO₄/KH₂PO₄ buffer, pH 7.4, containing 20 μ M neocuproine to prevent decomposition of GSNO. To remove nitrite, samples were treated with 0.1 ml ammonium sulfamate (100 mM) before acidification with 6M HCl, pH ~2–3 (Saville, 1958), and immediately analyzed by HPLC. The HPLC procedure was adapted from a method described previously (Mayer *et al.*, 1995). Samples (0.1 ml) were injected onto a C₁₈ reversed-phase column (250 \times 4 mm; Merck, Darmstadt, Germany) with a C₁₈ precolumn (4 \times 4 mm; Merck). Elution was performed isocratically at a flow rate of 0.75 ml/min with a 20 mM Na₂HPO₄/NaH₂PO₄ buffer, pH 7.4, containing 5% (v/v) methanol and 20 μ M neocuproine. Absorbance was monitored continuously at 338 nm (LiChroGraph L 4250; Merck) to detect S-nitrosothiols. The method was calibrated daily with authentic GSNO freshly dissolved in 50 mM K₂HPO₄/KH₂PO₄ buffer, pH 7.4, containing 20 μ M neocuproine. Calibration yielded linear responses of peak area versus GSNO concentration (0.5 μ M to 10 μ M). The detection limit was 0.5

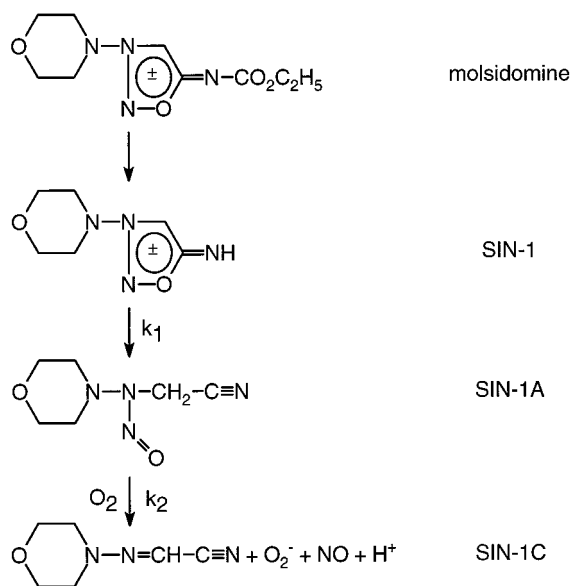


Fig. 1. Metabolism of molsidomine.

μM . GSNO was not detectable in control samples containing up to 0.1 mM NaNO_2 .

Electrochemical detection of NO release from GSNO. Release of NO from GSNO was measured with a Clark-type electrode (Iso-NO; World Precision Instruments, Berlin, Germany), which was connected to an Apple Macintosh computer by an analog-to-digital converter (MacLab; World Precision Instruments, New Haven, CT) (Pfeiffer *et al.*, 1998). The output current was recorded at 0.6 Hz under constant stirring at 37° . Two-point calibration of the electrode was performed daily according to the procedure recommended by the manufacturer. The sensitivity of the electrode was 0.5–0.66 nM/pA. Samples (1 ml) containing SIN-1 (1–20 μM) and GSH (1 mM) were incubated in water-jacketed open plastic vials in 50 mM $\text{K}_2\text{HPO}_4/\text{H}_3\text{PO}_4$ buffer, pH 7.4, containing 20 μM neocuproine. In some experiments, the incubation period was 10 min to allow a comparison with the sGC activity measurements. NO was released from GSNO by injecting 5 μl of a $\text{Cu}(\text{NO}_3)_2$ solution (10 mM final). The formation of NO was quantified from the initial release rates with the use of the CHART MacLab ver. 3.4 software program for Apple Macintosh (AD Instruments, Hastings, UK). Calibration curves were recorded with authentic GSNO (50 nM to 5 μM). Intra- and interassay variabilities were 6.6 ± 2.0 and $9.3 \pm 3.8\%$ (each $n = 3$), respectively.

Results

Decomposition kinetics of SIN-1 were measured by light absorbance spectroscopy at 37° and pH 7.4. Fitting of the data by single exponentials (eq. 1) yielded rate constants (k_1) of $6.4 \pm 1.1 \times 10^{-2}/\text{min}$ and $7.1 \pm 0.6 \times 10^{-2}/\text{min}$ in the absence and presence of 1 mM GSH, respectively. Decay of SIN-1A (i.e., formation of SIN-1C) was fitted according to eq. 2. The respective rate constants (k_2) were $3.1 \pm 0.3 \times 10^{-2}/\text{min}$ and $2.4 \pm 0.2 \times 10^{-2}/\text{min}$ in the absence and presence of GSH, respectively. These rate constants were used to model the concentration time courses shown in Fig. 2.

To probe the involvement of GSH in the stimulation of purified sGC by SIN-1, we recorded concentration-response curves in the absence and presence of GSH (1 mM) (Fig. 3A). In the absence of the thiol, sGC was activated by SIN-1 (1 μM) from 0.04 ± 0.03 to $1.3 \pm 0.1 \mu\text{mol}/(\text{mg} \times \text{min})$ cGMP (\circ). Presence of GSH (\bullet) potentially enhanced the efficacy of SIN-1 to $9.00 \pm 0.42 \mu\text{mol}/(\text{mg} \times \text{min})$ cGMP at 100 μM . The EC_{50}

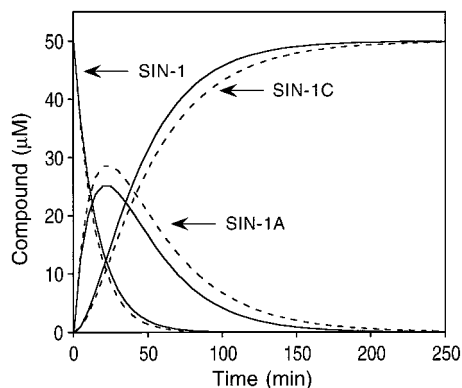


Fig. 2. Decomposition kinetics of SIN-1. Solutions of SIN-1 (50 μM) were incubated in the absence (solid lines) and presence (dotted lines) of GSH (1 mM). Absorbance changes were measured at 270 and 300 nm, and the rate constants k_1 and k_2 (see Fig. 1) were obtained from fitting the data as described under Experimental Procedures. The concentrations of SIN-1, SIN-1A, and SIN-1C as a function of time were plotted by substituting the rate constants k_1 and k_2 in the appropriate rate equations. The rate constants (k_1 , k_2) represent mean values \pm standard error of three experiments.

value was $0.92 \pm 0.07 \mu\text{M}$ SIN-1. The EC_{50} value of GSH to potentiate sGC activation by SIN-1 was $0.12 \pm 0.01 \text{ mM}$ (Fig. 3A, inset).

The enzyme became even more sensitive to SIN-1 in the presence of SOD ($\text{EC}_{50} = 0.11 \pm 0.03 \mu\text{M}$; \square). In addition, SOD slightly increased maximal sGC activity. The concentration-response curve of SIN-1 recorded in the presence of both GSH and SOD was between those of the single compounds ($\text{EC}_{50} = 0.48 \pm 0.03 \mu\text{M}$). The minor increase of basal enzyme activity by SOD was inhibited by reduced hemoglobin or 1*H*-[1,2,4]oxa-diazolo[4,3-*a*]quinoxalin-1-one (Schrammel *et al.*, 1996) (data not shown), which indicated that it was due to the stabilization of environmental NO (Friebe *et al.*, 1996).

Next we investigated the effects of other sulfhydryl-containing compounds. sGC was stimulated with 10 μM SIN-1 and assayed for cGMP formation in the presence of GSH, L-cysteine, DTT, and D,L-penicillamine (1 mM each). Enzyme activity was significantly increased from 1.1 ± 0.2 to 10.2 ± 0.4 and $5.1 \pm 0.4 \mu\text{mol}/(\text{mg} \times \text{min})$ cGMP in the presence of GSH and L-cysteine, respectively. The activities measured in the presence of DTT ($1.9 \pm 0.3 \mu\text{mol}/(\text{mg} \times \text{min})$ cGMP) and D,L-penicillamine ($3.0 \pm 0.2 \mu\text{mol}/(\text{mg} \times \text{min})$ cGMP) were not significantly different from controls (Fig. 3B).

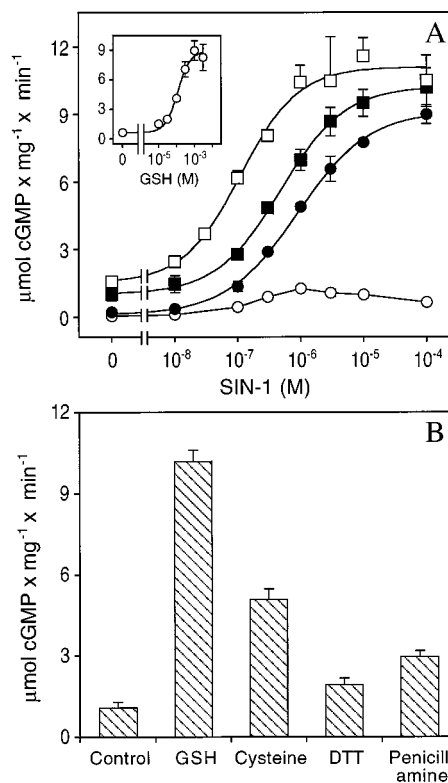


Fig. 3. Effect of thiols and SOD on the activation of sGC by SIN-1. A, Purified sGC (50 ng) was incubated with increasing concentrations of SIN-1 and assayed for cGMP formation in the absence of additives (\circ), in the presence of 1 mM GSH (\bullet) or 500 units/ml SOD (\square), or in the presence of both SOD and GSH (\blacksquare). Statistical significance of the EC_{50} values: GSH versus SOD, $p < 0.0001$; GSH versus GSH + SOD, $p < 0.002$; SOD versus SOD + GSH, $p < 0.02$. Inset, GSH dependence of sGC stimulation by 10 μM SIN-1. B, Purified sGC (50 ng) was incubated with SIN-1 (10 μM) and assayed in the presence of GSH, L-cysteine, DTT, and D,L-penicillamine (1 mM each). Statistical significance versus control: GSH, $p < 0.0001$; L-cysteine, $p < 0.01$; DTT, $p < 0.9$; D,L-penicillamine, $p < 0.4$. Data are mean values \pm standard error of three experiments performed in duplicate.

Previous studies described a fast reaction between peroxynitrite and CO_2 that effectively outcompeted other biological reactions of peroxynitrite (Lyman and Hurst, 1995). To probe whether NO/O_2^- produced by SIN-1 is similarly sensitive to CO_2 , we compared the effect of NaHCO_3 (10 mM) on sGC activation by SIN-1 and authentic peroxynitrite. As shown in Fig. 4A, peroxynitrite increased the enzyme activity up to $2.4 \pm 0.5 \mu\text{mol}/(\text{mg} \times \text{min})$ cGMP. NaHCO_3 potently antagonized this stimulation ($\sim 70\%$ inhibition at $100 \mu\text{M}$ peroxynitrite). By contrast, NaHCO_3 had only a small effect on sGC activation by SIN-1 (15–20% inhibition; Fig. 4B). A similar effect ($\sim 25\%$ inhibition) was observed when sGC was activated with DEA/NO (data not shown), which suggested a direct inhibition of sGC by NaHCO_3 or CO_2 .

We proposed recently that sGC activation by authentic peroxynitrite involves S-nitrosation of GSH followed by copper-catalyzed release of NO from the intermediate GSNO (Mayer et al., 1995). To assess whether a similar nitrosative pathway accounts for sGC activation by SIN-1, we studied the effects of specific copper chelators known to block this NO release (Fig. 5). The phenanthroline derivative neocuproine, which preferentially chelates Cu(I) ions (Diehl and Smith, 1958) antagonized sGC activation by SIN-1 with an IC_{50} value of $14.7 \pm 4.2 \mu\text{M}$ (●). Similar results were obtained with the analogue bathocuproine disulfonic acid (data not shown). By contrast, the Cu(II)-specific drug cuprizone (Peterson and Bollier, 1955) was less effective (25% inhibition at 1 mM; Fig.

5, ○). DEA/NO-stimulated sGC activity was affected neither by neocuproine (Fig. 5, ■) nor by cuprizone (Fig. 5, □).

HPLC analysis showed that the reaction of SIN-1 and GSH yielded a product with an absorbance maximum at 338 nm that coeluted with authentic GSNO (Fig. 6A). Additional evidence for the formation of a thionitrite was obtained in electrochemical experiments showing that the product of SIN-1 and GSH released NO upon treatment with $\text{Cu}(\text{NO}_3)_2$ (Fig. 6B). The shape of the NO signal was identical to that obtained by the addition of $\text{Cu}(\text{NO}_3)_2$ to authentic GSNO (data not shown). In the presence of 1 mM GSH, the yield of GSNO formation from SIN-1 ($1 \mu\text{M}$) was $9.62 \pm 1.92\%$ ($n = 3$). Release of NO was insensitive to NaHCO_3 . By contrast, the release of NO from the product of peroxynitrite and GSH was abolished by NaHCO_3 (data not shown), a finding that agrees well with the effects of carbonate on sGC activity in the presence of peroxynitrite and SIN-1.

Discussion

The objective of the present study was to characterize the molecular mechanism of sGC activation by the vasodilator SIN-1, a drug that produces NO and O_2^- in a 1:1 stoichiometry. We especially focused on the possible involvement of GSH, the most abundant intracellular thiol. Our data show that stimulation of purified bovine lung sGC by SIN-1 is strongly dependent on GSH. This observation seems to conflict with previous papers claiming a thiol-independent enzyme activation (Feelisch et al., 1989; Noack and Feelisch, 1989). However, the present experiments were performed with highly purified sGC, whereas crude tissue extracts, most likely containing thiols, were used in the previous studies.

GSH is not required for the activation of sGC by the NO donor DEA/NO (Mayer et al., 1995) and does not significantly affect the decomposition kinetics of SIN-1 (this study). Thus, it is likely that an activator of sGC was formed in a reaction of the thiol with the NO/O_2^- released from SIN-1. This activator was identified as GSNO, a thionitrite that releases NO in the presence of trace amounts of Cu^+ ions. Formation of GSNO from NO and O_2^- is apparently caused by a novel, as

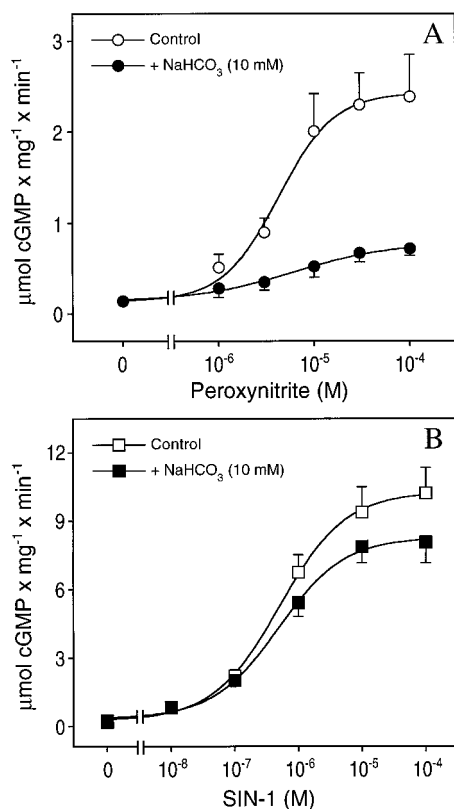


Fig. 4. Effect of NaHCO_3 on activation of sGC by SIN-1 and peroxynitrite. Purified sGC (50 ng) was incubated with increasing concentrations of A, peroxynitrite, or B, SIN-1, and assayed for cGMP formation in the absence (filled symbols) or presence (open symbols) of NaHCO_3 (10 mM) as described under Experimental Procedures. Data are mean values \pm standard error of three experiments performed in duplicate.

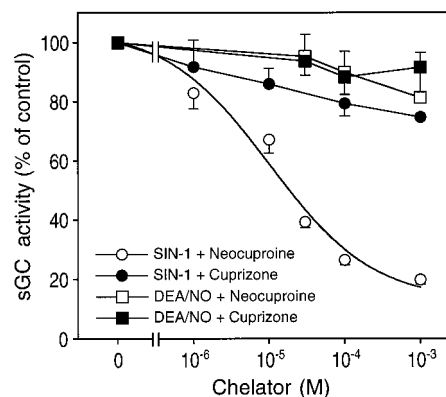


Fig. 5. Effect of copper ion chelators on stimulation of sGC by SIN-1 or DEA/NO in the presence of 1 mM GSH. Purified sGC (50 ng) was incubated with SIN-1 ($10 \mu\text{M}$) in the presence of varying concentrations of neocuproine (●) and cuprizone (○), or with $1 \mu\text{M}$ DEA/NO in the presence of varying concentrations of neocuproine (■) and cuprizone (□) and assayed for cGMP formation as described in Experimental Procedures. Data are mean values \pm standard error of three experiments performed in duplicate.

yet unrecognized nitrosation reaction that is clearly different from the nitrosation of GSH by peroxynitrite described previously (Mayer *et al.*, 1995). The reaction triggered by SIN-1 occurred with at least 20-fold higher efficiency and was not appreciably inhibited by CO₂, which almost completely inhibited nitrosation of GSH by authentic peroxynitrite.

The precise mechanism of NO/O₂⁻-mediated S-nitrosation remains to be clarified. Because GSH nitrosation by NO/O₂⁻ partially outcompetes the very rapid, nearly diffusion-controlled formation of peroxynitrite, GSNO formation must involve a comparably fast reaction. We propose that thiyl radicals (GS[•]) originating from the oxidation of GSH with O₂, H₂O₂, O₂⁻, or peroxynitrite combine with NO to form GSNO.

The GSH-dependence of sGC activation by SIN-1 was overcome by SOD, which suggests that the effect of GSH on sGC stimulation by SIN-1 may not be essential in biological systems. However, GSH markedly antagonized the effect of SOD [i.e., the leftward shift of the SIN-1 concentration-response curve (compare Fig. 3A)], which suggests that GSNO formation partially outcompetes both peroxynitrite formation and O₂⁻ scavenging by SOD.

The pharmacological implications of the proposed pathway remain to be established. The chemical route responsible for sGC activation by SIN-1 will be predominantly determined by the tissue levels of GSH and SOD (Fig. 7). As major

components of the cellular defense machinery against oxidative stress, both GSH and SOD occur physiologically at high concentrations. However, the levels of GSH and SOD may be significantly depressed in pathological conditions, such as myocardial ischemia/reperfusion injury (Ferrari *et al.*, 1991). According to the present data, such a state would favor the intracellular formation of peroxynitrite from SIN-1 and result in tissue damage from oxidative and nitrating chemistry (Beckman and Koppenol, 1996). Against this, another study reported an increase of GSH and a decrease of SOD during ischemia and reperfusion (Bridges *et al.*, 1992). According to our model, this would switch the outcome of SIN-1 to formation of GSNO.

The Cu⁺-induced decomposition of GSNO has been well characterized *in vitro* (Dicks *et al.*, 1996; Gorren *et al.*, 1996; Butler and Rhodes, 1997). The availability of free Cu⁺ ions is probably limited under physiological conditions, but a marked mobilization of redox-active copper in the coronary flow was found after prolonged cardiac ischemia (Chevion *et al.*, 1993). Moreover, efficient enzymatic mechanisms of GSNO decomposition may exist. A Cu⁺-dependent enzymatic activity was reported to catalyze the decomposition of GSNO in blood platelets (Gordge *et al.*, 1996), and diverse enzymes, including GSH peroxidase (Freedman *et al.*, 1995), γ-glutamyl transpeptidase (Hogg *et al.*, 1997), and thioredoxin reductase (Nikitovic and Holmgren, 1996), were shown to catalyze reactions that eventually resulted in NO release from GSNO.

In summary, we propose that NO/O₂⁻ released from SIN-1 mediates a CO₂-insensitive conversion of GSH to GSNO, a thionitrite that activates sGC by the Cu⁺-catalyzed release of NO *in vitro*. Our results may provide novel insights into the molecular mechanism of sydnonimine-based nitrovasodilators.

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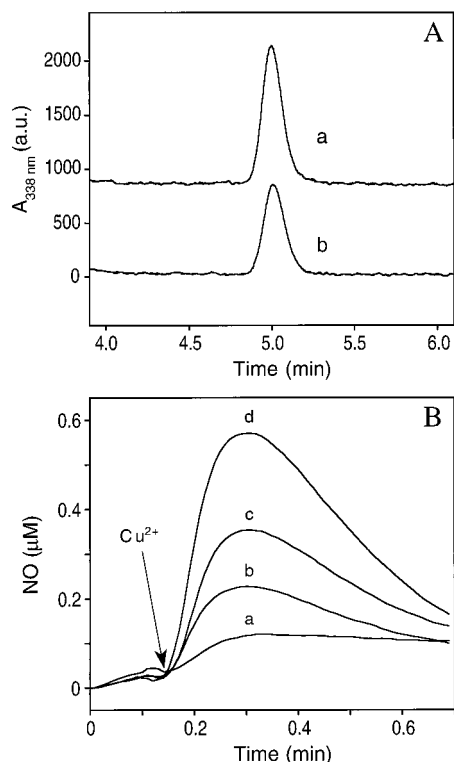


Fig. 6. HPLC and electrochemical characterization of GSNO. A, Samples containing SIN-1 (0.1 mM) and GSH (1 mM) were incubated at 37° for 10 min under sGC assay conditions (see Experimental Procedures) followed by addition of ammonium sulfamate (10 mM final), acidification, and immediate analysis by HPLC (see Experimental Procedures). Shown are chromatograms of authentic GSNO (a) and of the product of SIN-1 (0.1 mM) and GSH (1 mM) (b). Data are representative of five experiments. B, Samples containing 1 μM (trace a), 5 μM (trace b), 10 μM (trace c), and 20 μM (trace d) SIN-1 were incubated in the presence of 1 mM GSH followed by addition of Cu(NO₃)₂ (10 mM final; arrow) and determination of the released NO with a Clark-type electrode. Traces are representative of three experiments.

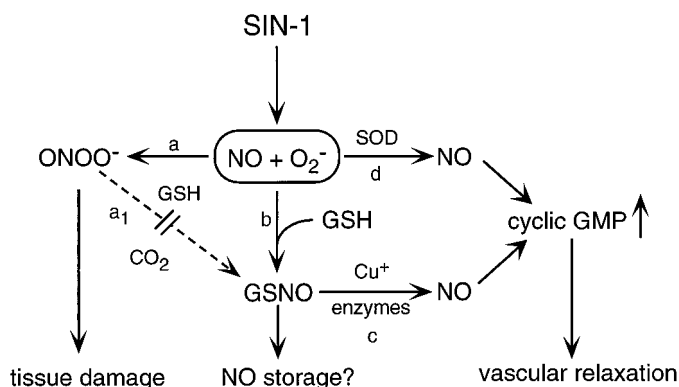


Fig. 7. NO/O₂⁻ signaling pathways. In the absence of SOD and GSH (path a), NO/O₂⁻ released from SIN-1 combines to peroxynitrite, which causes tissue damage by the formation of cytotoxic intermediates. Nitrosation of GSH by peroxynitrite (path a1) occurs at low yields (<1%) and is inhibited by CO₂. Nitrosation of GSH by NO/O₂⁻ (path b) is considerably more efficient (~10%). GSNO may serve as endogenous storage or transport form of NO. Release of NO from GSNO (path c) may be catalyzed by trace metals or by enzymes. Free NO binds with high affinity to the heme of sGC, which leads to accumulation of cGMP. At low concentrations of GSH or in its absence, SOD (path d) shifts the pathway toward free NO and cGMP production.

the kinetic data. We also acknowledge gratefully the excellent technical assistance of Jürgen Malkewitz.

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