Superoxide-mediated Decomposition of Biological S-Nitrosothiols*

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Incubation of S-nitrosocysteine or S-nitrosogluthathione (5-100 μM) in the presence of a generator of superoxide (xanthine/xanthine oxidase) resulted in a time-dependent decomposition of S-nitrosothiols and accumulation of nitrite/nitrate in reaction mixtures. Quantitatively, the amounts of nitrite/nitrate represented >90% of nitrosamine equivalent of S-nitrosothiols degraded during the incubation. The reaction rates were unaffected by the presence catalase (1 unit/ml). Kinetic analysis showed that the degradation of S-nitrosothiols in the presence of superoxide proceeded at second order rate constants of 76,900 s⁻¹ M⁻¹ (S-nitrosocysteine) and 12,800 s⁻¹ M⁻¹ (S-nitrosogluthathione), respectively, with a stoichiometric ratio of 1 mol of S-nitrosothiol per 2 mol of superoxide. The findings provide the evidence for the involvement of superoxide in the metabolism of S-nitrosothiols. Furthermore, substantially slower reaction rates of superoxide with S-nitrosothiols relative to the reaction rate with NO are consistent with the contention that the transient formation of S-nitrosothiols in biological systems may protect NO from its rapid destruction by superoxide, thus enabling these compounds to serve as carriers or buffers of NO.

S-Nitrosothiols (RSNOs)† have been shown previously to elicit biochemical and physiological effects similar to those elicited by endothelium-derived relaxing factor, including stimulation of soluble guanylate cyclase, vascular relaxation, and inhibition of platelet aggregation (1–3). Such effects have been, for the most part, attributed to the release of NO or related reactive nitrogen oxide species. However, biochemical mechanisms leading to the dissociation of S-NO bond under in vivo conditions are at this time largely unknown. Under in vitro conditions, RSNOs are reasonably stable (over a period of several hours) in physiological buffers in the presence of a chelator of transition metals. The breakdown of S-NO bond can be induced by UV light (4) and certain metal ions such as Hg²⁺ or Cu⁺ (5, 6); however, neither of these reactivities can be considered to be of physiological importance. Additional reactivities of RSNOs which may be operative under in vivo conditions include thiol- and glutathione peroxidase-mediated decomposition, respectively (7, 8). Available evidence suggests that thiol-mediated degradation of RSNOs results in the formation of nitroxyl anion rather than NO (9), whereas the reaction between RSNOs and glutathione peroxidase causes the deactivation of the enzyme due to the modification of selenocysteine residue at its active center (10).

In this report, we present the evidence that RSNOs formed by low molecular weight biological thiol (L-cysteine and glutathione) are degraded in the presence of superoxide. Since the propensity for the generation of superoxide under aerobic conditions is a ubiquitous property of biological systems, the interaction of superoxide with RSNOs is likely to represent a biological route of RSNO catabolism which is not confined to specific tissues or organs. Furthermore, relatively slow rates of superoxide-mediated decomposition of RSNOs indicate that this process may be of physiological importance in mediation of more prolonged, tonic effects of RSNOs exerted simultaneously by the modification of fluxes of oxygen-derived free radicals and by reactive nitrogen oxide species released by the decomposition of S-NO bond.

EXPERIMENTAL PROCEDURES

Materials—Ammonium sulfamate, catalase, L-cysteine, diethylenetriamine pentaacetic acid (DTPA), reduced glutathione, N-ethylemaleimide, nitro blue tetrazolium (NBT), superoxide dismutase (Cu,Zn form), xanthine, xanthine oxidase (XO), specific activity 0.11 unit/mg of protein at pH 7.5), and sulfanilamide were purchased from Sigma. Dihydrorhodamine 123 (DHR) was a product of Calbiochem, La Jolla, CA. All other chemicals were of analytical grade and obtained from standard vendors. The solutions were prepared in deionized, ultrafiltered water with resistance >18 megohms. Spectrophotometric measurements were carried out using a Hitachi U-2000 UV/VIS spectrophotometer. Fluorometric analysis was performed on a Perkin-Elmer Fluorescent Spectrophotometer, model 204-A.

Preparation of RSNOs—Stock solutions of S-nitrosocysteine (CYSNO) and S-nitrosogluthathione (GSNO) were prepared fresh for each experiment by incubating 10 mM thiold with 10 mM NaNO₂ in 20 mM HCl, 1 mM DTPA for 15 min at room temperature in the dark followed by the addition of 1 mM ammonium sulfamate to remove unreacted nitrite. The concentration of RSNOs was determined from the absorbance at 336 nm (molar absorptivity 900 M⁻¹ cm⁻¹). Working solutions were prepared by dilution of stock solutions into 50 mM potassium phosphate buffer (pH 7.8). These solutions were then reacted with 20 μM of HgCl₂ and the second photometric reading was taken either after 10 min (CYSNO) or 15 min (GSNO) incubation. Different incubation times reflect the different sensitivities of S-NO bonds of respective RSNOs to mercuric salt in the presence of DTPA. RSNO concentration was determined from HgCl₂-induced decrement in photometric signal using molar absorption coefficient of 50,000 M⁻¹ cm⁻¹. Control samples were processed identically except for the absence of HgCl₂. No significant degradation of RSNOs was observed in control samples at reaction times used in the study. For simultaneous analysis of nitrite/RSNO and nitrate, the incubations were carried out in a final volume of 2 ml.

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† The abbreviations used are: RSNO(s), S-nitrosothiols; CYSNO, S-nitrosocysteine; DHR, dihydorhodamine 123; DTPA, diethylenetriamine pentaacetic acid; GSNO, S-nitrosogluthathione; KPB, potassium phosphate buffer; NBT, nitro blue tetrazolium; XO, xanthine oxidase.
determination of nitrate utilized the Cd-mediated reduction to nitrite (12) followed by detection in Griess assay. Reliable determination of nitrate in reaction mixtures containing xanthine/XO required the removal of hydrogen peroxide to prevent an additional formation of nitrate resulting from the decomposition of residual amounts of RSNOs in the course of analysis. Hydrogen peroxide was removed by the incubation of samples for 10 min with 0.5 mM 2-mercaptoethanol followed by the addition of 2.5 mM N-ethylmaleimide to eliminate the unreacted thiol. Decomposition of RSNOs was carried out by the addition of 0.2 mM HgCl₂ to the samples supplemented by 200 mM HCl, 100 μM amonium sulfate. Under such conditions, the nitrosoum equivalent of RSNO was converted quantitatively (>99%) to nitrogen (13).

Assay of Superoxide by NBT Reduction (14, 15)—The rate of superoxide-mediated reduction of NBT in competition experiments with CYSNO and GSNO was measured by continuous monitoring of absorbance at 560 nm (molar absorptivity 11,000 M⁻¹ cm⁻¹) in a media containing 50 mM KPB, 1 mM DTPA, pH 7.8, 50 μM xanthine, 56 μM NBT and various concentrations of RSNOs in a final volume of 1 ml. The reaction was initiated by addition of XO (25–40 μg of protein/ml) to yield the rate of absorbance change in the absence of RSNO between 0.015 and 0.025 units/min. The measurements proceeded for 5 min and the reaction rate was determined from the change of absorbance between 60 and 300 s to allow for the equilibration of superoxide concentration during the first minute after the addition of XO. The determination of the rates of superoxide generation in experiments on superoxide-mediated degradation of RSNOs was conducted similarly except for the adjustment of reaction times as mandated by the protocols of RSNO degradation and the increase in xanthine concentration to 200 μM to prevent substrate depletion in assays employing longer incubation times. Under such conditions, the activity of XO represented approximately 80% of that seen with 50 μM xanthine due to the susceptibility of the enzyme to substrate inhibition (16). Superoxide flux was calculated based on the stoichiometry of 2 mol of superoxide/1 mol of monoformazan (17). The rates of superoxide production detected in NBT reduction assay corresponded to the rates of urate formation within the margin of error <15%.

Assay of Uric Acid—Formation of urate in the reaction between xanthine and XO was determined in 50 mM KPB, 1 mM DTPA, pH 7.8 (final volume 1 ml) by continuous monitoring of absorbance at 295 nm (molar absorptivity 11,000 M⁻¹ cm⁻¹); Ref. 16).

Assay of Rhodamine 123—Oxidation of DHR was monitored by measuring the fluorescent intensity of rhodamine 123 (excitation/emission wavelengths 500 and 530 nm, respectively; Ref. 18) in media containing 50 mM KPB, 1 mM DTPA, pH 7.8, 200 μM xanthine, XO (40–45 μg of protein/ml), 50 μM DHR, and 100–150 μM RSNO in a final volume of 2.5 ml. Stock solution of DHR was prepared in dimethyl formamide as described previously (19).

Data Analysis—Unless indicated otherwise, data are presented as mean ± S.E. of measurement with the number of experiments indicated in parentheses. Significance of effects was determined by analysis of variance with post-hoc Scheffe’s test.

RESULTS AND DISCUSSION

Incubation of either CYSNO or GSNO in reaction mixtures consisting of 50 mM KPB, 1 mM DTPA, pH 7.8, 200 μM xanthine and different concentrations of XO resulted in a degradation of RSNOs which was more pronounced at higher rates of superoxide production (Fig. 1). These observations indicate that the rate of RSNO degradation was proportional to the concentration of superoxide in reaction mixtures, the latter being related to the magnitude of superoxide flux and to the rates of superoxide degradation either by spontaneous dismutation or in reaction with RSNO. No significant degradation of RSNOs was observed when the reaction mixtures were supplemented with SOD (5 units/ml) or when the incubations were carried out in the presence of catalase. The rate of formation of uric acid by xanthine/XO under similar reaction conditions was unaffected by up to 500 μM CYSNO or GSNO (not shown).

Inclusion of catalase at concentrations up to 1 unit/ml failed to elicit a significant change in the rate of superoxide-mediated decomposition of either RSNO; higher concentrations of the enzyme caused a partial inhibition. Such an inhibition could be attributed to the reduction in superoxide flux (most likely due to the contamination of catalase by superoxide dismutase) as indicated by the findings that the presence of catalase at concentrations >1 unit/ml inhibited the superoxide-mediated reduction of NBT (Fig. 2). These observations are consistent with the notion that the degradation of RSNOs observed in the presence of xanthine/XO resulted from the reaction of RSNOs with superoxide as opposed to the reaction with the species formed in the course of its further catabolism (hydrogen peroxide, hydroxyl radicals). Additional support for such a contention is provided by findings that hydrogen peroxide (up to 1 mM) did not promote the degradation of RSNOs (20).

Superoxide-mediated decomposition of RSNOs was accompanied by the accumulation of nitrite and nitrate in reaction mixtures. Quantitatively, the sum of concentrations of nitrite and nitrate represented >90% of nitrosoum equivalent of RSNOs degraded during the incubation. The proportion of nitrite in reaction mixtures (34–38% of total nitrite/nitrate content) was found to be remarkably stable for either CYSNO or GSNO, irrespective of the initial RSNO concentration, magnitude of superoxide flux, and presence or absence of catalase.

Time course of the reaction of superoxide with RSNOs at constant, non-limiting rates of superoxide production (5–10-fold excess relatively to the rates of RSNO degradation) is illustrated in Fig. 3A. Under such conditions, the actual concentrations of superoxide are determined primarily by the rate of spontaneous dismutation and can be anticipated to be relatively constant over the time period of the assay.² Plot of the natural logarithms of

\[^{2}\text{S. Alyerani and P. Kostka, unpublished observations.}\]

\[^{3}\text{The relative proportion of superoxide consumed in the reaction with the RSNO (RSNO:O}_2\text{)} in relation to the overall flux at steady state (V = v}_{\text{RSNO}\text{+v}_{\text{Ramn}}\text{)} can be expressed by the relationship (21), v}_{\text{RSNO}}/V = k_{\text{RSNO}}[\text{RSNO}]^2/k_{\text{Ramn}}[\text{O}_2]^2 + k_{\text{Ramn}}[\text{O}_2]^2,\text{ where v}_{\text{Ramn}}\text{ represents the rate of spontaneous dismutation. Under conditions when k}_{\text{Ramn}}[\text{RSNO}]^2/k_{\text{Ramn}}[\text{O}_2]^2,\text{ the contribution of RSNO degradation to the overall rate of superoxide catabolism can be regarded as being negligible and the relationship will be simplified to v}_{\text{RSNO}} = k_{\text{RSNO}}[\text{RSNO}],\text{ where k}_{\text{RSNO}} = k_{\text{RSNO}}[\text{O}_2]\text{ and n is the reaction order in respect to RSNO. The model assumes first order kinetics in respect to superoxide as supported by the findings showing a lack of direct effect of catalase on the reaction rate.}\]
residual RSNO concentrations at different time intervals of incubation followed straight lines for either CYSNO or GSNO (Fig. 3B), indicating that under such conditions the reaction obeys a pseudo-first order kinetics with respect to RSNO concentrations. Such a kinetics would imply a bimolecular reaction between superoxide and RSNOs governed by the rate equation, \( v = k [O_2][RSNO] \). The determination of rate constants for such a reaction by conventional methods of kinetic analysis is hampered by uncertainty about the actual concentrations of superoxide in reaction mixtures. An alternative approach undertaken in this study consisted of the examination of relative rates of NBT reduction at different concentrations of RSNOs (22, 23). Bimolecular reaction between superoxide and RSNOs would predict a linear relationship between the reciprocal values of relative rates of NBT reduction in the presence of RSNOs and the ratios of concentrations of RSNOs to NBT, with the slope equal to the ratio of rate constants,

\[
\frac{v}{V_0} = 1 + \frac{k_{RSNO}}{k_{NBT}}[RSNO]/[NBT] \tag{Eq. 1}
\]

where \( V_0 \) and \( v \) are the rates of NBT reduction in the absence and presence of RSNO, and \( k_{RSNO} \) and \( k_{NBT} \) are second order rate constants of superoxide-mediated RSNO decomposition and NBT reduction, respectively. Experimental data were found to be consistent with such a kinetic model (Fig. 4). Using \( k_{NBT} \) of \( 6 \times 10^4 \text{ M}^{-1} \text{s}^{-1} \) (16), the values of \( k_{RSNO} \) determined from the competition experiments were \( 7.69 \pm 0.64 \times 10^4 \text{ M}^{-1} \text{s}^{-1} \) (CYSNO, \( n = 4 \)) and \( 1.28 \pm 0.05 \times 10^4 \text{ M}^{-1} \text{s}^{-1} \) (GSNO, \( n = 4 \)), respectively.

Table I shows the magnitude of superoxide-mediated decomposition of RSNOs when the reactions were carried out at non-limiting concentrations of RSNOs (initial concentrations approximately 100 \( \mu \text{M} \)). The values of \( k_{CYSNO} \) and \( k_{GSNO} \) would predict that under such conditions the rate of spontaneous dismutation of superoxide at fluxes used in this study would be quantitatively insignificant (second order rate constant for spontaneous dismutation at pH 7.8 is approximately 79,000 \text{ M}^{-1} \text{s}^{-1}, Ref. 24), with near-quantitative consumption of superoxide in reaction with RSNOs. As illustrated by data in Table I, the magnitude of superoxide-mediated degradation at high initial concentrations of RSNOs was not significantly different between GSNO and CYSNO. The ratios between the concentrations of RSNOs consumed in the reaction and the superoxide flux were found to be significantly different from 1 \(( p < 0.01 \) while non-significantly different from 0.5 \(( p > 0.2 \) and remained unchanged by further increase in the initial RSNO concentrations (not shown). Such ratios are consistent with the stoichiometric requirement of consumption of 2 mol of superoxide in reaction with 1 mol of RSNO.

The implications of 2:1 stoichiometry for the reaction mechanism of superoxide-mediated decomposition of RSNOs are at this time unknown and await further investigations. One of the possible mechanisms may involve the initial formation of NO by superoxide-mediated breakdown of S–NO bond. Since NO

![Fig. 2. Effect of catalase on superoxide-mediated decomposition of CYSNO and NBT reduction, respectively.](image)

![Fig. 3. Time course of RSNO degradation at a constant rate of superoxide production. A, reaction mixtures contained 50 mM KPB, 1 mM DTPA, pH 7.8, 200 \( \mu \text{M} \) xanthine, XO (50 \( \mu \text{g} \) of protein/ml; superoxide flux 2.1 \( \mu \text{M} \text{min}^{-1} \) and either CYSNO (circles) or GSNO (triangles). The mixtures were analyzed for RSNO content (ordinate) at time intervals as indicated on the abscissa. Results are means of duplicates for each time interval. B, plot of natural logarithm of residual RSNO concentrations as a function of reaction time.](image)
reactions with superoxide at nearly diffusion limited rate (25), such a mechanism would lead to the consumption of 2 molecules of superoxide and result in the formation of peroxynitrite as a principal metabolite of nitrosourea equivalent of RSNO. Indirect support for such a mechanism is provided by findings that nitrate is a predominant end-product of nitrosonium moiety of RSNOs (see above). The main pathways leading to the formation of nitrate under our experimental conditions may include either the hydrogen peroxide-mediated oxidation of nitrosourea ion (or related species with reactivities similar to those of acidified nitrite) or the decomposition of peroxynitrite (26). The participation of the former pathway of nitrate production is unlikely since the experimental manipulations which suppressed the accumulation of hydrogen peroxide (presence of catalase, increased initial concentrations of RSNOs) failed to influence the nitrate/nitrite ratios. In this context, it may be of interest to note that the relative proportion of nitrate in reaction mixtures observed in this study (62–66%) was nearly identical to that observed by Ischiropoulos et al. (27) in the study of peroxynitrite formation by activated macrophages.

The potential formation of peroxynitrite in the reaction of superoxide with RSNOs was evaluated by monitoring the oxidation of DHR in the course of incubation of RSNOs with xanthine/XO. It has been shown previously that the oxidation of DHR requires the presence of a strong oxidant such as hydroxyl radicals, whereas superoxide or hydrogen peroxide alone was ineffective in oxidizing this compound (18, 19). Recently, Miles et al. (19) have reported that DHR could be oxidized by the product formed during the spontaneous decomposition of spermine/NO adduct in the presence of superoxide. Such a product was presumed to be peroxynitrite. We have observed a similar oxidation of DHR during superoxide-mediated decomposition of RSNOs. The results obtained with CYSNO are illustrated in Fig. 5. Incubation of CYSNO in the presence of XO caused a time-dependent increase in the fluorescent signal of rhodamine 123 in the reaction mixtures supplemented by XO (50 μM DHR, 1 unit/ml of catalase). The formation of rhodamine 123 in the absence of XO was reduced to approximately 10–15% of that seen in the presence of CYSNO and was likely related to the generation of hydroxyl radicals in the xanthine/XO system. No appreciable formation of rhodamine 123 was observed when XO was omitted from the reaction mixtures.

In conclusion, the findings of this study provide evidence for the involvement of superoxide in the metabolism of CYSNO and GSNO. The findings are particularly relevant to the studies of physiological and pharmacological effects of RSNOs as the relative contributions of different routes of RSNO degradation with ensuing generation of reactive nitrogen oxide species in biological systems will be influenced by the fluxes of oxygen-derived free radicals. At the same time, the biological responses elicited by RSNOs will be related in part to the alterations in the metabolic effects exerted by superoxide and related free radical species formed in the course of its catabolism.

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