Free Radical Research, May 2010; 44(5): 552–562

Nitrosation-modulating effect of ascorbate in a model dynamic system of coexisting nitric oxide and superoxide

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(Received date: 8 September 2009; In revised form date: 1 February 2010)

Abstract

The coexistence of nitric oxide and superoxide leads to complex oxidative and nitrosative chemistry, which has been implicated in many pathophysiological conditions. The present study investigated the role of ascorbate in affecting the kinetics of nitrosative chemistry in a model dynamic snystem of coexisting nitric oxide and superoxide. SIN-1 (3-morpholinosydnonimine) was used to elicit various degrees of nitroxidative stress in a reaction buffer and DAN (2,3-diaminonaphthalene) was used as a probe for *N*-nitrosation reaction. The nitrosation kinetics in the absence and presence of ascorbate was followed by measuring the formation of the fluorescent product over time. Computational modelling was used to provide quantitative or semi-quantitative insights into the studied system. The results show that ascorbate effectively quenches *N*-nitrosation reaction, which could be partially attributed to the free radical scavenging and repairing effect of ascorbate. Computational modelling reveals an interesting temporal distribution of superoxide, nitric oxide and peroxynitrite. The model predicts that peroxynitrite is the most predominant species in the SIN-1 system. Furthermore, ascorbate might alter the system dynamics by removing superoxide and, thereby, increasing the availability of nitric oxide.

Keywords: *SIN-1, ascorbate, nitric oxide, superoxide, kinetics,* in silico *modelling, simulations, mathematical model*

Introduction

Nitric oxide ($^{\bullet}NO$) and superoxide ($O_2^{-\bullet}$) are the two important free radicals in biological systems. Simultaneous generation of $^{\bullet}NO$ and $O_2^{-\bullet}$ may occur in many physiological and pathophysiological conditions [1]. When $^{\bullet}NO/O_2^{-\bullet}$ are coexistent in conditions such as during inflammation, they react at a near-diffusion-limited rate to form peroxynitrite, which subsequently undergoes rapid decomposition to form reactive oxygen and nitrogen species [2–6]. Together, nitric oxide, superoxide, peroxynitrite and their derived reactive chemical species form a complex reaction system that governs important chemical reactions of biological significances, e.g. oxidation, nitration and nitrosation [1,7,8].

Nitrosation has been extensively studied as one of the key biochemical reactions associated with nitric oxide and its related species [9]. Nitrosation of proteins or DNA may result in functional modifications of these molecules [10–19]. Two major mechanisms have been proposed for a nitrosation reaction. The first mechanism involves $^{\bullet}NO$ auto-oxidation and the subsequent formation of N_2O_3 that donates a nitrosonium equivalent (NO^+) to nucleophilic residues such as amines and thiols [20,21]. The second mechanism requires the concomitant presence of $^{\bullet}NO$ and $O_2^{-\bullet}$, the formation of reactive oxidative species from the $^{\bullet}NO/O_2^{-\bullet}$ reaction, and the subsequent oxidative attack of a target residue (RH) to form a radical species ($R^{\bullet}$) that directly combines with $^{\bullet}NO$ [22,23]. In a system of concurrent nitric oxide and superoxide, both mechanisms may operate simultaneously, in a flux-dependent, competitive manner [22]. The presence of scavenging molecules for reactive species (e.g. superoxide dismutases) in such a system, therefore, may alter nitrosation kinetics [24,25].

Ascorbic acid (ascorbate, vitamin C) had long been recognized as a nitrosation inhibitor, mostly in acidic, stomach-like biologically relevant conditions [26–29].

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2010 Informa UK Ltd. (Informa Healthcare, Taylor & Francis AS)
DOI: 10.3109/10715761003667570

Earlier studies mainly demonstrated that ascorbate inhibited nitrite-mediated *N*-nitrosation reaction in acidic conditions, which has been implicated in carcinogenesis and cancer prevention [30–33]. Recently, however, ascorbate was shown to be pro-nitrosative when lipids are present in a similar acidic condition [34]. While the role of ascorbate in nitrite-derived, acid-catalysed *N*-nitrosation has been most extensively studied, there is relatively less information regarding the effect of ascorbate on nitrosation reactions mediated by nitric oxide and associated reactive species, especially under neutral physiological pH conditions. Kosaka et al. [35] first reported that ascorbate modulated macrophage-mediated *N*-nitrosation at neutral pH. Their data suggests that ascorbate inhibits *N*-nitrosation via nitrite independent, N_2O_3 associated pathways. Ascorbate was later used as a scavenging agent for N_2O_3 in the studies aiming at dissecting nitrosating mechanisms in cells [36,37].

The role of ascorbate as a nitrosation modulating agent in a system of concomitant generation of nitric oxide and superoxide is less well characterized. In such a system, ascorbate may inhibit nitrosation by acting as a scavenger for superoxide, peroxynitrite, $^{\bullet}NO_2$ and/or other radical species [38–43]. This present study, therefore, aims to investigate how ascorbate would affect nitrosation kinetics in a system that simulates the coexistence of nitric oxide/superoxide. SIN-1 (3-morpholinosydnonimine) was used as a nitric oxide/superoxide/peroxynitrite donor and the effect of ascorbate on SIN-1 mediated nitrosation kinetics was determined. The results show that ascorbate delayed nitrosation with a lag period that is a linear function of ascorbate concentrations. Computational modelling has been applied to gain insights into the dynamics of the studied system.

Materials and methods

Materials

SIN-1 (3-morpholinosydnonimine HCl), 2,3-diaminonaphthalene (DAN), L(+) ascorbic acid, diethyltriaminepentaacetic acid (DTPA), catalase, tetrabutylammonium hydrogen sulphate, sodium nitrite and all other chemicals were purchased from Sigma Chemical Company (St. Louis, MO).

SIN-1 mediated nitrosation kinetics

A fluorometric method using DAN as the nitrosation probe was adopted with slight modifications [25]. Briefly, reactions were performed in 96-well microplates (Nunc, Denmark) at 25 and 37°C. The fluorescence intensity of the nitrosation product, 2,3-naphthotriazole (NAT), was measured using a flourometric microplate reader (Fluoroskan Ascent FL, Thermo Electron) at excitation and emission wavelengths of 380 and 460 nm, respectively. The reaction buffer consists of DAN (3.15 μM), DTPA (0.1 mM), catalase (120 U/ml) and phosphate-buffered saline (pH 7.4; 10 mM phosphate buffer containing 138 mM NaCl and 2.7 mM KCl). Reactions were initiated by adding SIN-1 (5 mM stock solution in DMSO) into 300 μL reaction buffers containing various concentrations of ascorbic acid (0–10 μM). The fluorescence intensity after the addition of SIN-1 (at various concentrations: 100, 50, 25 and 12.5 μM) was measured at 10-min intervals up to 300 min. The concentrations of formed 2,3-naphthotriazole (NAT) were determined using synthesized NAT standards [44].

Kinetics of SIN-1 decomposition

The kinetics of SIN-1 decomposition in the reaction buffer at different temperatures (22, 25, 30 and 37°C) was measured using an HPLC method [25]. Briefly, an initial concentration of 100 μM SIN-1 in reaction buffer (pH 7.4) was prepared for the kinetic measurement. At each time interval (10, 30 or 60 min), an aliquot of 50 μL sample was taken and injected directly to the HPLC system (Shimadzu LC-6A, Japan; Rheodyne 7125, USA) for the determination of SIN-1 concentrations. The analytical method consists of a reversed phase column (Inertsil ODS-80A, 5 μm, 4.6×250 mm), a mobile phase (10 mM sodium acetate buffer (pH 3.1)/acetonitrile/methanol=98/1/1.5, flow rate =1 ml/min) and UV detection (Soma S-3702, Japan) at 238 nm. The rate constant for the first step of SIN-1 decomposition (k_1) was directly estimated from the slope of the log-linear concentration vs time plot.

HPLC determination of ascorbic acid

The kinetics of ascorbate decay in the reaction buffer at 37°C was measured by an HPLC method described by Veltman et al. [45]. The method consists of a reversed phase column (Inertsil ODS-80A, 5 μm, 4.6×250 mm), a mobile phase (7 mM tetrabutylammonium hydrogen sulphate in Milli-Q water, flow rate=1 ml/min) and UV detection (Soma S-3702, Japan) at 254 nm. The kinetic experiment was initiated by adding ascorbate (final concentration of 10 μM) in reaction buffer containing SIN-1 (100 μM, 50 μM and 25 μM). An aliquot of 50 μL sample was then taken at the pre-defined sampling times and immediately injected into the HPLC system (Shimadzu LC-6A, Japan; Rheodyne 7125, USA). The concentrations of ascorbate were determined using authentic ascorbate standards.

Computational modelling

Numerical simulations were carried out using Mathematica 6 (Wolfram Research, Champagne, IL).

The Mathematica built-in function NDSolve was used for numerically solving the differential equations (StartingStepSize → 0.1).

Results

SIN-1 mediated oxidation and nitrosation chemistry is initiated by the first-step decomposition reaction of SIN-1 in aqueous solution; therefore, to better characterize the studied system, we conducted a formal kinetic study using HPLC and measured the rate of the first-step decomposition of SIN-1. The results show temperature dependency of the rate constant, which follows the Arrhenius relationship (activation energy (E_a) = 32 kcal/mole; pre-exponential factor (A) = 1.7×10^{19} s^{-1}). The measured rate constant at 37°C is 0.030 ± 0.002 min^{-1} (5.0×10^{-4} s^{-1}, $n=3$).

The kinetic profiles of SIN-1-mediated *N*-nitrosation (i.e. the formation of 2,3-naphthotriazole, NAT) in the absence and presence of ascorbate (1, 5 and 10 μM, 37°C, $n=3$) are shown in Figure 1. The degree of nitrosation in the studied system was intensified by increasing initial concentrations of SIN-1 (12.5–100 μM). For lower degrees of nitrosation (Figures 1A and B), 10 μM ascorbate was sufficient to bring about a total inhibitory effect on nitrosation kinetics; whereas for higher degrees of nitrosation (Figures 1C and D), nitrosation kinetics is mainly delayed by ascorbate at the concentration ranges studied. Notably, three major phases can be identified in Figure 1D—the initial lag phase, the acceleration phase and the slow decaying phase. At initial [SIN-1] = 100 μM, ascorbate can only prolong the lag phase of nitrosation (i.e. right shifting of curves, Figure 1D); other phases, however, are less affected by ascorbate. When the lag times were plotted against ascorbate concentrations, a linear relationship could be obtained (Figure 2).

It is of interest to note that NAT fluorescence diminished after reaching a maximum, especially in the system that underwent intensive nitrosation (Figure 1D). Indeed, the results of additional experiments indicate that the authentic NAT was inherently unstable in the reaction buffer (Figure 3)—its concentration decays initially and then reaches equilibrium. Remarkably, SIN-1 was able to accelerate the initial degradation of NAT (Figure 3).

Ascorbate concentrations were followed over time and the results in Figure 4 show that the rate of ascorbate decomposition increases with increasing nitroxidative stress. These data suggest that ascorbate was consumed by reacting with the reactive nitrogen and oxygen species derived from SIN-1. There is, however,

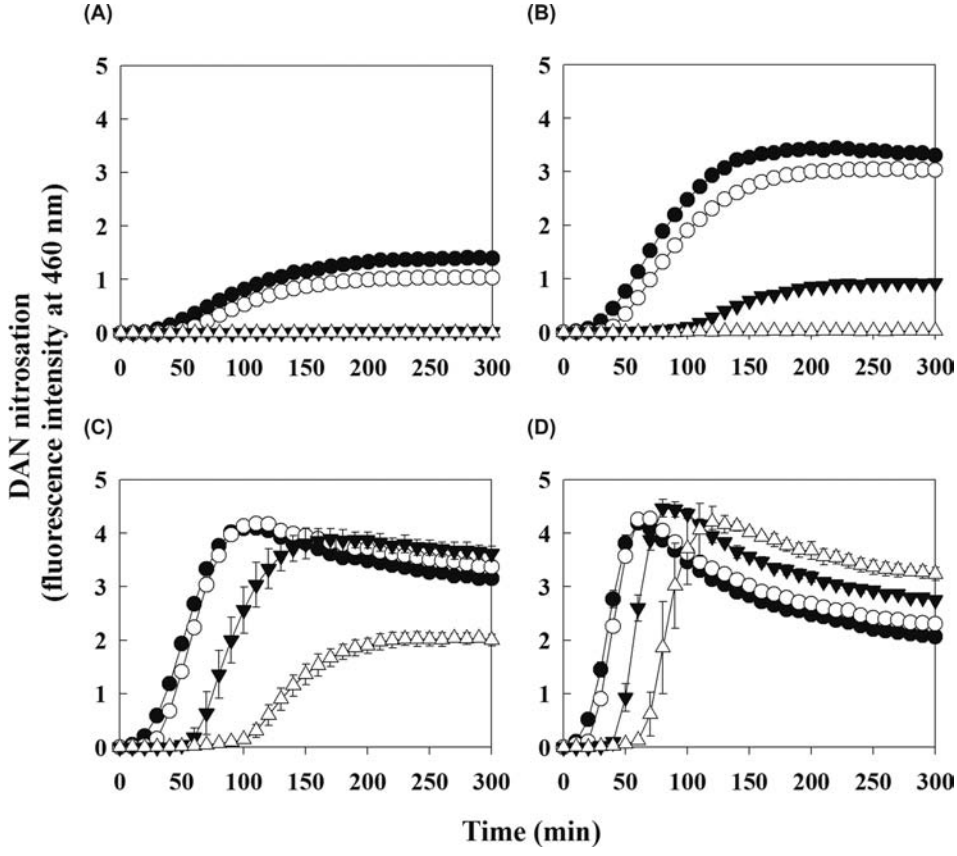


Figure 1. The effect of ascorbate on SIN-1-mediated *N*-nitrosation kinetics ($n=3$). (A) [SIN-1] = 12.5 μM; (B) [SIN-1] = 25 μM; (C) [SIN-1] = 50 μM; (D) [SIN-1] = 100 μM. Ascorbate concentrations = 0 (solid circle), 1 μM (open circle), 5 μM (solid triangle) and 10 μM (open triangle).

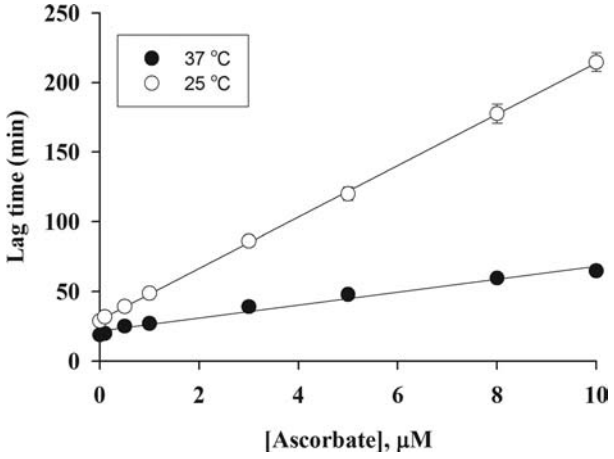


Figure 2. Linear dependence of the lag time on ascorbate concentrations. *N*-nitrosation kinetics mediated by 100 μM SIN-1 was measured at 25 and 37°C. The lag time was estimated by drawing a tangent to the slope of the acceleration phase of kinetic profiles (as in Figure 1D) and extrapolating it to the time-axis.

a discrepancy between the estimated time for complete decay of ascorbate ($t_{c=0}$) and the lag time for nitrosation initiation (t_{lag}) (Figure 4). The time differences ($\Delta t = t_{lag} - t_{c=0}$) are estimated to be 43 min, 61 min and >189 min for different initial SIN-1 levels (i.e. 100, 50 and 25 μM, respectively). In other words, the nitrosation-inhibitory effect of ascorbate is extensively extended beyond the decomposition time of ascorbate, suggesting that other chemical species derived from ascorbate may operate. It can be seen, however, that the two-electron oxidation product of ascorbate, dehydroascorbate (DHA), had little effect on nitrosation kinetics (Figure 5).

To offer a quantitative understanding of the SIN-1 model system, a base model of SIN-1-derived reaction chemistry was proposed (Scheme 1). This model takes into account the mechanisms of the release of superoxide and nitric oxide from SIN-1 and assembles the current knowledge of chemical reactions associated with reactive nitrogen and oxygen species. The detailed chemical reactions and the rate constants are listed in Table I. Simulations were conducted by numerically solving the rate equations, according to Scheme 1 and Table I, as a set of ordinary differential equations (Appendix; online version only). The simulation results show that the proposed base model can satisfactorily describe the nitrosation kinetics observed under different levels of nitroxidative stress elicited by SIN-1 (Figure 6).

The effect of ascorbate on SIN-1-mediated nitrosation kinetics was modelled. The ascorbate model was developed by incorporating the reactive-species-scavenging reactions of ascorbate (Table II) into the base model. It appears that the ascorbate model can capture, in a semi-quantitative manner, the essence of the time delaying effect of ascorbate on nitrosation kinetics (Figures 7A) as well as the essence of rapid decaying of ascorbate (Figure 7B).

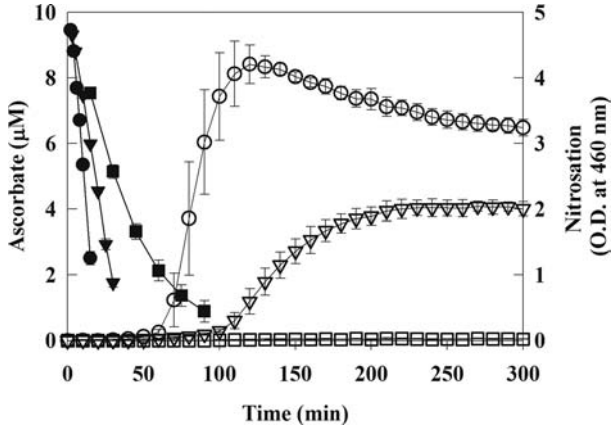


Figure 4. Decomposition kinetics of 10 μM ascorbate (solid symbols) and the corresponding nitrosation kinetics (open symbols) in the reaction system with various SIN-1 concentrations (circles, 100 μM; triangles, 50 μM; squares, 25 μM; $n=3$).

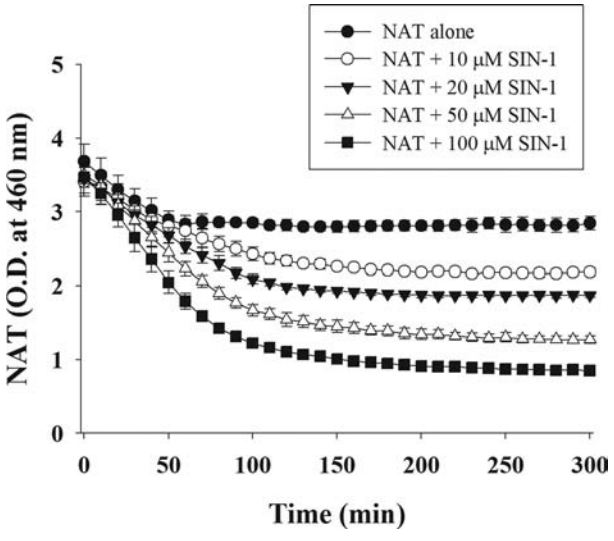


Figure 3. Degradation kinetics of NAT (2,3-naphthotriazole; 0.5 μM) in the absence and presence of SIN-1 ($n=3$).

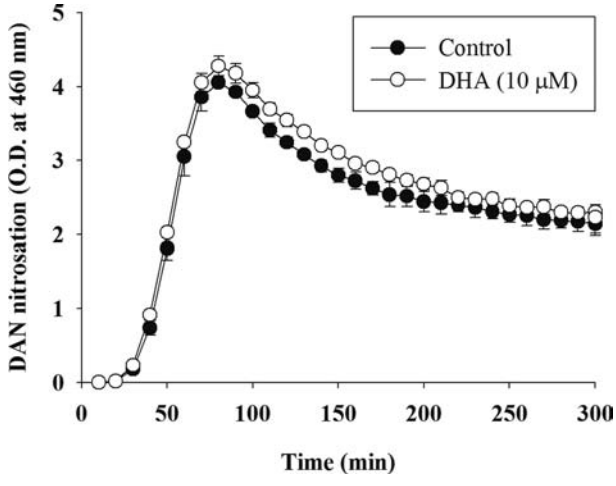
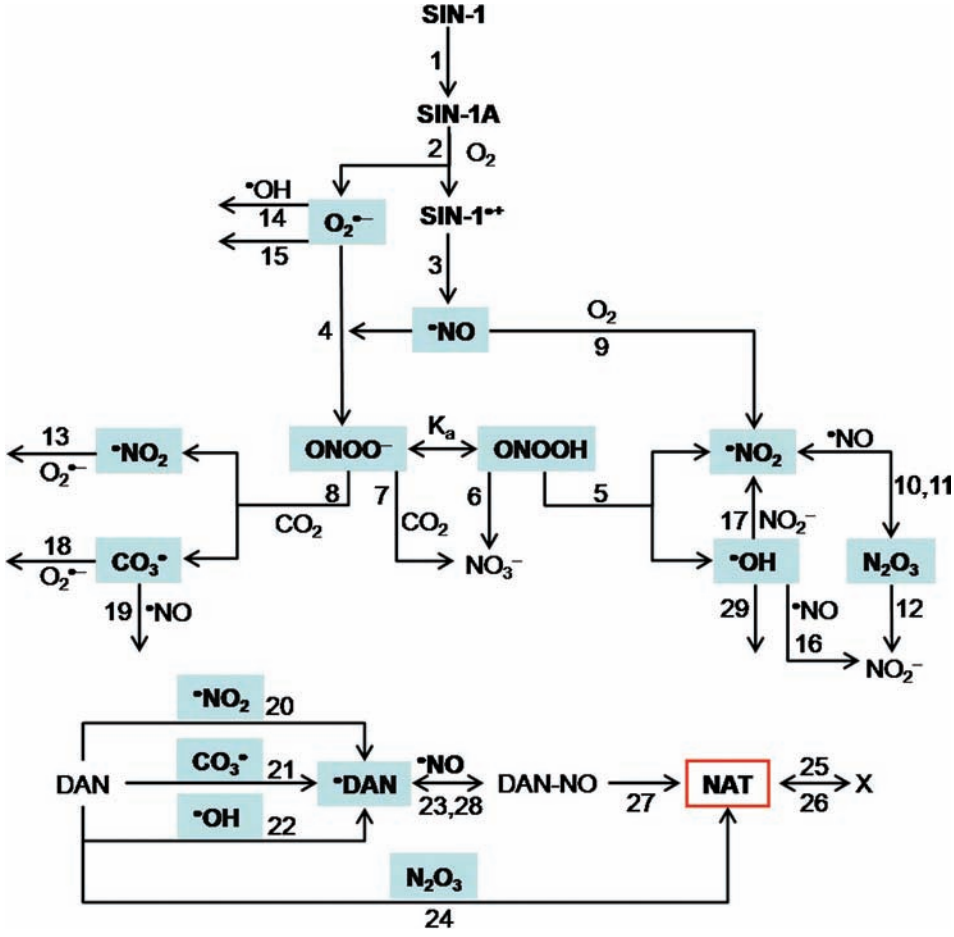


Figure 5. Effect of dehydroascorbate (DHA) on SIN-1-mediated *N*-nitrosation kinetics ($n=3$).



Scheme 1. The base model of the SIN-1 reaction system. The number denotes a particular reaction step. The exact reactions and their rate constants are listed in Table I. DAN, 2,3-diaminonaphthalene; NAT, 2,3-naphthotriazole; X, unknown product.

Discussion

SIN-1 (3-morpholinosydnonimine) has been widely used as a nitric oxide- or peroxynitrite-releasing agent in biochemical and pharmacological studies [46–58]. Chemically, SIN-1 releases nitric oxide and superoxide through a consecutive 3-step mechanism [59–61]. Since nitric oxide and superoxide react in a diffusion-limited manner to form peroxynitrite, SIN-1 would be intuitively considered as a peroxynitrite donor. Alternatively, SIN-1 could act mainly as a nitric oxide donor under an anaerobic condition [61] or in the presence of strong oxidants [62]. Our current understanding, therefore, is that SIN-1 can be a donor of either nitric oxide or peroxynitrite, depending on experimental conditions. Because our current knowledge is somewhat qualitative in nature, to better understand the SIN-1 reaction system, we need to study the dynamics of the system. For example, a relevant question may be: what are the kinetic profiles (or the relative abundance) of various reactive species in the SIN-1 system. This present study investigated the dynamics of SIN-mediated *N*-nitrosation by perturbing the system with ascorbate and applied computational modelling to comprehend the complexity of the system.

Low micromolar concentrations of ascorbate are shown to effectively quench SIN-1-mediated *N*-nitrosation kinetics (Figure 1). This seems to be consistent with a previous study showing low IC_{50} of ascorbate (6–7 μM) for SIN-1-mediated oxidation of dihydrorhodamine 123 [41]. In addition, our data show that oxidized ascorbate (dehydroascorbate) did not significantly alter DAN nitrosation (Figure 5). Thus, the ability of ascorbate to scavenge or repair reactive oxidative species may play a major role in inhibition of nitrosation reaction. Furthermore, the results of additional experiments indicate that the observed action of ascorbate was not resulting from an artificial reaction between ascorbate/dehydroascorbate and DAN (not shown) [63].

Computational modelling

SIN-1-mediated reaction chemistry involves many reactive species derived from nitric oxide and superoxide and the mutual reactions of various reactive species form a complex reaction network [64–66]. While the dynamic behaviour of the input and output of the SIN-1 system can be measured experimentally, it is

Table I. Reactions, rate constants and parameters used in the base model.*

No	Reaction	Rate constant	Remarks/reference
1	SIN-1 → SIN-1A	$k_1 = 5.0\times10^{-4}$ (s^{-1})	Experimentally determined
2	SIN-1A + O_2 → SIN-1$^{\bullet+}$ + $O_2^{\bullet-}$	$k_2 = 6.7$ ($M^{-1}\,s^{-1}$)	Estimated
3	SIN-1$^{\bullet+}$ → $^{\bullet}NO$ + SIN-1C + H^+	$k_3 = 5.0\times10^{-3}$ (s^{-1})	Estimated
4	$^{\bullet}NO + O_2^{\bullet-} \rightarrow ONOO^-$	$k_4 = 1.9\times10^{10}$ ($M^{-1}\,s^{-1}$)	[71]
5	$ONOOH \rightarrow {}^{\bullet}NO_2 + {}^{\bullet}OH$	$k_5 = 2.3\times10^{-1}$ (s^{-1})	[72]
6	$ONOOH \rightarrow NO_3^- + H^+$	$k_6 = 5.7\times10^{-1}$ (s^{-1})	[72]
7	$ONOO^- + CO_2 \rightarrow NO_3^- + CO_2$	$k_7 = 2.0\times10^{4}$ ($M^{-1}\,s^{-1}$)	[73]
8	$ONOO^- + CO_2 \rightarrow {}^{\bullet}NO_2 + CO_3^{\bullet-}$	$k_8 = 1.0\times10^{4}$ ($M^{-1}\,s^{-1}$)	[73]
9	$2\,{}^{\bullet}NO + O_2 \rightarrow 2\,{}^{\bullet}NO_2$	$k_9 = 2.0\times10^{6}$ ($M^{-2}\,s^{-1}$)	[74]
10	$^{\bullet}NO + {}^{\bullet}NO_2 \rightarrow N_2O_3$	$k_{10} = 1.1\times10^{9}$ ($M^{-1}\,s^{-1}$)	[75]
11	$N_2O_3 \rightarrow {}^{\bullet}NO + {}^{\bullet}NO_2$	$k_{11} = 8.1\times10^{4}$ (s^{-1})	[76]
12	$N_2O_3 + H_2O \rightarrow 2NO_2^- + 2H^+$	$k_{12} = 2.0\times10^{3}$ (s^{-1})	[77]
13	$O_2^{\bullet-} + {}^{\bullet}NO_2 \rightarrow O_2NOO^-$	$k_{13} = 4.5\times10^{9}$ ($M^{-1}\,s^{-1}$)	[75]
14	$O_2^{\bullet-} + {}^{\bullet}OH \rightarrow O_2 + OH^-$	$k_{14} = 1.0\times10^{10}$ ($M^{-1}\,s^{-1}$)	[78]
15	$2O_2^{\bullet-} + 2H^+ \rightarrow O_2 + H_2O_2$	$k_{15} = 2.5\times10^{5}$ ($M^{-1}\,s^{-1}$)	[79]
16	$^{\bullet}NO + {}^{\bullet}OH \rightarrow NO_2^- + H^+$	$k_{16} = 1.0\times10^{10}$ ($M^{-1}\,s^{-1}$)	[75]
17	$NO_2^- + {}^{\bullet}OH \rightarrow {}^{\bullet}NO_2 + OH^-$	$k_{17} = 5.3\times10^{9}$ ($M^{-1}\,s^{-1}$)	[75]
18	$CO_3^{\bullet-} + O_2^{\bullet-} + H^+ \rightarrow HCO_3^- + O_2$	$k_{18} = 4.0\times10^{8}$ ($M^{-1}\,s^{-1}$)	[80]
19	$CO_3^{\bullet-} + {}^{\bullet}NO + OH^- \rightarrow HCO_3^- + NO_2^-$	$k_{19} = 3.5\times10^{9}$ ($M^{-1}\,s^{-1}$)	[81]
20	$^{\bullet}NO_2 + DAN \rightarrow DAN^{\bullet} + NO_2^- + H^+$	$k_{20} = 4.6\times10^{7}$ ($M^{-1}\,s^{-1}$)	Model aromatic amine: *p*-phenylenediamine [82]
21	$CO_3^{\bullet-} + DAN \rightarrow DAN^{\bullet} + HCO_3^-$	$k_{21} = 5.0\times10^{8}$ ($M^{-1}\,s^{-1}$)	Model aromatic amine: aniline [82]
22	$^{\bullet}OH + DAN \rightarrow DAN^{\bullet} + OH^- + H^+$	$k_{22} = 1.0\times10^{8}$ ($M^{-1}\,s^{-1}$)	Model aromatic amine: *p*-phenylenediamine [83]
23	$^{\bullet}NO + DAN^{\bullet} \rightarrow DANNO$	$k_{23} = 1.0\times10^{10}$ ($M^{-1}\,s^{-1}$)	[65]
24	$N_2O_3 + DAN \rightarrow NAT + NO_2^- + H^+$	$k_{24} = 1.8\times10^{8}$ ($M^{-1}\,s^{-1}$)	[76]
25	NAT → X	$k_{25} = 1.0$–3.3×10^{-3} (s^{-1})	Estimated
26	X → NAT	$k_{26} = 9.2\times10^{-5}$–$5.3\times10^{-4}$ (s^{-1})	Estimated
27	DANNO → NAT	$k_{27} = 3.3\times10^{-4}$ (s^{-1})	Estimated
28	DANNO → $^{\bullet}NO$ + $DAN^{\bullet}$	$k_{28} = 1.7\times10^{1}$ (s^{-1})	Estimated
29	$^{\bullet}OH + DMSO \rightarrow CH_3S(O)OH + CH_3^{\bullet}$	$k_{29} = 7.0\times10^{9}$ ($M^{-1}\,s^{-1}$)	[82]

*Initial concentrations: [SIN-1] = 100, 50, 25 and 12.5 μM; [DAN] = 3.15 μM; [O_2] = 100 μM [84]; [CO_2] = 10 μM [6]; [DMSO] = 0.43 M. pKa values: pKa (ONOOH) = 6.8 [85]; pKa (Ascorbic acid) = 4.04 [86].

somewhat a daunting task to conduct experiments for determining multiple highly reactive reactions inside the system. The difficulty arises from the fact that the reactive species are unstable, mutually reactive and short-lived, that they exist at low concentrations and that the analytical methods employed often lack required sensitivity and specificity or the method itself would affect the dynamics of the system [67–69].

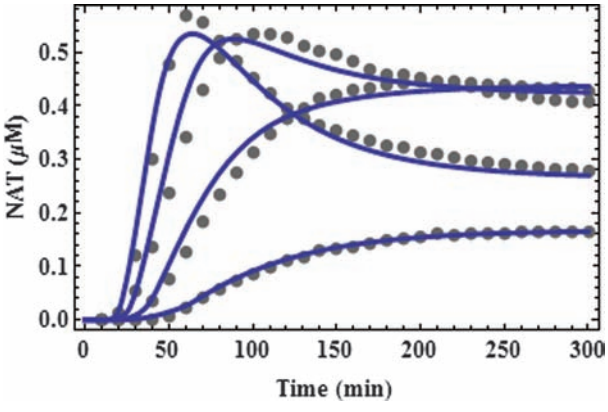


Figure 6. The base-model fitting of *N*-nitrosation kinetics mediated by various SIN-1 concentrations (from left to right: 100, 50, 25 and 12.5 μM). Circles: observed data; lines: simulated data.

Computational modelling, therefore, was used to provide quantitative (or semi-quantitative) insights into the SIN-1 model system. As can be seen from Scheme 1 and Table I, the complexity of the SIN-1 system is evident from the fact that some 29 reactions are required to describe the system. These reactions were considered mechanistically probable in the SIN-1 system and the rate constants for most reactions are available from the literature. In the model-building process, those established reactions with reported rate constants were first assembled as the core of the model, to which assumed reactions were added and tested one at a time to possibly obtain a parsimonious model. To increase the validity of our base model, multiple experimental data obtained under different levels of nitroxidative

Table II. Reactions and rate constants associated with ascorbate's action in the SIN-1 model system.

No.	Reaction	Rate constant	Remarks/reference
I	$ASC^- + O_2^{\bullet-} + H^+ \rightarrow ASC^{\bullet-} + H_2O_2$	$k_I = 2.7\times10^5$ ($M^{-1}\,s^{-1}$)	[38]
II	$ASC^- + ONOOH \rightarrow ASC^{\bullet-} + {}^{\bullet}NO_2$	$k_{II} = 2.35\times10^2$ ($M^{-1}\,s^{-1}$)	[42]
III	$ASC^- + {}^{\bullet}NO_2 \rightarrow ASC^{\bullet-} + NO_2^-$	$k_{III} = 3.5\times10^7$ ($M^{-1}\,s^{-1}$)	[82]
IV	$ASC^- + DAN^{\bullet} \rightarrow ASC^{\bullet-} + DAN$	$k_{IV} = 1.0\times10^8$ ($M^{-1}\,s^{-1}$)	Estimated
V	$2ASC^{\bullet-} + H^+ \rightarrow ASC^- + DHA$	$k_V = 6.5\times10^7$ ($M^{-1}\,s^{-1}$)	[82]
VI	$ASC^{\bullet-} + O_2^{\bullet-} + 2H^+ \rightarrow DHA + H_2O_2$	$k_{VI} = 2.6\times10^8$ ($M^{-1}\,s^{-1}$)	[82]
VII	$ASC^- + {}^{\bullet}OH \rightarrow ASC^{\bullet-} + H_2O$	$k_{VII} = 1.6\times10^9$ ($M^{-1}\,s^{-1}$)	[82]
VIII	$ASC^- + CO_3^{\bullet-} \rightarrow ASC^{\bullet-} + HCO_3^-$	$k_{VIII} = 1.1\times10^9$ ($M^{-1}\,s^{-1}$)	[82]

stress were fitted; and the result seems satisfactory (Figure 6).

Our simulation data, for the first time, reveal an interesting temporal distribution of the three major species, i.e. superoxide, peroxynitrite and nitric oxide, of the SIN-1 system (Figure 9A). It can be seen that superoxide is dominant only initially and within a short period of time (Figure 9A). In contrast, nitric oxide is significantly accumulated only after the concentration of superoxide is diminished to a critical level. This superoxide/nitric oxide-switching phenomenon could be understood as follows. First, superoxide is the first reactive species to be released from SIN-1. Secondly, nitric oxide and superoxide reacts at a diffusion-limited rate. Thirdly, superoxide is also consumed by reacting with downstream reactive species (e.g. reactions 13 and 18, Scheme 1 and Table I). Thus, figuratively speaking, there is a competition between superoxide and nitric oxide—i.e. the prevailing species would consume the less prevailing one. Among the three species, peroxynitrite is predicted to be the most predominant species, given that it has the highest value in the area under the concentration-time curve (AUC) and has a sustained presence in the system (Figure 9A).

The greatest challenge of the present study was to find a quantitative model to simultaneously describe the nitrosation-inhibitory effect and the decomposition kinetics of ascorbate. Our modelling strategy, therefore, was to include in the model only the established actions of ascorbate on free radical scavenging and repairing (Reactions I–VIII, Table II). Although the departure of the model simulation from the experimental data is evident (Figure 7), it is important to note that our model can epitomize the quintessence of the experimental result (Figure 8). The discrepancy described, however, underscores that there is a gap in our current understanding of the action of ascorbate in the nitric oxide/superoxide-coexisting system. Specifically, more

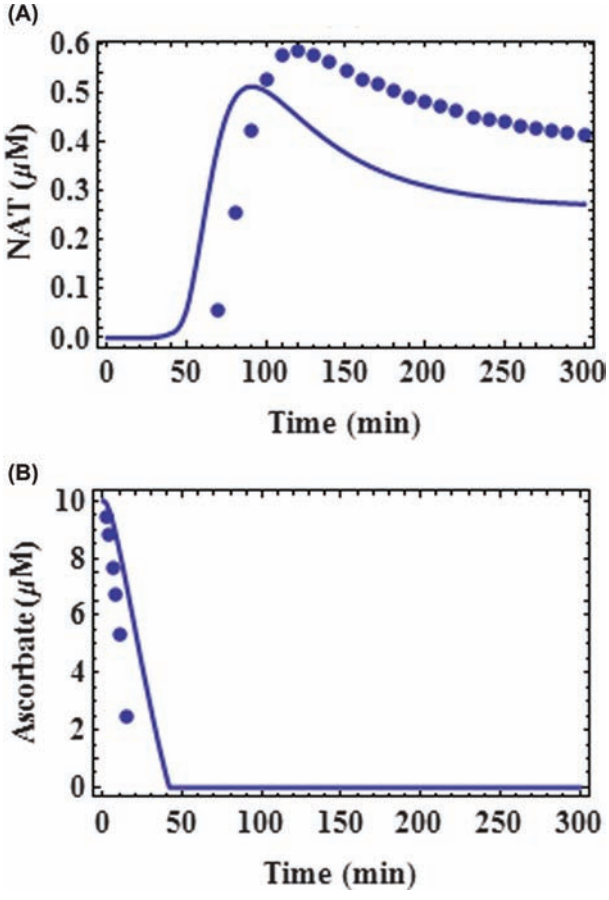


Figure 7. Comparison of simulated (lines) and observed (symbols) kinetic profiles in the presence of 10 μM ascorbate. (A) *N*-nitrosation; (B) ascorbate decomposition. [SIN-1] = 100 μM.

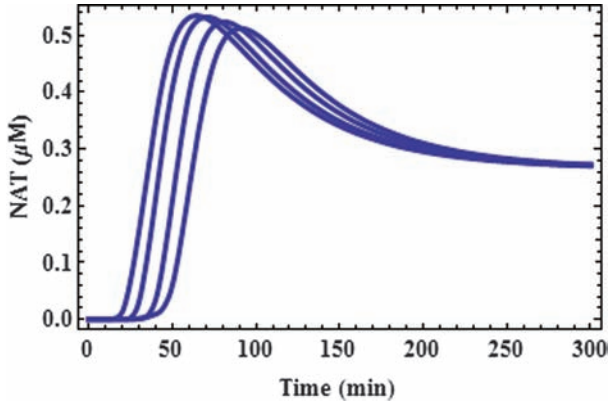


Figure 8. Simulated kinetic profiles of *N*-nitrosation in the presence of various concentrations of ascorbate (from left to right: 0, 1, 5 and 10 μM). [SIN-1] = 100 μM.

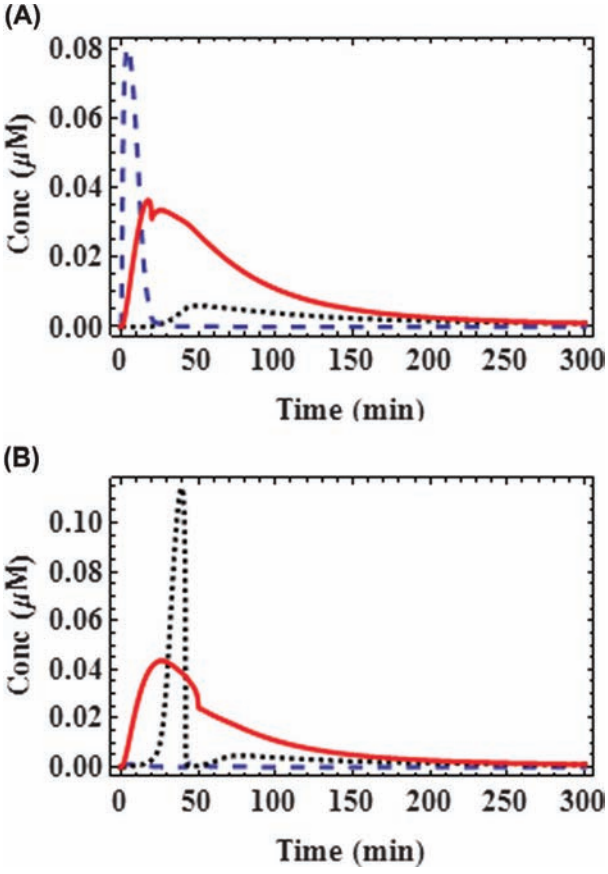


Figure 9. Simulated kinetic profiles of superoxide (dashed lines), nitric oxide (dotted lines) and peroxynitrite (solid lines). [SIN-1] = 100 μM. (A) control (the base model); (B) in the presence of 10 μM ascorbate.

rapid decomposition of ascorbate and longer initiation time for nitrosation than predicted, as shown in Figure 7, suggest that intermediates or products derived from ascorbate oxidation might be operative. Experimentally, our data indicate that dehydroascorbate did not alter nitrosation kinetics (Figure 5). Furthermore, the simulation result shows that the ascorbyl radical forms and disappears rapidly (not shown), suggesting that it might contribute insignificantly to the action of ascorbate. Kytzia et al. [70] have shown that the ascorbyl radical reacts in a reversible manner with nitric oxide. A plausible mechanism, therefore, is the formation of an ASC-NO adduct via the following reaction [70].

$$^{\bullet}NO + ASC^{\bullet -} \rightleftharpoons ASCNO^{-}$$

Further simulations, however, show that nitrosation kinetics was not substantially altered even when the rate constant for the NO-ascorbyl radical reaction was raised to a diffusion-limited range (i.e. $\sim 10^{10}\,M^{-1}\,s^{-1}$). The reason is that the nitric-oxide-scavenging reaction of the ascorbyl radical is a really reversible reaction, whereas the self-dismutation reaction of the ascorbyl radical is an (nearly) irreversible process (Reaction V, Table II).

Dimethyl sulphoxide (DMSO) was used to dissolve SIN-1 and DAN in the preparation of stock solutions. The final DMSO concentration in the reaction buffer is estimated to be 3.05% (0.43 M). Since DMSO reacts extremely rapid with hydroxyl radicals (Reaction 29, Table I), it is important to clarify how DMSO would affect the system dynamics studied. Computationally, by setting [DMSO] = 0 in the mathematical model, the result shows that the profile of nitrosation kinetics is not significantly altered (only 2.2% change in the extent of nitrosation). Moreover, by experimentally reducing the final DMSO concentration to 0.05%, we did not observe a significant change in nitrosation kinetics. These results suggest that hydroxyl radicals are not the major radical species for initiating oxidative reaction in our model system (Step 22, Scheme 1). Instead, we calculate that the major oxidative pathways are controlled by nitrogen dioxide and carbonate radicals (Steps 20 and 21); both pathways contribute to ~97% of DAN oxidation. According to Reaction 29 (Table I), methyl radical will be formed and involved in trapping other radical species or promoting one electron oxidations or, more probably, both. However, because of the insignificant contribution of hydroxyl radicals discussed above, the reactions of methyl radicals can possibly be disregarded.

Conclusions

The concept invoked in the present study is that SIN-1 should not be treated as a simple releasing agent of nitric oxide, superoxide or peroxynitrite. Rather, a dose of SIN-1 in an aqueous solution signifies a complex dynamic system of multiple reactions involving reactive nitrogen and oxygen species. Levels of the three species (nitric oxide, superoxide and peroxynitrite) in such a system, therefore, are not only dependent on their release and formation kinetics, but on the kinetics of their self-consuming and degradation reactions. Our base model predicts that in the SIN-1 system an initial burst and then rapid declining of superoxide occurs. The peak formation of peroxynitrite follows that of superoxide; and peroxynitrite is predicted, among the three species, to be the most dominant species. Nitric oxide, on the contrary, has a delayed but sustained, lower-level, presence. Furthermore, by perturbing the system with ascorbate, the capricious and volatile nature of the system is revealed. Our study demonstrates that ascorbate can effectively inhibit SIN-1-mediated nitrosation kinetics, which could be partially attributed to the free radical scavenging and repairing effect of ascorbate. Notably, ascorbate might alter the system dynamics by removing superoxide and, thereby, increasing the availability of nitric oxide. An important implication of the present study, therefore, is that, for those who would use SIN-1 in their experiments, they should be

reminded that it is not just nitric oxide or peroxynitrite, but the entire dynamic system that is administered. The temporal pattern of coexistence between competing reactive nitrogen and oxygen species and the redox status of the system studied should be considered in the interpretation of experimental results.

Declaration of interest: The study was supported by grants from the National Science Council, Taiwan, ROC. (NSC 92-2320-B016-053 and 93-2320-B-0-16-049). The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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This paper was first published online on Early Online on 26 February 2010.