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On the Mechanism of NO Release from Sydnonimines

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Summary: The vasodilator and antiaggregatory properties of sydnonimines like SIN-1 are thought to be due to their marked stimulatory action on soluble guanylate cyclase. Enzyme activation and consecutive cyclic GMP accumulation is mediated by the liberation of nitric oxide (NO) from the open-ring A forms of sydnonimines. The purpose of the present study was to investigate the mechanism of NO release from sydnonimines in direct comparison to their stimulatory effect at the target enzyme, soluble guanylate cyclase. All sydnonimines tested were found to spontaneously liberate NO, the rate of which closely correlated with the extent of enzyme activation. NO release occurred nonlinearly with time and became maximal at high sydnonimine concentration. The in vitro stability of the A forms neither correlated with the measured rate of NO release nor with enzyme activation, indicating that a direct stimulation of guanylate cyclase by the A forms is rather unlikely. Besides NO, all sydnonimines generated NO_2^- and NO_3^- at a nearly equimolar rate. The addition of cysteine induced a marked shift from NO_3^- to NO_2^- with a small reduction in NO release, which is paralleled by a weak rightward shift of the EC_{50} at the guanylate cyclase. All tested sydnonimines were found to consume molecular oxygen at rates that closely corresponded to the measured rates of NO formation. By a molar comparison, the amounts of consumed oxygen are clearly higher, as would be expected for the oxidative conversion of NO to NO_2^- and NO_3^-. Oxygen seems to be additionally involved in the induction of NO formation while being converted to superoxide (O_2^-). In accordance with an autocatalytic processs, O_2^- further enhances sydnonimine decomposition, since in the presence of superoxide dismutase (SOD) the rate of SIN-1C and NO_2^-/NO_3^- formation from SIN-1A was reduced, whereas the rate of NO liberation seemingly increased. O_2^- has, however, no influence on the rate of hydrolysis of SIN-1 to SIN-1A. At the level of guanylate cyclase, the presence of SOD induced a leftward shift of the concentration–response curve to SIN-1, in agreement with an enhancement of efficacy of NO by blocking the NO-scavenging effect of O_2^-. An additional O_2^- generation markedly enhanced SIN-1A decomposition to NO_2^-/NO_3^- and reduced the apparent rate of NO formation. We conclude from our results that oxygen plays a key role in the decomposition of sydnonimines and thus in the formation of NO as their pharmacodynamically active principle. Oxygen attack most probably occurs by one-electron abstraction from the A form of the respective sydnonimine compound. Oxygen is thereby converted to O_2^- while the radical cation of the sydnonimine A form decomposes with the release of NO. Sydnonimines such as SIN-1 thus not only appear to be donors of EDRF by releasing NO but also of EDCF while simultaneously producing O_2^-, a behavior that has recently been demonstrated for endothelial cells, activated macrophages, and neutrophils. **Key Words:** Nitric oxide (NO, EDRF)—Sydnonimine—SIN-1—Oxygen—Superoxide (EDCF)—Guanylate cyclase.

Numerous biological effects have been reported with sydnonimines (1–3), among which their vasodilator and antiaggregatory effects seem to be of major therapeutic relevance (4–8). The clinically used prodrug molsidomine is the pharmacologically most well-documented sydnonimine. Its active metabolite SIN-1A arises from a hydroxyl-induced nonenzymatic ring opening of hepatically formed SIN-1 (9–11). Its pharmacodynamic activity is attributed to the marked stimulatory effect on the soluble isoenzyme of guanylate cyclase (EC 4.6.1.2) (7,12–14). The consecutive intracellular accumulation of cyclic guanosine monophosphate (cyclic GMP) in turn mediates relaxation of the smooth muscle. Neither molsidomine itself nor its metabolite without the N–NO group, SIN-1C, displays vasoactive properties in vitro or stimulates guanylate cyclase (6,14).

Several hypotheses have been proposed to explain the enzyme-stimulating action of sydnoni-

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mines. One of these suggests that an active metabolite of SIN-1 such as nitric oxide (NO) elicits activation of soluble guanylate cyclase (6,7). Whether or not any splitting off of NO from the sydnonimine molecule could, however, take place under physiological conditions had not been demonstrated at that time. Therefore, a direct stimulation of the N–NO-containing A forms was regarded as the most reasonable mode of action for sydnonimines (14) until 1987, when the first direct demonstration of NO release from SIN-1 was published (15). In that study, we reported that SIN-1 fits the correlation between NO-formation rate and activation of guanylate cyclase, valid for a number of organic nitrates. In contrast to organic nitrates, NO formation from SIN-1 does not require the presence of cysteine, but occurs spontaneously. The formation of NO accounts for the pharmacodynamic action of all nitrovasodilators (16–18). Nitrovasodilators thus may mimic a physiological process in the blood vessels by providing a factor that is also produced by endothelial cells. Indeed, this endothelium-derived relaxing factor (EDRF) most probably is identical with or at least gives rise to nitric oxide (17,19,20).

The objective of the present study was to investigate the mechanism of NO release from sydnonimines and, in particular, the potential role of oxygen in initiating this reaction. The different rates by which metabolites are generated during the in vitro decomposition of sydnonimines will be compared to the stimulatory action of these compounds on the target enzyme, soluble guanylate cyclase.

METHODS

Guanylate cyclase

A crude extract from rat liver was prepared according to Steurer and Schütz (21) and Ignarro et al. (22). The activity of the soluble guanylate cyclase in the high-speed supernatant was determined by radioimmunoassay (RIA) with a commercial RIA kit for cyclic GMP (Amersham/Buchler, Braunschweig, F.R.G.) according to Kimura et al. (23). Typical assay conditions: 50 mM phosphate buffer (pH 7.40), 4 mM $MgCl_2$, 1 mM GTP, 30 μg of protein, incubation time of 15 min, at 37 ± 0.05°C.

Nitric oxide

The formation of nitric oxide was monitored by using an indirect difference-spectrophotometric method based on the NO-induced oxidation of oxyhemoglobin to methemoglobin (15,24), which had been validated by chemiluminescence (25). All measurements were performed in 50 mM phosphate buffer, pH 7.70, at 37 ± 0.05°C. Values are expressed as initial kinetics calculated from the slope of the linear extinction vs. time gradient obtained shortly after the lag phase, which is due to hydrolysis of the sydnonimine compounds to the respective A forms.

Nitrite/nitrate

Both anions were determined simultaneously without prior extraction step by a high-pressure liquid chromatography (HPLC) technique described in detail elsewhere (15). All incubations were carried out in 50 mM phosphate buffer, pH 7.70, at 37 ± 0.05°C. With the exception of the N-nitroso forms of the sydnonimines (A forms), all compounds showed a much weaker rate of anion formation during the first 2 to 4 min, after which NO_2^- and NO_3^- was generated linearly. Reported values refer to the linear kinetics calculated from the slope of the resulting concentration vs. time gradient. A single kinetic determination consisted of at least six points obtained by a delay time of 4–7 min.

Determination of molsidomine metabolites

Metabolites generated by the in vitro decomposition of SIN-1 and related compounds were determined simultaneously by HPLC. The incubations were carried out in 50 mM phosphate buffer, pH 7.40, at 37°C. Measurements were performed on a Bondapak C-18 column (30 cm × 3.9 mm) with a mobile phase comprised of aqueous NH_4 acetate buffer, pH 3.1, acetonitrile, and methanol (93:6:2) at a flow rate of 1 ml/min. The detection wavelength was set at 238 nm. A calibration was performed every day with freshly prepared aqueous standard solutions. Retention times for molsidomine metabolites were SIN-1, 5.0 min; SIN-1A, 8.6 min; and SIN-1C, 10.6 min.

Superoxide

Generation of superoxide (O_2^-) was measured by the superoxide dismutase-inhibitable reduction of both cytochrome c and nitroblue tetrazolium (NBT) (26,27). Cytochrome c was used in a final concentration of 100 μM in the presence of 100 μM Na_2EDTA. Incubations were performed in 50 mM phosphate buffer, pH 7.70, at 37°C while continuously recording the absorbance at 550 nm for 1 h. Although the measurement of O_2^- production with the cytochrome c method is of higher reliability than that with NBT (27), values obtained with the first method do not display the correct rates of O_2^- formation from sydnonimines. This is because all sydnonimines tested possessed a marked oxidative effect toward reduced cytochrome c. This oxidative action was demonstrated by incubating SIN-1 with fully reduced cytochrome c under the same conditions. Upon addition of SIN-1, absorbance at 550 nm rapidly decreases to blank values (n = 2). Whether this effect is due to an oxidative property of sydnonimines themselves or to the generation of metabolites other than O_2^- is unknown.

The reduction of NBT was monitored in the presence of Na_2EDTA, both at a final concentration of 100 μM in 50 mM phosphate buffer, pH 7.70, at 37°C. An increase in the absorbance at 560 nm was continuously recorded for at least 1 h. Superoxide dismutase (SOD, EC 1.15.1.1, 100 U/ml) prevented the increase in absorbance if present at the start of incubation or terminated a further increase when added after 5, 10, 20, or 30 min (n = 12). Since the reduction of NBT by O_2^- does not proceed stoichiometrically, all values were referred to SIN-1 used as the "internal standard." All values have been corrected for the O_2^--independent reduction measured under the same conditions.

Oxygen consumption

Measurements of oxygen consumption were performed polarographically by means of a Clark electrode (28). Briefly, air-saturated phosphate buffer (50 mM, pH 7.40)

was filled into a closed all-glass system and maintained at 37°C while continuously stirring. Freshly prepared aqueous solutions of sydnonimines were added by means of a Hamilton syringe and oxygen consumption was monitored by digital recording (Ysi 5300, Yellow Springs Instr., Yellow Springs, OH, U.S.A.). The rate of O_2 consumption was calculated from the slope of the resulting gradient arising within the first 5 min and converted to absolute values by assuming that the oxygen concentration in air-saturated aqueous buffer is 197 μM at 37°C.

Substances

All sydnonimines and related *N*-nitroso compounds were a kind gift of Cassella AG (Frankfurt, F.R.G.). Because of their known photolytic and hydrolytic instability (9), all compounds were stored dry, cool, and protected from light prior to use. SIN-1A was stored under nitrogen at −80°C. S-nitroso-*N*-acetylpenicillamine was synthesized according to Field et al. (29); glyceryl trinitrate and isosorbide-5-mononitrate were a kind gift of Schwarz Pharma AG (Monheim, F.R.G.). Aqueous solutions of all compounds were prepared freshly before measurements were made. Xanthine oxidase from buttermilk (1 U/mg of protein suspension) and xanthine were from Serva (Heidelberg, F.R.G.). All thiols, ferricytochrome c from horse heart (type IV), and superoxide dismutase from bovine erythrocytes were purchased from Sigma (Taufkirchen, F.R.G.). All other chemicals were of the highest grade available.

Statistics

All data are expressed as means ± SEM. Differences were assessed by using Student's two-tailed *t* test for paired observations. *p* Values of less than 0.05 were regarded as denoting statistically significant differences. Calculation of the concentrations at which guanylate cyclase stimulation is half-maximal (EC_{50} values) was assessed by using the logit transformation according to Hafner et al. (30).

RESULTS

Enzyme stimulation by different sydnonimines

The activity of soluble guanylate cyclase was enhanced markedly in a thiol-independent manner with all tested sydnonimines. Figure 1 depicts the guanylate cyclase-stimulating potencies of six different sydnonimines in comparison to a relatively stable *S*-nitrosothiol and to glyceryl trinitrate and isosorbide-5-mononitrate. The relative enzyme-stimulating activity of sydnonimines ranged from

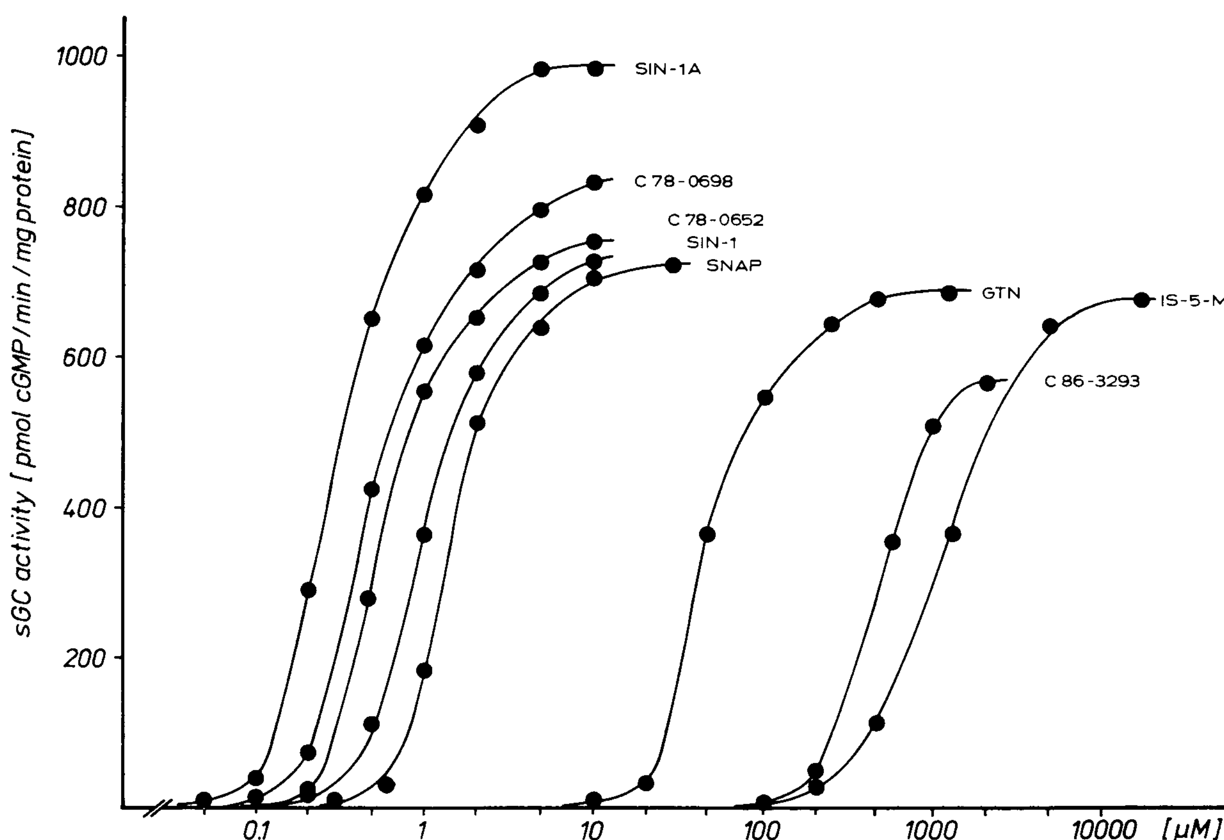


FIG. 1. Concentration–response curves for five sydnonimines in comparison to S-nitroso-*N*-acetyl-D,L-penicillamine (SNAP), glyceryl trinitrate (GTN), and isosorbide-5-mononitrate (IS-5-N). The concentration–response curves were performed as described in the Methods section, with the exception that those for the organic nitrates were recorded in the presence of 5 mM L-cysteine; $n = 3$. The chemical structure of the investigated compounds is given in the Appendix. The potency of several sydnonimines to stimulate soluble guanylate cyclase (sGC) differs by a factor of 1,000.

compounds with an even higher potency than *S*-nitrosothiols to those with an activity comparable to organic nitrates. The maximal achievable stimulation of soluble guanylate cyclase was not the same for the different compounds; compounds requiring a smaller concentration for half-maximal enzyme activation elicited a higher maximal effect.

When SIN-1 was incubated in the presence of the SH-containing amino acid L-cysteine (5 m*M*), which is known to be an essential costimulator of guanylate cyclase activation by organic nitrates (31,32), no further stimulatory but, on the contrary, a weak inhibitory effect with a shift of the concentration–response curve to the right was observed ($n = 3$).

Oxygen consumption

All tested sydnonimines consumed molecular oxygen (O_2) at different rates. The tendency to consume oxygen was linked with the decomposition of the individual sydnonimines. SIN-1A, incubated in a closed system, no longer decayed when the concentration of dissolved oxygen fell to zero ($n = 2$). The initial rates of O_2 consumption were compared with those of NO formation (Fig. 2). A close correlation existed between both parameters for the 23 sydnonimine compounds tested. By molar comparison, the rate of oxygen consumption is higher than that of NO formation. For SIN-1, for example, the initial rate of oxygen consumption is about 2.8-fold higher than NO formation (mean values, $n = 24$). The molar ratio of the amounts of oxygen consumed and of SIN-1 completely decomposed was 0.9 ($n = 4$).

Formation of superoxide

The formation of superoxide anions (O_2^-) was determined during sydnonimine decomposition. The O_2^- formation depended on the concentration of oxygen in the incubation medium. In buffer solutions that had been deoxygenated previously by ultrasonication and bubbling with argon (and can be regarded as oxygen-free), superoxide formation from SIN-1 was nearly abolished, whereas in buffers gassed with pure oxygen, an acceleration of O_2^- production was seen compared to air-saturated solutions ($n = 2$). The relative rates of O_2 consumption and O_2^- formation were correlated (Fig. 3).

Formation of NO

All sydnonimines spontaneously released NO. This process proceeded shortly after their addition to neutral or weakly alkaline (pH 7.4) aqueous buffer. The time delay for the induction of a measurable NO release depended on the concentration

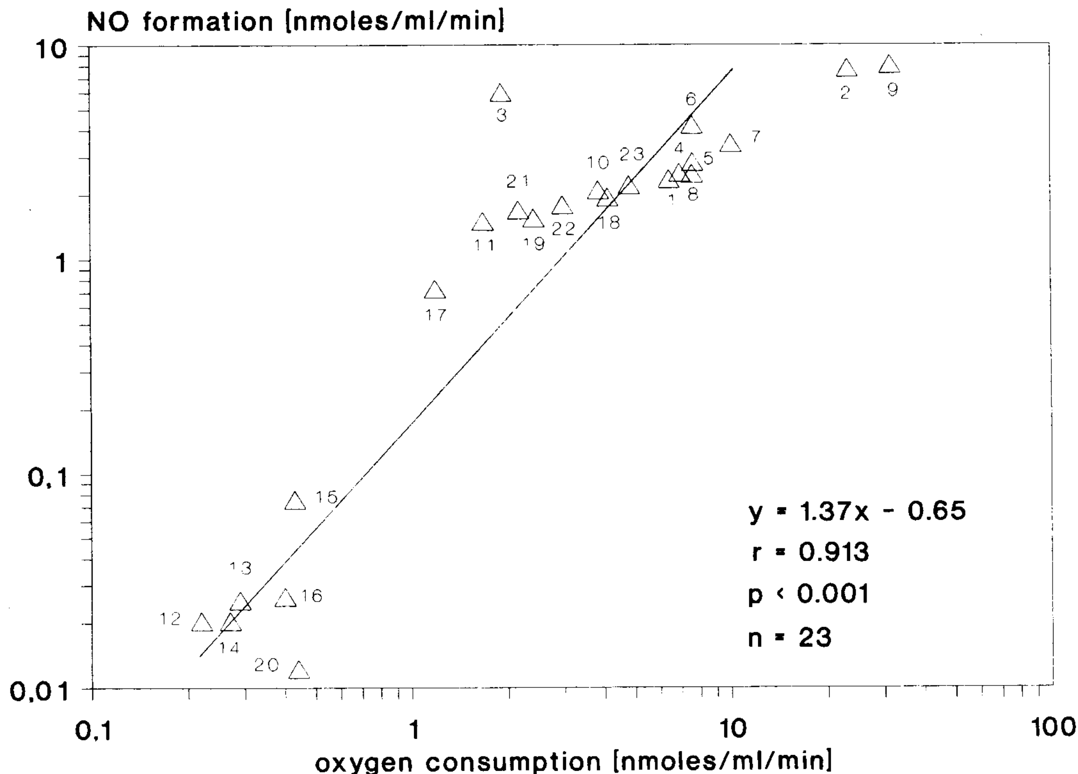


FIG. 2. Correlation between NO formation and oxygen consumption for different sydnonimines. Assay conditions are given in the Methods section. All sydnonimines were used in a final concentration of 500 μ*M*. The initial formation rates were calculated from the slopes of the linear part of recordings obtained within the first 1 to 5 min. Each point represents the mean of three separate kinetic runs for oxygen consumption and NO release. A list of the 23 tested compounds is given in the Appendix.

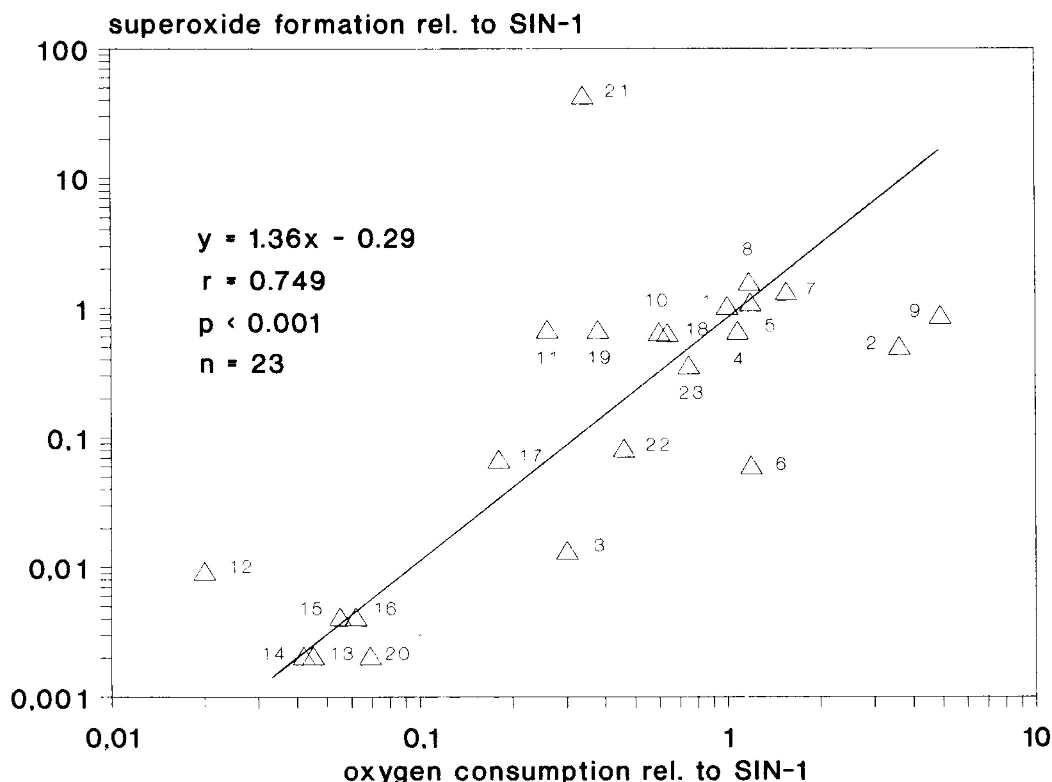


FIG. 3. Correlation between superoxide formation and oxygen consumption for different sydnonimines. Assay conditions are given in the Methods section. All sydnonimines were used in a final concentration of 500 μM. The rate of superoxide formation was measured by means of the SOD-inhibitable reduction of NBT. As SIN-1 was chosen as the internal standard, the rates of both oxygen consumption and superoxide formation from SIN-1 were arbitrarily taken as 1. Measured values from all other sydnonimine compounds are relative rates referred to that of SIN-1.

of sydnonimine, the incubation temperature, and the pH. The absolute rate of NO liberation from SIN-1 was strictly pH dependent: shifting the pH from 6.5 to more alkaline values markedly enhanced the velocity of NO generation. The rate of NO formation from SIN-1A did not significantly differ in the pH range 5–10 ($n = 6$).

The hydrolytic step involved in the formation of SIN-1A from SIN-1 predicts a nonlinear relationship between the concentration of sydnonimine and the resulting rate of NO liberation. Thus, the NO liberation became maximal at a sydnonimine concentration of about 1 m*M*; the same was true for SIN-1A (Fig. 4). Compound C 78-0698, a thiomorpholinyl analogue of SIN-1, showed a higher rate of NO formation than SIN-1, although its A form is more stable than SIN-1A (33). The stability difference between C 78-0698A and SIN-1A could be verified by use of HPLC ($n = 4$). The rate of NO formation closely correlated with the activation of the enzyme.

The kinetic measurements of NO generation from SIN-1 were repeated in the presence of 100 U/ml of SOD; a 30% increase in the apparent rate of formation of NO was noticed ($n = 3$, Fig. 5). At the level of the soluble guanylate cyclase, the presence of 100 U/ml of SOD produced a shift to the left of the concentration–response curve to SIN-1. Upon additional O_2^- supply by the O_2^--generating system xanthine/xanthine oxidase, the apparent rate of NO formation was reduced by approximately 45% ($n = 3$). Cysteine decreased the initial rate of NO release from SIN-1 by nearly 10% ($n = 3$) and that from C 86-3293 by 45% ($n = 2$).

Formation of nitrite and nitrate

There was a striking uniformity between the rate of nitrite and that of nitrate formation for most sydnonimines (Table 1). Addition of thiols to the incubation buffer produced a marked shift of the nitrite/nitrate ratio to higher values. Spontaneously generated nitrite and nitrate did not account for the enzyme stimulation, because C 78-0698 displayed a higher stimulating potency on guanylate cyclase than SIN-1 while producing less nitrite and nitrate.

The presence of SOD significantly reduced the rates of both NO_2^- and NO_3^- production from their A forms, while at the same time inducing a shift from NO_3^- to NO_2^- (Fig. 5). SOD did not reduce the final amount of generated anions [the total NO_2^-/NO_3^-

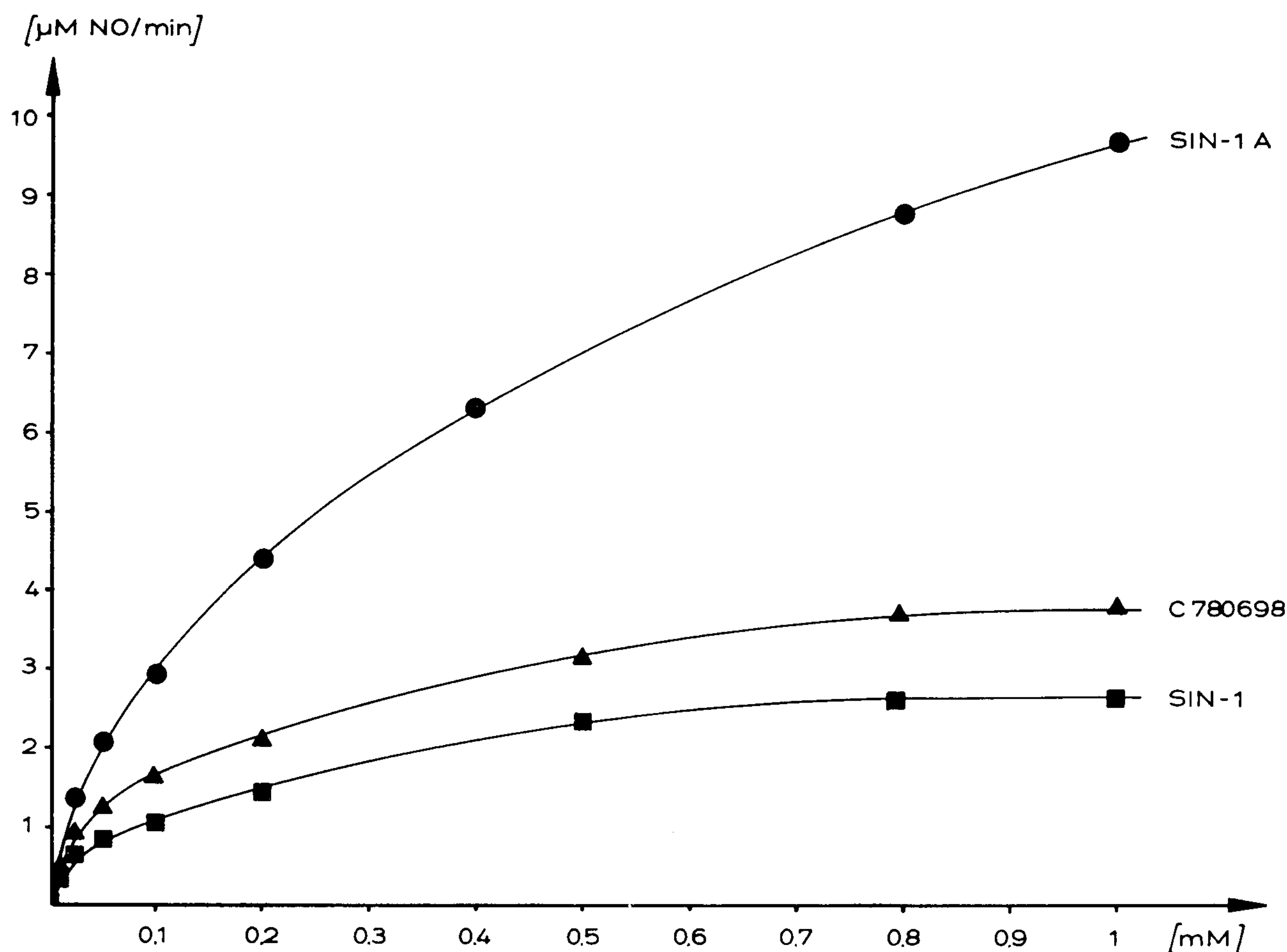


FIG. 4. Relationship between sydnonimine concentration and the initial rate of NO release. Experimental conditions: 100 m*M* phosphate buffer, pH 7.70, 37 ± 0.05°C, 5 μ*M* HbO_2; each point represents the mean of four separate kinetic runs. Up to a certain maximum, the rate of NO formation increases nonlinearly with increasing concentrations of the sydnonimines.

concentration at the end of a 90 min incubation of 500 μ*M* SIN-1 was not significantly different from that obtained in the absence of SOD (n = 2)]. On the other hand, the formation rates of NO_2^- and NO_3^- from SIN-1 and C 78-0698 were accelerated transiently (within the first 5 min) in the presence of an O_2^--generating system (xanthine/xanthine oxidase), but were reduced in the further course of incubation.

Formation of the A and C forms of sydnonimines

The action of SOD on the spontaneous decomposition of SIN-1 and C 78-0698 was followed by monitoring the formation of their A and C metabolites. SOD reduced the formation of the C from the A forms. By stabilizing, for example, generated SIN-1A, the formation of SIN-1C was reduced in the presence of SOD (Fig. 6). The scavenger of O_2^- did not influence the rate of hydrolysis of a given sydnonimine to its respective A form or the total amount of metabolites formed (evaluated from the loss of SIN-1 and C 78-0698 and the sum of metabolite formation at the end of each incubation run).

The sum of the generated A and C metabolites never reached the theoretical 100% value. In all cases, about 10% of the initial sydnonimine concentration was not recovered (n = 7).

DISCUSSION

In this study, we used physicochemical and enzymatic methods to investigate the mechanism of NO release from sydnonimines. The data presented clearly show that molecular oxygen plays a key role in the initiation of the decomposition of sydnonimines. In addition to nitrite and nitrate, superoxide anions are generated during the liberation of NO, which markedly modulate the metabolic pattern of sydnonimine breakdown and thus the extent of stimulation of soluble guanylate cyclase that they cause. As can be concluded from the marked differences in the potency of enzyme stimulation, the intensity

TABLE 1. *Formation rates for nitrite, nitrate, and nitric oxide from the in vitro decomposition of SIN-1 and C 78-0698 in comparison to half-maximal activation of soluble guanylate cyclase (EC_{50}) in the presence and absence of cysteine*

Compounds	NO_2^- (μM/min)	NO_3^- (μM/min)	NO_2^-/NO_3^- ratio	NO (μM/min)	EC_{50} (μM)
SIN-1	2.75	2.73	1.01	2.39	1.23
SIN-1 + cysteine	4.69	0.48	9.77	2.28	1.98
C 78-0698	0.76	0.66	1.15	3.81	0.62
C 78-0698 + cysteine	1.41	0.15	9.41	3.63	1.23

Assay conditions: 100 m*M* phosphate buffer, pH 7.70, 37 ± 0.05°C; NO_2^-/NO_3^- measurement: sydnonimines 0.2 m*M* ± L-cysteine 1 m*M*, $n = 3$; NO measurement: sydnonimines 1 m*M* ± L-cysteine 5 m*M*, $n = 3$; guanylate cyclase activation: ± L-cysteine 5 m*M*, $n = 3$.

of metabolite formation must differ fundamentally among the different compounds.

All sydnonimine compounds were found to generate spontaneously nitric oxide, the rate of which closely correlated with their stimulatory action on isolated soluble guanylate cyclase. The NO release from sydnonimines occurred nonlinearly with time. The initially slower liberation of NO can be explained by the hydrolytic step that is required for the formation of the NO-generating A form from the sydnonimine molecule, since the same lag phase was not seen with SIN-1A or with C 78-0652. The assumption that NO splitting off from the A form of sydnonimines occurs independently from pH (9) is confirmed by the nearly uniform rate of NO release from SIN-1A between pH 5 and 10. That NO formation becomes maximal at high sydnonimine concentrations cannot be attributed to a rate-limiting hydrolysis because SIN-1A showed a similar effect. Rather, this phenomenon may be due to a limited diffusion of dissolved oxygen in the incubation buffer when very high sydnonimine concentrations are used.

In contrast to organic nitrates, the sydnonimine compounds tested not only differed with respect to their EC_{50} values on soluble guanylate cyclase, but also in the maximally achievable extent of enzyme activation. This may be due to the extremely short half-life of the active principle for enzyme stimulation, NO. Because of its intriguing instability, the *velocity* of nitric oxide formation is probably a much more important determinant for guanylate cyclase

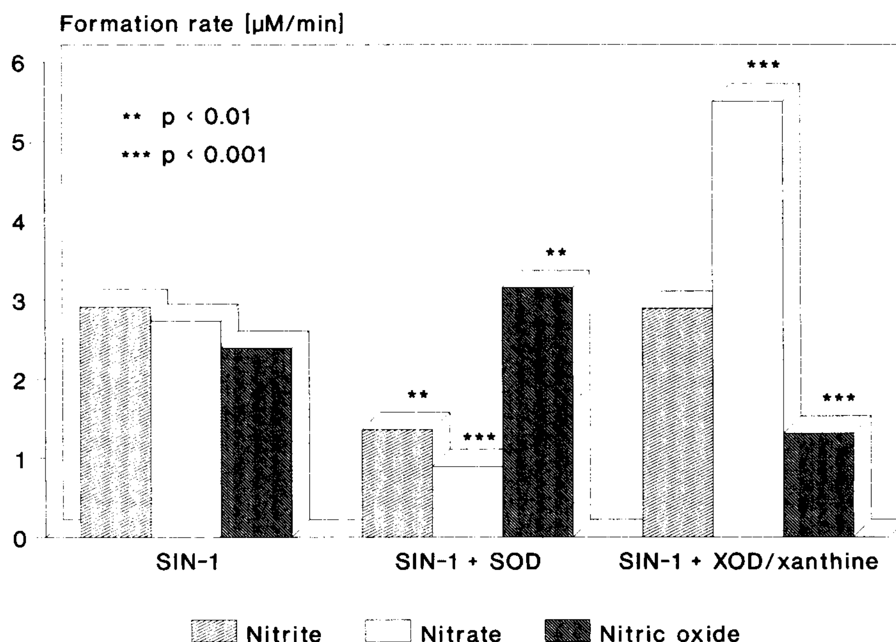


FIG. 5. Influence of superoxide dismutase (SOD) and xanthine/xanthine oxidase (XOD) on formation of nitrite, nitrate, and nitric oxide from SIN-1. Incubation conditions: 100 m*M* phosphate buffer, pH 7.70, 37 ± 0.05°C. NO_2^-/NO_3^- measurement: SIN-1 0.2 m*M*; NO measurement: SIN-1 0.5 m*M*; SOD 100 U/ml, XOD 50 mU/ml, xanthine 50 nmol/ml; the data are means from three to four kinetic runs and are all compiled from the initial formation rates within the first 1 to 5 min of incubation.

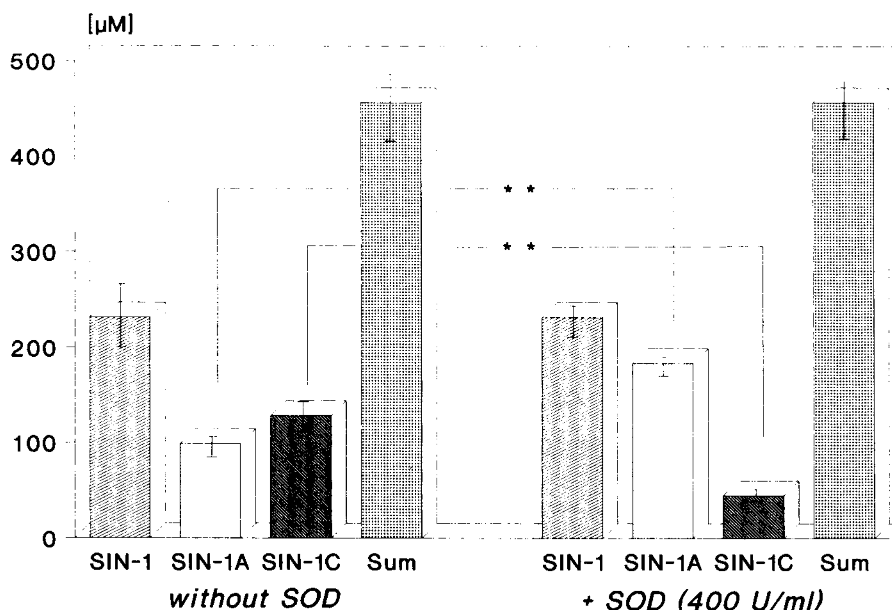


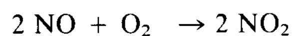
FIG. 6. In vitro decomposition of SIN-1 to SIN-1A and SIN-1C. Incubations were carried out in 100 m*M* phosphate buffer, pH 7.40. The data are mean concentrations ± SEM after 15 min of incubation of 500 µ*M* SIN-1 at 37°C. Mean values of SIN-1 decomposition and total metabolite formation in the presence of SOD are not significantly different from those obtained in its absence, whereas the formation of SIN-1C is markedly reduced in favor of an increased stability of SIN-1A when superoxide formation is diminished by SOD ($n = 3$; $^{**}p < 0.01$).

activation than the *concentration* of NO achieved in the incubation medium and may exert its influence not only on the catalytic but also on the regulatory subunit of soluble guanylate cyclase.

The in vitro stabilities of the A forms of sydnonimines did not correlate with the measured rates of NO release or with the activation of the enzyme, confirming that a direct stimulation of soluble guanylate cyclase by the NO-containing A forms is unlikely (32). Previous studies revealed that, simultaneously with the release of nitric oxide, NO_2^- and NO_3^- occur as metabolic products of sydnonimine breakdown. The formation rates for both anions are similarly dependent on concentration and pH, as is true for the NO formation (results not shown). NO_2^- and NO_3^-, however, do not account for the activation of guanylate cyclase, because C 78-0698 displays a higher stimulating potency than SIN-1 while producing less nitrite/nitrate. The fact that C 78-0698 produces a higher rate of NO release while being apparently more stable in its A form indicates that the extent of NO formation cannot be deduced from a simple comparison of stability constants of intermediary compounds without knowledge of side reactions that determine the further fate of the released NO.

While searching for a reasonable mechanism for the NO release, it was observed that all sydnonimines appeared to be extremely susceptible to the presence of molecular oxygen. We did not succeed in preparing an aqueous solution absolutely free of oxygen, in which SIN-1A could have been completely prevented from decomposition; a small initial decrease in SIN-1A concentration was always noticed that may, at least in part, explain the nearly identical rate of initial NO formation from SIN-1 under air-saturated conditions and those previously regarded as oxygen-free (25). Trace amounts of oxygen are seemingly sufficient to induce the breakdown of sydnonimines. Once induced, the breakdown process becomes autocatalytic: although superoxide formed during the initial stage of the exposure to oxygen does not have any influence on the hydrolytic formation of the A forms of sydnonimines, it markedly enhances the rate of their decomposition and by this the rate of NO formation. This implies the necessity of defining the partial pressure of oxygen when pharmacological studies with sydnonimines are performed.

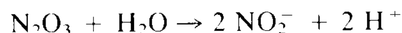
With one exception (C 78-0698), all tested sydnonimines were found initially to consume molecular oxygen at rates that are 1.5- to 10-fold higher than that of NO formation (with C 78-0698, NO formation is about threefold higher than oxygen consumption). By a molar comparison, however, the amounts of consumed oxygen are higher, as would be expected for the oxidative conversion of NO to NO_2^- and NO_3^- according to the following equations:



$$2\,NO + O_2 \rightarrow 2\,NO_2$$

$$2\,NO_2 \rightleftharpoons N_2O_4$$

$$NO_2 + NO \rightleftharpoons N_2O_3$$



$$N_2O_3 + H_2O \rightarrow 2\ NO_2^- + 2\ H^+$$

$$N_2O_4 + H_2O \rightarrow NO_2^- + NO_3^- + 2\ H^+$$

The ratio of the molar amounts of oxygen consumed and SIN-1A degraded to NO and SIN-1C should theoretically be 0.5, but a quotient of 0.9 was found experimentally. These results indicate that O_2 is not only consumed by the oxidative conversion of generated NO to NO_2^- and NO_3^-, but also by an additional process, which most probably is responsible for the initiation of NO formation. The correlation between O_2 consumption and O_2^- formation points to a chemical mechanism in which molecular oxygen initiates the decomposition of the A forms of sydonimines, most probably by one-electron abstraction from the nitrogen that is connected to the N–NO group, as suggested by Bohn and Schönafinger (34). During this reaction, oxygen is reduced to superoxide and SIN-1A is oxidized to an unstable radical cation that decomposes with the release of NO. Furthermore, superoxide may facilitate this process, as indicated by the outcome of kinetic studies on the decomposition of SIN-1 in the presence and absence of SOD.

This is the first report on superoxide formation during sydnonimine breakdown. Simultaneously generated superoxide anions further reduce the half-life of released NO, presumably by a chemical reaction that has been described to occur under alkaline conditions (35). The observed leftward shift of the concentration–response curve to SIN-1 in the presence of SOD is in agreement with an enhancement of the efficacy of NO by blocking the NO-scavenging effect of O_2^-. In view of the coupled production of both species, one could wonder whether or not the measured rates of NO liberation are the true ones. We believe that this is not the case, since the balance between the formation rates of both NO and O_2^- determines what can be measured. This means that the true rates of NO generation will inevitably be underestimated when high amounts of O_2^- are produced at the same time. The same will probably hold true for all reported rates of superoxide liberation in biological systems where both species are formed at the same time.

From our study, which originally focused on the nonenzymatic mechanism of NO release from sydnonimines, it becomes clear that sydnonimines share the simultaneous release of nitric oxide and superoxide in common with physiological mechanisms, which have recently been demonstrated for endothelial cells, activated macrophages, and neutrophils (36–39). SIN-1 thus appears to be not only a donor of endothelium-derived relaxing factor (EDRF) but also of endothelium-derived contracting factor (EDCF), if superoxide may be regarded as the latter (40). Because the individual sydnonimines have a somewhat different balance between the formation of NO and O_2^-, it may be important to test the therapeutic usefulness of compounds that form nitric oxide and superoxide at different rates.

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APPENDIX

The compounds used in this study were as follows: 1) SIN-1: 3-morpholinosydnonimine-*N*-ethylcarbamide; 2) C 78-0652: 2-(*N*-nitrosomorpholinamino)isobutyronitrile; 3) C 78-0698: 3-(thiomorpholino-*S*,*S*-dioxide)sydnonimine hydrochloride; 4) C 78-0757: 3-(4-methylsulfonylpiperazino)sydnonimine hydrochloride; 5) C 79-0862: 3-(4-dimethylaminosulfonylpiperazino)sydnonimine hydrochloride; 6) C 79-0901: 3-(thiomorpholino-*S*-oxide)sydnonimine hydrochloride; 7) C 79-0954: 3-[4-(4-tolylsulfonyl)-piperazino]sydnonimine hydrochloride; 8) C 80-1192: 3-(4-ethoxycarboxypiperazino)sydnonimine hydrochloride; 9) C 80-1239: 3-(*N*-methyl-*N*-tetrahydrothien-3-yl)sydnonimine hydrochloride; 10) C 85-3116: 3-dimethylaminosydnonimine hydrochloride; 11) C 85-3118: 3-piperidinosydnonimine hydrochloride; 12) C 86-3293: 3-(2,2,6,6-tetramethylpiperidino)sydnonimine hydrochloride; 13) C 87-3754: 3-(2,6-dimethylpiperidino)sydnonimine hydrochloride; 14) C 87-3787: 3-(2,2,6,6-tetramethylmorpholino)sydnonimine hydrochloride; 15) C 88-3793: 3-(4-isopropyl-2,2,6,6-tetramethylpiperazino)sydnonimine hydrochloride; 16) C 88-3797: 3-(2,6-dimethylthiomorpholino-*S*,*S*-dioxide)sydnonimine hydrochloride; 17) C 88-3798: 3-(*N*-*t*-butyl)-2-hydroxyethylamino)sydnonimine hydrochloride; 18) C 88-3833: 3-(2,2-dimethyl-4-isopropylpiperazino)sydnonimine hydrochloride; 19) C 88-3834: 3-(2,5,-dimethylpyrrolidino)sydnonimine hydrochloride; 20) C 88-3864: 3(2-hydroxycyclohexylamino)sydnonimine ethylcarbamide; 21) C 88-3877: 3-(*N*,*N*-dibenzylamino)sydnonimine hydrochloride; 22) C 88-3901: 3-cyclohexylaminosydnonimine hydrochloride; 23) and C 88-3913: 3-(2-hydroxyethylamino)sydnonimine hydrochloride.

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