Reaction of Superoxide and Nitric Oxide with Peroxynitrite
IMPLICATIONS FOR PEROXYNITRITE-MEDIATED OXIDATION REACTIONS
IN VIVO

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Running title: Oxidation by Simultaneous Generation of ·NO and O₂⁻

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Summary. Peroxynitrite (ONOO⁻/ONOOH), the product of the diffusion-limited reaction of nitric oxide (‘NO) with superoxide (O₂⁻), has been implicated as an important mediator of tissue injury during conditions associated with enhanced ‘NO and O₂⁻ production. Although several groups of investigators have demonstrated substantial oxidizing and cytotoxic activities of chemically synthesized peroxynitrite, others have proposed that the relative rates of ‘NO and O₂⁻ production may be critical in determining the reactivity of peroxynitrite formed in situ (Miles, A.M. et al. (1995) J.Biol.Chem. 271, 40-47). In the present study, we examined the mechanisms by which excess O₂⁻ or ‘NO production inhibits peroxynitrite-mediated oxidation reactions. Peroxynitrite was generated in situ by co-addition of a chemical source of ‘NO, spermineNONOate, and an enzymatic source of O₂⁻, xanthine oxidase with either hypoxanthine or lumazine as a substrate. We found that the oxidation of the model compound dihydrorhodamine (DHR) by peroxynitrite occurred via the free radical intermediates, ‘OH and ‘NO₂, formed during the spontaneous decomposition of peroxynitrite and not via direct reaction with peroxynitrite. The inhibitory effect of excess O₂⁻ on the oxidation of DHR could not be ascribed to the accumulation of the peroxynitrite scavenger urate produced from the oxidation of hypoxanthine by xanthine oxidase. A biphasic oxidation profile was also observed upon oxidation of NADH by simultaneous generation of ‘NO and O₂⁻. Conversely, the oxidation of glutathione, which occurs via direct reaction with peroxynitrite, was not affected by excess production of ‘NO. We conclude that the oxidative processes initiated by the free radical intermediates formed from the decomposition of peroxynitrite are inhibited by excess production of ‘NO or O₂⁻ while
oxidative pathways involving a direct reaction with peroxynitrite are not altered. The physiological implications of these findings are discussed.

Introduction

Peroxynitrite (ONOO\(^-\)/ONOOH), the product of the diffusion-limited reaction of nitric oxide (\(^{\cdot}\)NO) with superoxide (\(\text{O}_2^{\cdot -}\)), may represent an important mediator of inflammation-induced tissue injury and dysfunction by virtue of its ability to nitrate and oxidize biomolecules (1). Although the idea that peroxynitrite is a potent oxidant \emph{in vivo} is well-supported by studies utilizing chemically synthesized peroxynitrite (2), others have proposed that the relative rates of \(^{\cdot}\)NO and \(\text{O}_2^{\cdot -}\) production may be critical in determining the amount and ultimately the reactivity of peroxynitrite formed \emph{in vivo} (3-6). This contention was outlined in a study by Miles and co-workers that examined oxidation reactions derived from the co-generation of \(^{\cdot}\)NO and \(\text{O}_2^{\cdot -}\) at neutral pH (4). Consistent with the formation of peroxynitrite, these investigators reported that the oxidation of the model compound dihydrorhodamine (DHR) to its fluorescent derivative rhodamine was maximum in the presence of equimolar fluxes of \(^{\cdot}\)NO and \(\text{O}_2^{\cdot -}\). However, when one free radical was produced in excess of the other one, the oxidation of DHR was inhibited, possibly via secondary reactions occurring directly between peroxynitrite and excess \(^{\cdot}\)NO or \(\text{O}_2^{\cdot -}\). These observations as well as data recently published (5;7-9) suggest that significant oxidation (and nitration) by peroxynitrite might never be attained \emph{in vivo} since the timing and amplitude of \(^{\cdot}\)NO and \(\text{O}_2^{\cdot -}\) synthesis in tissues are distinctly different and the production of these free radicals may never occur at equimolar fluxes.

While the aforementioned results raised important questions with regard to the formation and reactivity of peroxynitrite \emph{in vivo}, a number of mechanistic and methodological limitations were recently brought to light that may limit the scope of these
findings (10;11). Mechanistically, the direct reaction of ‘NO with peroxynitrite is too slow to account for the inhibition of DHR oxidation observed with excess ‘NO as originally suggested (12;13). Recent studies also indicate that the cis- and not the trans-conformer of peroxynitrite is formed at neutral pH, which rules out any difference in reactivity for ‘NO and O$_2^-$ between “authentic” alkaline cis-peroxynitrite and peroxynitrite formed in situ (10). As to the methodological approach, one problem was related to the accumulation of urate formed from the oxidation of hypoxanthine by xanthine oxidase utilized to generate O$_2^-$: Urate might scavenge peroxynitrite and consequently inhibit peroxynitrite-mediated oxidation reactions. Questions were also raised regarding the ability to match fluxes of ‘NO and O$_2^-$ over long periods (hours).

To gain further insights into the mechanism by which excess ‘NO or O$_2^-$ might inhibit peroxynitrite-mediated oxidation reactions, we characterized the kinetic of DHR oxidation by chemically synthesized peroxynitrite. We circumvented the aforementioned methodological problems by using an alternate substrate for xanthine oxidase to eliminate the production of urate and shorter incubation times to insure equal rates of ‘NO and O$_2^-$ production. In addition, we systematically evaluated the effect of excess ‘NO on the peroxynitrite-mediated oxidation of three different model compounds: DHR, NADH, and glutathione. Our results indicate that the inhibition of peroxynitrite-mediated oxidation reactions by excess ‘NO or O$_2^-$ occurs via scavenging of hydroxyl radical (‘OH) and nitrogen dioxide (‘NO$_2$), which are formed from the decomposition of peroxynitrite. The physiological relevance of our findings is discussed.

**Experimental Procedures**

**Materials.** Catalase, Cu,Zn-superoxide dismutase, and xanthine oxidase were purchased from Roche Molecular Biochemicals (Indianapolis, IN). Dihydrorhodamine
123 (DHR) and rhodamine 123 (RH) were purchased from Molecular Probes Inc. (Eugene, OR). SpermineNONOate was obtained from Cayman Chemicals (Ann Arbor, MI). Human recombinant manganese superoxide dismutase was obtained from Kabi-Pharmacies AB (Uppala, Sweeden). All other chemicals were purchased from Sigma Chemical Co. (St Louis, MO).

**Kinetics of peroxynitrite decomposition and DHR oxidation.** The decomposition of peroxynitrite in the presence of DHR was determined every 100 ms by stop-flow absorbance spectrophotometry at 302 nm, using a Shimadzu UV-1601 PC spectrometer (Shimadzu Scientific Instrument Inc., Columbia, MD) equipped with a SFA-20 Rapid Kinetic Instrument (Hi-Tech limited, Salisbury, UK). All solutions were purged with helium and degased immediately before use to minimize the presence of adventitious bicarbonate. Reactions were initiated at 27 °C by mixing equal volumes of a solution consisting of 200 mM phosphate buffer (pH 7.4), and 0.2 mM diethyltriaminepentaacetic acid (DTPA) with a solution of peroxynitrite (0 to 200 µM) diluted immediately before use in distilled water. The oxidation of DHR (0 to 150 µM in 100 mM phosphate buffer) to rhodamine 123 in the presence of 10 µM peroxynitrite was also studied at 27 °C and pH 7.4 by measuring the increase in fluorescence every 100 ms in a rapidly stirred, temperature-controlled cuvette. Fluorescence measurements were obtained with an Aminco/Bowman Series 2 luminescence spectrometer (SLM Instruments Inc., Rochester, NY) using an excitation and emission wavelength of 500 and 536 nm, respectively. The kinetics of peroxynitrite decomposition and rhodamine 123 formation were fitted to a pseudo-first-order reaction equation by non-linear regression (SPSS inc., Chicago, IL).

**Generation of Superoxide, Hydrogen Peroxide, and/or Nitric Oxide.** Superoxide was generated at 37 °C using 500 µM hypoxanthine and various concentration of xanthine oxidase (0 to 20 mU/ml) in phosphate-buffered saline (PBS; pH 7.4) containing 100 µM
DTPA. The initial rates of $O_2^{-}$ generation were determined spectrophotometrically by measuring the superoxide dismutase-inhibitable reduction of cytochrome c at 550 nm. In some experiments, 500 µM lumazine was used in place of hypoxanthine as an alternate substrate for xanthine oxidase to avoid urate formation. Nitric oxide was generated using the spontaneous decomposition of the ‘NO donor spermineNONOate (0-400 µM). SpermineNONOate solutions were prepared each day as a 10 mM stock solution in ice-cold 10 mM NaOH and stored on ice until used. The initial rate of ‘NO generation *via* spermineNONOate decomposition at 37 °C and pH 7.4 was determined electrochemically using a ‘NO-specific electrode (World Precision Instruments, Sarasota, FL).

*Oxidation of DHR by simultaneous generation of ‘NO and $O_2^{-}$*. The oxidation of DHR to rhodamine was determined at 37 °C by measuring the increase in rhodamine fluorescence every 5 seconds in a rapidly stirred and temperature-controlled cuvette. Typical experiments contained 50 µM DHR, 100 µM DTPA, 500 µM hypoxanthine, and various concentrations of xanthine oxidase (0 to 20 mU/ml) and/or spermineNONOate (0 to 400 µM) in phosphate-buffered saline (pH 7.4; 1 ml final volume). All solutions were purged with helium and degased immediately before use to minimize carbonate contamination. DHR was prepared as a 25 mM stock solution in nitrogen-purged dimethyl formamide and kept stored in the dark at –20 °C until used. Fluorescence measurements were obtained with an Aminco/Bowman Series 2 luminescence spectrometer (SLM Instruments Inc., Rochester, NY) using an excitation and emission wavelength of 500 and 536 nm, respectively. Autofluorescence, which did not exceed 10 % of the total fluorescence, was corrected for by the inclusion of parallel blanks and the concentration of rhodamine 123 was calculated using rhodamine 123 standards.
HPLC analysis of DHR oxidation products. In some experiments, products obtained from the oxidation of DHR by `NO and O$_2^-$` were quantified by reversed phase high performance liquid chromatography (HPLC). Briefly, samples were submitted to filtration followed by extraction with acetone and re-suspension in acetonitrile. The samples were then injected onto a 250 x 4.6 mm 5 µm octadecyl silane C$_{18}$ ultrasphere column (Beckman Coulter Inc., Fullerton, CA) isocratically running at a flow rate of 0.5 ml/min with 75% acetonitrile and 1% acetic acid in distilled water. Dihydrhodamine and rhodamine were detected at 290 nm and the identity of each peak was confirmed by co-elution with authentic standards. Recovery of standards through this procedure was better than 80%. No other oxidation product other than rhodamine could be detected from the oxidation of DHR by `NO and O$_2^-$` and both the fluorescence and the HPLC methods gave identical results. Therefore only one parameter, the increase in fluorescence will be shown.

Oxidation of NADH by simultaneous generation of `NO and O$_2^-$`. The oxidation of NADH was determined at 37 °C by measuring the decrease in NADH fluorescence every 5 seconds in a rapidly stirred and temperature-controlled cuvette. Typical experiments contained 50 µM NADH, 100 µM DTPA, 500 µM hypoxanthine, and various concentrations of xanthine oxidase (0 to 20 mU/ml) and/or spermineNONOate (0 to 200 µM) in phosphate-buffered saline (pH 7.4; 1 ml final volume). Fluorescence measurements were obtained using an excitation and emission wavelength of 339 and 460 nm, respectively. NADH concentrations were determined from NADH standard curves. All solutions were purged with helium and degased immediately before use.

Oxidation of glutathione by simultaneous generation of `NO and O$_2^-$` and detection of oxidation and nitrosation products. One ml reaction volumes containing 1 mM GSH, 100
µM DTPA, 500 µM hypoxanthine and various concentrations of xanthine oxidase (0 to 20 mU/ml) and/or spermine NONOate (0 to 400 µM) in phosphate-buffered saline were incubated for 15 min at 37 °C. All solutions were purged with helium and degased immediately before use. Samples were rapidly subjected to ultrafiltration using centrifugal filter units with a molecular weight cutoff of 10,000 (Millipore, Bedford, MA, USA). The ultrafiltrates were then immediately injected onto a 250 x 4.6 mm 5 µm octadecyl silane C18 ultrasphere column (Beckman Coulter Inc., Fullerton, CA) isocratically running at a flow rate of 0.5 ml/min with 0.5% acetonitrile and 1% acetic acid in distilled water. Glutathione, glutathione disulfide, and S-nitrosoglutathione were detected at 220 and 334 nm and the identity of each peak was confirmed by co-elution with authentic standards with a detection limit of 50 nmoles.

Statistics. For groups of three or more, the data were analyzed by one-way analysis of variance, and when a significant difference was suggested, the Tukey test was used as a post-hoc test. Comparisons restricted to two groups were analyzed using the Student’s t-test. A probability value of less than 0.05 was considered to represent a statistically significant difference.

Results

Oxidation of DHR by peroxynitrite. The reaction of DHR with peroxynitrite was studied under limiting concentrations of peroxynitrite at pH 7.4 and 27 °C (Fig.1). In the presence of a 5 to 15 fold excess of DHR, the increase in rhodamine concentration was first-order. The apparent rate constant for the reaction was independent of the initial concentration of DHR and it was within experimental error identical to that of the self-decomposition of peroxynitrite at the same pH and temperature (Table I). These results indicated that the oxidation of DHR by peroxynitrite stemmed from its reaction with the intermediates formed during the self-decomposition of peroxynitrite and not from its direct reaction with peroxynitrite (14).
To confirm that the oxidation of peroxynitrite was indirect, we performed a series of competition experiments between NADH, DHR, and glutathione as shown in Fig. 2. NADH and glutathione were selected because their reactivity towards peroxynitrite is different. NADH reacts with \( \cdot \text{OH} \) and \( \cdot \text{NO}_2 \) formed during the self-decomposition of peroxynitrite while glutathione directly reacts with peroxynitrite at neutral pH (7;15). Thus, NADH should inhibit competitively only those reactions that involve \( \cdot \text{OH} \) and \( \cdot \text{NO}_2 \) and not peroxynitrite itself. In accordance, we found that NADH did not significantly inhibit the oxidation of glutathione (50 \( \mu \text{M} \)) by peroxynitrite (20 \( \mu \text{M} \); Fig. 2). However, NADH completely inhibited the oxidation of DHR (50 \( \mu \text{M} \)) by peroxynitrite (20 \( \mu \text{M} \); IC\(_{50}\): 4.96 \( \mu \text{M} \)) indicating that the main species oxidizing DHR were \( \cdot \text{OH} \) and/or \( \cdot \text{NO}_2 \).

Oxidation of DHR in the presence of constant \( \cdot \text{NO} \) production and various fluxes of \( \text{O}_2^- \).

The oxidation of DHR by peroxynitrite generated in situ was previously studied by co-addition of a chemical source of \( \cdot \text{NO} \), spermineNONOate, and an enzymatic source of \( \text{O}_2^- \), xanthine oxidase with hypoxanthine as a substrate (4). The advantage of this approach is the ability to evaluate DHR oxidation at different \( \cdot \text{NO}/\text{O}_2^- \) ratios. In the following experiments, we sought to re-evaluate this system in light of the recent studies suggesting a number of artefactual limitations (10;11). One problem was related to the accumulation of the peroxynitrite scavenger urate formed from the oxidation of hypoxanthine by xanthine oxidase that might inhibit the oxidation of DHR by peroxynitrite. Questions were also raised regarding the depletion of oxygen in solution upon oxidation of hypoxanthine, which in turn may affect the rate of \( \text{O}_2^- \) production, reduce steady state levels of peroxynitrite, and diminish DHR oxidation yields over time. To address these issues, we used lumazine as an alternative substrate for xanthine oxidase that does not form a peroxynitrite scavenger upon oxidation (16). In preliminary
experiments, we verified that violapterin, the oxidation product of lumazine, did not inhibit the oxidation of DHR by peroxynitrite (data not shown). We also utilized shorter incubation times to insure that oxygen depletion was not a limiting factor for maintaining equal rates of ‘NO and $O_2^-$ production.

Based on the superoxide dismutase-inhibitable reduction of cytochrome c, we observed a concentration-dependent generation of $O_2^-$ upon co-incubation of increasing concentration of xanthine oxidase with either hypoxanthine (500 $\mu$M) or lumazine (500 $\mu$M) (Fig. 3A). The rates of $O_2^-$ generation for both substrates were linear for at least the first 10 min of incubation (data not shown). In the presence of lumazine, the amount of xanthine oxidase was adjusted to obtain the same rates of $O_2^-$ generation as for hypoxanthine (Fig 3A). The initial rate of ‘NO generation via spermineNONOate decomposition at 37 °C in phosphate-buffered saline (pH 7.4) containing 100 $\mu$M DTPA was determined electrochemically using an ‘NO-specific electrode. A linear relationship was found between the initial rate of ‘NO release and spermineNONOate concentrations (0-400 $\mu$M; data not shown).

Fig. 3B illustrates a typical experiment where DHR (50 $\mu$M) in phosphate-buffered saline and 100 $\mu$M DTPA was pre-incubated at 37°C with a combination of either hypoxanthine (HX; 500 $\mu$M) and xanthine oxidase (XO; 1 mU/ml) or lumazine (LU; 500 $\mu$M) and xanthine oxidase (XO; 4 mU/ml). The rates of $O_2^-$ production in the presence of hypoxanthine and lumazine were 0.68 ± 0.01 and 0.69 ± 0.05 $\mu$M/min, respectively. Superoxide generation alone resulted in a low dose-dependent oxidation of DHR to rhodamine that was subtracted from the signal obtained in the presence of spermineNONOate to calculate the rate of rhodamine formation upon co-generation of $O_2^-$ and ‘NO. After addition of spermineNONOate (25 $\mu$M; 0.64 ± 0.01 $\mu$M/min ‘NO
generated), the formation of rhodamine was linear over the 150 s time frame examined. The concentration of spermineNONOate utilized was selected to establish a flux of ‘NO that would match the fluxes of O_2^-. Under these specific conditions (equimolar fluxes of ‘NO and O_2^-), the rates of rhodamine formation were 0.28 ± 0.04 and 0.26 ± 0.04 μM/min in the presence of hypoxanthine and lumazine, respectively. The efficiencies of rhodamine formation, relative to O_2^- fluxes, were 39.43 and 37.68 %, which was in good agreement with the yields of DHR oxidation by chemically synthesized peroxynitrite (14).

As illustrated in Fig. 4A, it was evident that rates of rhodamine formation increased sharply from negligible amounts in the absence of O_2^- to a maximum of 0.31 ± 0.04 μM/min and 0.26 ± 0.04 μM/min for hypoxanthine and lumazine, respectively. These maxima were attained for O_2^- fluxes that were equal to those of ‘NO (0.64 ± 0.01 μM/min). Most importantly, excess O_2^- production over ‘NO completely inhibited rhodamine formation in a fashion that was identical for both hypoxanthine and lumazine. Fig. 4B shows that neither O_2^- nor ‘NO alone at fluxes of ~0.7 μM/min were capable of producing more than 0.04 μM/min rhodamine. In contrast, rates of rhodamine formation of ~0.3 μM/min were attained upon generation of equimolar fluxes of ‘NO and O_2^- . The addition of superoxide dismutase (0.5 mg/ml) but not catalase (15 μg/ml) inhibited the oxidation of DHR by more than 90%. All together, our results demonstrated that the inhibition of DHR oxidation seen in the presence of excess O_2^- over ‘NO could not be ascribed to the scavenging of peroxynitrite by urate produced from the oxidation of hypoxanthine by xanthine oxidase. This is because the utilization of lumazine, which
does not form a peroxynitrite scavenger upon oxidation by xanthine oxidase, was also inhibitory in the presence of excess $\text{O}_2^-$. $\text{NO}$.  

**Oxidation of DHR in the presence of constant $\text{O}_2^-$ production and various fluxes of $\text{NO}$.**

The oxidation of DHR with increasing fluxes of $\text{NO}$ in the presence of a constant flux of $\text{O}_2^-$ is shown in Fig 5A. The rate of rhodamine formation increased from undetectable levels to a maximum rate of $0.73 \pm 0.08 \mu M/min$ for a flux of $\text{NO}$ ($1.49 \pm 0.03 \mu M/min$) that was approximately two-fold higher than the flux of $\text{O}_2^-$ applied to the system ($0.73 \pm 0.07 \mu M/min$). Increasing $\text{NO}$ fluxes above $1.5 \mu M/min$ produced a decrease in the rate of rhodamine formation such that when the flux of $\text{NO}$ approached $4 \mu M/min$, there was an $\sim 80\%$ reduction in rhodamine formation. No other oxidation product other than rhodamine could be detected from the oxidation of DHR by $\text{NO}$ and $\text{O}_2^-$ using HPLC (see Experimental Procedures) and both the fluorescence and the HPLC methods gave identical results (data not shown). Earlier, Kooy and co-workers proposed that the one-electron oxidation product of DHR might reversibly reduce molecular oxygen to form $\text{O}_2^-$. (14). It is possible that excess $\text{NO}$ may drive this reaction by reacting with $\text{O}_2^-$ forming more peroxynitrite which would increase the rate of rhodamine formation and shift the maximum to higher $\text{NO}/\text{O}_2^-$ ratios. Thus, to investigate whether the oxidation of DHR by peroxynitrite generated $\text{O}_2^-$, we determined $\text{O}_2^-$ production by measuring the superoxide dismutase inhibitable reduction of cytochrome $c$ spectrophotometrically as depicted in Fig. 5B. We found that addition of incremental amounts of DHR ($0-150 \mu M$) resulted in a statistically significant increase in the amount of $\text{O}_2^-$ formed upon
incubation with peroxynitrite (20 μM) suggesting that the reaction of DHR with peroxynitrite indeed generated O$_2^{-}$.

**Effect of the simultaneous generation of ‘NO and O$_2^{-}$ on the oxidation of NADH and GSH.** To further evaluate the physiological relevance of excess ‘NO production on peroxynitrite-mediated oxidation reactions, we examined the oxidation of NADH by O$_2^{-}$ in the presence of various fluxes of ‘NO (Fig. 6). The efficiency of oxidation of NADH at equimolar fluxes of ‘NO (0.37 ± 0.06 μM/min) and O$_2^{-}$ (0.40 ± 0.02 μM/min) was 88 ± 19 %. This was consistent with the oxidation of NADH occurring via a chain reaction in which the one-electron oxidation product of NADH reduces molecular oxygen to yield O$_2^{-}$ and subsequent oxidation of NADH by O$_2^{-}$ (7). We found that the rate of NADH oxidation in the presence of a constant flux of O$_2^{-}$ (0.40 ± 0.02 μM/min) increased to a maximum of 0.89 ± 0.13 μM/min for a flux of ‘NO of 1.17 ± 0.19 μM/min. In a fashion similar to DHR, increasing ‘NO fluxes above 1.2 μM/min produced a decrease in NADH oxidation to levels observed in the absence of O$_2^{-}$. Increasing fluxes of ‘NO in the absence of xanthine oxidase resulted only in minimal oxidation.

The oxidation of glutathione to glutathione disulfide with increasing fluxes of ‘NO in the presence or the absence of a constant flux of O$_2^{-}$ is shown in Fig 7. In the absence of O$_2^{-}$, glutathione disulfide increased from undetectable levels in the absence of ‘NO to 40 μM for a flux of ‘NO of ~8.0 μM/min (15 min incubation time). In the presence of a constant flux of O$_2^{-}$, the amounts of glutathione disulfide increased in a dose-dependent manner over fluxes of ‘NO ranging from 0 to ~8.0 μM/min. In fact, the amounts of glutathione disulfide formed were indistinguishable from those obtained in
the absence of $O_2^-$ when $\cdot$NO fluxes reached $\sim 0.7 \mu M/min$ or higher. These results indicated that the oxidation of glutathione was not inhibited by increasing fluxes of $\cdot$NO. This is consistent with previous studies showing that glutathione directly reacts with peroxynitrite at neutral pH, in contrast to NADH and DHR, thus precluding any inhibitory effect of $\cdot$NO via $\cdot$OH and $\cdot$NO$_2$ scavenging (15). Although $\cdot$NO reacts with peroxynitrite itself (5;13), this reaction is too slow to inhibit the oxidation of glutathione in the presence of $\cdot$NO and $O_2^-$.

It is important to note that S-nitrosoglutathione was also formed in micromolar concentrations with a shift to lower amounts formed when $O_2^-$ was also produced due to the formation of peroxynitrite as previously shown (data not shown) (17).

**Discussion**

Peroxynitrite ($\text{ONOO}^-/\text{ONOOH}$) is presumably formed *in vivo* through the diffusion-limited reaction of $\cdot$NO with $O_2^-$. Upon protonation to peroxynitrous acid ($\text{ONOOH}$; $pK_a = 6.8$), peroxynitrite anion ($\text{ONOO}^-$) rapidly decomposes at physiological pH to form nitrate ($\text{NO}_3^-$; Fig. 8) as well as free hydroxyl radical ($\cdot$OH) and nitrogen dioxide ($\cdot$NO$_2$). Recent studies indicate that the yield of $\cdot$OH and $\cdot$NO$_2$ formation approximates 25 to 35% in the absence of competitive reactions (18;19). Thus, peroxynitrite-induced oxidation reactions include those directly mediated by peroxynitrite itself as well as those mediated indirectly by $\cdot$OH and $\cdot$NO$_2$ (Fig. 8). In physiological settings where bicarbonate abounds, the formation of $\cdot$OH and $\cdot$NO$_2$ is limited by the reaction of peroxynitrite with carbon dioxide to form the nitrosoperoxocarbonate anion ($\text{ONOOCO}_2^-$; Fig. 8), 33 % of which decomposes to carbonate radical ($\text{CO}_3^-$) and nitrogen dioxide ($\cdot$NO$_2$) (20). Another important aspect related to the production of peroxynitrite *in vivo* is the fact that the timing and location of
‘NO and O$_2^-$ production in tissues are different and have only a limited overlap such that peroxynitrite formation always occurs in excess of either ‘NO or O$_2^-$ (21). The oxidative chemistry associated with the production of peroxynitrite in situ has been most often examined by using 3-morpholinosydnonimine (SIN-1) (22) or pulse radiolysis (7) to generate equimolar fluxes of ‘NO and O$_2^-$·. These methods do not allow for the examination of the effect of excess production of one free radical over the other one. The use of NONOates and xanthine oxidase as a source of ‘NO and O$_2^-$· has provided a unique opportunity to examine the oxidative and nitrative chemistry associated with peroxynitrite that would be expected to occur in vivo, i.e. at different ‘NO/O$_2^-$· ratios (3-5).

However, a number of methodological limitations have been recently raised questioning the validity of the results obtained with this experimental approach (10;11). Mainly, oxidation reactions might be inhibited by the progressive accumulation of the peroxynitrite scavenger urate formed from the oxidation of hypoxanthine by xanthine oxidase. It is important to note that the first product formed from the oxidation of hypoxanthine is xanthine such that the accumulation of urate is considerably minimized, at least during the first few minutes of the experiment (16). Both xanthine and urate have similar absorption spectrum, which may lead to misleading information if an increase in absorbance at 292 nm is taken as an indication of urate formation. In fact, the oxidation of the model compound DHR over time was linear (Fig. 3), which was inconsistent with the progressive accumulation of urate that would have resulted in the curving of the slope over time. Most importantly, substituting hypoxanthine with lumazine to eliminate the production of urate did not alter the biphasic oxidation profile of DHR in the presence of ·NO and increasing fluxes of O$_2^-$· (Fig. 4). Thus, our results clearly indicated that the production of urate could not account for the inhibitory effect of
excess $O_2^\cdot$ production on the oxidation of DHR by peroxynitrite generated \textit{in situ}.

Another possibility would be that the decrease in DHR oxidation with increased fluxes of $O_2^\cdot$ or ·NO might involved the interaction of $O_2^\cdot$ or ·NO with a free radical intermediate of DHR to yield adducts with diminished fluorescence (14). However, we failed to detect any other oxidation product than rhodamine utilizing HPLC techniques (data not shown).

It is important to note that excess ·NO production also inhibited the oxidation of NADH, which is structurally different from DHR. This observation reinforces our opinion that the reaction pathways underlying the inhibitory effect of $O_2^\cdot$ and ·NO are not specific to DHR and might apply to a large variety of physiologically relevant targets.

Previous interpretations of the inhibition of the oxidation of DHR by excess ·NO or $O_2^\cdot$ were based on the assumption that peroxynitrite directly oxidized DHR (4;14) and that excess ·NO or $O_2^\cdot$ reacted with peroxynitrite with a consequential inhibition of DHR oxidation. However, recent studies indicate that the reaction of ·NO with peroxynitrite is too slow to account for the inhibition seen, at least at high fluxes of ·NO (5;13). Herein, we show that the oxidation of DHR to rhodamine takes place indirectly via reaction with ·OH and ·NO$_2$ formed during the self-decomposition of peroxynitrite (Fig. 1 and 2 and Table I). These results indicate that competing reactions involving excess ·NO or $O_2^\cdot$ with ·OH and/or ·NO$_2$ might represent important pathways by which the oxidative chemistry of peroxynitrite may be modulated. Both ·OH and ·NO$_2$ oxidize DHR (14) and the rate constants for the reaction of ·NO or $O_2^\cdot$ with ·OH and/or ·NO$_2$ are high enough to be relevant in the presence of excess ·NO or $O_2^\cdot$, as illustrated in Table II. Additional reactions such as the rapid reaction of nitrite (NO$_2^-$) with ·OH may also contribute to the inhibition associated with excess ·NO production (Table II).
Another interesting aspect was the observation that the efficiency of DHR oxidation was increased from approximately 40% at equimolar fluxes of ‘NO and O$_2^\cdot$ to almost 100 % upon generation of a two-fold excess of ‘NO over O$_2^\cdot$ and then decreased at higher ‘NO fluxes (Fig. 5). Similar results were obtained with NADH (i.e. a shift of the maximum to higher ‘NO/O$_2^\cdot$ ratios) that were reminiscent of simulation data suggesting that maximum oxidation yield of NADH in the presence of ‘NO and O$_2^\cdot$ might be attained when ‘NO is generated in excess over O$_2^\cdot$ (7). We detected the formation of O$_2^\cdot$ upon co-incubation of peroxynitrite with DHR (Fig. 5), which we attributed to the reversible reduction of molecular oxygen by the free radical intermediate formed from the oxidation of DHR with peroxynitrite (14;23). Excess ‘NO might drive this reaction by reacting with O$_2^\cdot$ forming additional peroxynitrite which would increase the rate of rhodamine formation and shift the maximum to higher ‘NO/O$_2^\cdot$ ratios. Thus, the reaction of physiologically relevant substrates with one-electron oxidants produced during the decay of peroxynitrite may yield O$_2^\cdot$. This may in turn alter oxidation yields when ‘NO and O$_2^\cdot$ are generated simultaneously but ultimately excess ‘NO will effectively inactivate any additional •OH and/or •NO$_2$ formed.

As already mentioned, the reaction of peroxynitrite with carbon dioxide (CO$_2$) is a major route for peroxynitrite activity in vivo (Fig. 8; (24)). We have already shown that the presence of CO$_2$ did not alter the oxidation profile of DHR suggesting that substantial inhibition of peroxynitrite-mediated oxidation reactions by excess ‘NO might occur in vivo even in the presence of carbon dioxide (25). In this case, the inhibitory pathways would be similar to that of CO$_2$-free peroxynitrite where excess ‘NO would scavenge CO$_3^\cdot$/NO$_2^\cdot$ instead of •OH/NO$_2$ (7). In either case, dinitrogen trioxide (N$_2$O$_3$), a potent nitrosating
agent, is produced from the reaction of ‘NO with ‘NO2, which supports the notion that the oxidative chemistry of peroxynitrite is decreased in favor of nitrosation reactions in the presence of excess ‘NO (17).

In addition to oxidation reactions, peroxynitrite promotes the nitration of aliphatic and aromatic residues including the amino acid tyrosine (26). Tyrosine is oxidized by the free radical intermediates derived from peroxynitrite to the tyrosyl radical, which then reacts with ‘NO2 to form 3-nitrotyrosine (8). Pfeiffer and Mayer reported that excess generation of O2•− over ‘NO inhibited the formation of 3-nitrotyrosine suggesting that peroxynitrite-mediated nitration reactions in vivo may also be inhibited by excess production of O2•− (5). The inhibition of tyrosine nitration is best explained by experimental and simulation data showing that O2•− compete with ‘NO2 for the tyrosyl radical intermediate such that the formation of 3-nitrotyrosine may be inhibited when the rates of O2•− generation exceed those of ‘NO (8;9). All together, this indicates that both oxidation and nitration reactions mediated by peroxynitrite are greatly influenced by the relative rates of ‘NO and O2•− production.

In conclusion, the finding that excess production of ‘NO or O2•− inhibits the oxidative chemistry associated with the production of peroxynitrite or CO2•−peroxynitrite in situ provides an additional pathway that affects the fate of peroxynitrite in vitro and in vivo. In biologically relevant systems, only a small fraction of peroxynitrite will undergo spontaneous decomposition because most peroxynitrite will be directly consumed through bimolecular reactions with a variety of molecules such as glutathione, cysteine, ascorbate, and bicarbonate. Whether the oxidative processes initiated by CO3•−/NO2 formed from the interaction of CO2 with peroxynitrite will be inhibited in vivo by excess
‘NO or \( \text{O}_2^- \)’ will depend on the presence and relative concentrations of biomolecules capable of competing with ‘NO or \( \text{O}_2^- \)’ for \( \text{CO}_3^-/\text{NO}_2^- \).

**Literature cited**


**Footnotes**

1The abbreviations used are: ‘NO, nitric oxide; O$_2^-$, superoxide anion; ONOO’, peroxynitrite anion; ONOOH, peroxynitrous acid; ‘OH, hydroxyl radical; ‘NO$_2$, nitrogen dioxide; CO$_3^-$, trioxocarbonate (1-); NO$_2^-$, nitrite; NO$_3^-$, nitrate; CO$_2$, carbon dioxide; ONOOCO$_2^-$, nitrosoperoxocarbonate anion; O$_2$NOO$^{-}$, peroxynitrate; CAT, catalase; DHR, dihydrorhodamine; DTPA; diethyltriaminepentaacetic acid; HX, hypoxanthine; LU, lumazine; SOD, superoxide dismutase; XO, xanthine oxidase.
Table I

Apparent rate constants for the reaction of DHR with peroxynitrite

<table>
<thead>
<tr>
<th>DHR (µM)</th>
<th>Rate (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.22 ± 0.02</td>
</tr>
<tr>
<td>50</td>
<td>0.25 ± 0.01</td>
</tr>
<tr>
<td>100</td>
<td>0.25 ± 0.04</td>
</tr>
<tr>
<td>150</td>
<td>0.24 ± 0.02</td>
</tr>
</tbody>
</table>

The reaction of DHR with peroxynitrite was studied under limiting concentrations of peroxynitrite at pH 7.4 and 27 °C as described under Experimental Procedures. Rate constants represent averages of five experiments.

Table II

Rate constants for the reaction of •OH and NO₂⁻ with •NO, O₂⁻, and nitrite.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>k (M⁻¹.s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>•NO₂ + •NO → N₂O₃</td>
<td>a1.1 x 10⁹</td>
</tr>
<tr>
<td>•OH + •NO → HNO₂</td>
<td>a1.0 x 10¹⁰</td>
</tr>
<tr>
<td>•NO₂ + O₂⁻ → O₂NOO⁻</td>
<td>a4.5 x 10⁹</td>
</tr>
<tr>
<td>•OH + O₂⁻ → OH⁻ + O₂</td>
<td>b1.0 x 10¹⁰</td>
</tr>
<tr>
<td>NO₂⁻ + •OH → •NO₂ + OH⁻</td>
<td>a5.3 x 10⁹</td>
</tr>
</tbody>
</table>

a rate constants (studied at 25 °C) were taken from reference (7). b rate constant (studied at 25 °C) was taken from reference (27).
Figure Legends

Fig. 1. *Time course of DHR oxidation to rhodamine by peroxynitrite.* Peroxynitrite (10 µM) was added to a rapidly stirred and temperature-controlled cuvette containing 150 µM dihydrorhodamine in 100 mM phosphate and 100 µM DTPA, pH 7.4, at 27°C. The formation of rhodamine was followed by fluorescence spectroscopy as described under Materials and Methods. The kinetics of rhodamine formation were fitted to a single exponential function by non-linear-regression and the apparent first order rate constants are presented in Table I. The solid line represents the non-linear regression fitting of data points (opened circles). Data are representative of five experiments.

Fig. 2. *Effect of NADH on peroxynitrite-mediated oxidation of DHR and glutathione.* Peroxynitrite (20 µM) and various concentrations of NADH (0-1 mM) in phosphate-buffered saline (pH 7.4) containing DTPA (100 µM) was incubated in the presence of either DHR (50 µM; opened circles) or glutathione (50 µM; closed triangles) at 37 °C for 15 min. The amount of rhodamine and glutathione disulfide formed was then determined by fluorescence spectroscopy and HPLC, respectively. Values represent the mean ± standard deviation (n=3).

Fig. 3. *Panel A. Superoxide production by xanthine oxidase in the presence of hypoxanthine or lumazine.* Hypoxanthine (500 µM) or lumazine (500 µM) were incubated with various concentrations of xanthine oxidase in phosphate-buffered saline and DTPA (100 µM) at 37 °C. The rate of superoxide production was determined by measuring the superoxide dismutase-inhibitable reduction of cytochrome c as described under Materials and Methods. Values represent the mean ± standard deviation (n=3).

*Panel B. Time course of rhodamine formation in the presence of simultaneous...*
generation of nitric oxide and superoxide. DHR (50 μM) in phosphate-buffered saline and 100 μM DTPA was pre-incubated at 37°C with a combination of either hypoxanthine (HX; 500 μM) and xanthine oxidase (XO; 1 mU/ml) or lumazine (LU; 500 μM) and xanthine oxidase (XO; 4 mU/ml). The rates of O₂⁻ production in the presence of hypoxanthine and lumazine were 0.68 ± 0.01 and 0.69 ± 0.05 μM/min, respectively. After approximately 100 s, 25 μM spermineNONOate (0.64 ± 0.01 μM/min ¹NO generated) was added to the reaction mixture and the formation of rhodamine was followed for approximately 150 s by measuring the increase in fluorescence as described under “Materials and Methods”. Control conditions represent the addition of spermine (25 μM) to a sample containing DHR (50 μM) in phosphate buffer saline and 100 μM DTPA. Tracings are representative of three separate experiments.

Fig.4. Oxidation of dihydrorhodamine to rhodamine in the presence of constant nitric oxide production and various fluxes of superoxide. Panel A. The rates of rhodamine formation obtained from the experiments illustrated in Fig. 3B were reported as a function of O₂⁻ production. Superoxide generation alone resulted in a low, dose-dependent oxidation of dihydrorhodamine to rhodamine 123 that was subtracted from the signal obtained in the presence of spermineNONOate to calculate the rate of rhodamine formation. Values represent the mean ± standard deviation (n=4). Panel B. Effect of superoxide dismutase (SOD) and catalase (CAT) on the oxidation of dihydrorhodamine in the presence of equimolar fluxes of ¹NO and O₂⁻. DHR (50 μM) in phosphate-buffered saline and 100 μM DTPA was incubated at 37°C with a combination of either hypoxanthine (500 μM) and xanthine oxidase (1 mU/ml) or lumazine (500 μM) and xanthine oxidase (4 mU/ml). Nitric oxide was generated with spermineNONOate
(25 μM). Rates of ‘NO and O_2^-’ were identical to those described in Figure 3B. Superoxide dismutase (0.5 mg/ml) and catalase (15 μg/ml) were added as described under Materials and Methods. Values represent the mean ± standard deviation (n=3).

Fig. 5. **Oxidation of dihydrorhodamine to rhodamine 123 in the presence of constant superoxide production and various fluxes of nitric oxide.** Panel A. DHR (50 μM) was pre-incubated with hypoxanthine (500 μM) and 1 mU/ml xanthine oxidase (0.73 ± 0.07 μM/min O_2^- generated) in phosphate-buffered saline and 100 μM DTPA at 37°C. After approximately 100 s, various concentration of spermineNONOate were added to the reaction mixtures and the formation of rhodamine 123 was followed for approximately 150 s by measuring the increase in fluorescence as described in Fig. 3. The rates of rhodamine 123 formation are reported as a function of ‘NO fluxes. Values represent the mean ± standard deviation (n=3). Panel B. Peroxynitrite-mediated superoxide production in the presence or the absence of DHR. Peroxynitrite (20 μM) was added to DHR (0-150 μM), 10 μM cytochrome c in phosphate-buffered saline (pH 7.4; 37°C) containing 100 μM DTPA in the presence or the absence superoxide dismutase 0.01 mg/ml. Cytochrome c^{2+} concentrations were determined by measuring the absorbance at 550 nm. For each concentration of DHR, the difference in absorbance between the sample with and without superoxide dismutase was used to calculate the amount of O_2^- trapped by cytochrome c. Values represent the mean ± standard deviation (n=3).

Fig. 6. **Oxidation of NADH in the presence of constant superoxide production and various fluxes of nitric oxide.** NADH (50 μM) was pre-incubated with hypoxanthine
(500 μM) and xanthine oxidase (0.40 ± 0.02 μM/min O₂⁻ generated) in phosphate-buffered saline and 100 μM DTPA at 37°C. After approximately 100 s, various concentration of spermineNONOate were added to the reaction mixtures and the oxidation of NADH was followed for approximately 150 s by measuring the decrease in fluorescence as described in Materials and Methods. The rates of NADH oxidation are reported as a function of ‘NO fluxes. Values represent the mean ± standard deviation (n=3).

Fig. 7. Oxidation of glutathione in the presence of constant superoxide production and various fluxes of nitric oxide. Glutathione (1 mM) was incubated with hypoxanthine (500 μM), increasing concentrations of spermineNONOate in phosphate-buffered saline and 100 μM DTPA for 10 min at 37°C in the presence or the absence of xanthine oxidase (0.53 ± 0.07 μM/min O₂⁻ generated). The reaction was terminated by addition of allopurinol (1 mM) and the amount of glutathione disulfide was determined as described under Materials and Methods. Values represent the mean ± standard deviation (n=3).

Fig. 8. Proposed mechanism of inhibition of peroxynitrite reaction pathways by excess nitric oxide.
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Fig. 5 Jourd'heuil et al.
Fig. 6 Jourd'heuil et al.
Fig. 7 Jourd'hui et al.
Fig. 8 Jourd'heuil et al.
Reaction of superoxide and nitric oxide with peroxynitrite. Implications for peroxynitrite-mediated oxidation reactions
David Jourd'heuil, Frances L. Jourd'heuil, Peter S. Kutchukian, Rabi A. Musah, David A. Wink and Matthew B. Grisham

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