Modulation of Superoxide-dependent Oxidation and Hydroxylation Reactions by Nitric Oxide*

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The rapid and spontaneous interaction between superoxide (O\textsubscript{2}\textsuperscript{-}) and nitric oxide (NO) to yield the potent oxidants peroxynitrite (ONOO\textsuperscript{-}) and peroxynitrous acid (ONOOH), has been suggested to represent an important pathway by which tissue may be injured during inflammation. Although several groups of investigators have demonstrated substantial oxidizing and cytotoxic activities of chemically synthesized ONOO\textsuperscript{-}, there has been little information available quantifying the interaction between O\textsubscript{2}\textsuperscript{-} and NO in the absence or the presence of redox-active iron. Using the hypoxanthine (HX)/xanthine oxidase system to generate various fluxes of O\textsubscript{2}\textsuperscript{-} and H\textsubscript{2}O\textsubscript{2} and the spontaneous decomposition of the spermine/NO adduct to produce various fluxes of NO, we found that in the absence of redox-active iron, the simultaneous production of equimolar fluxes of O\textsubscript{2}\textsuperscript{-} and NO increased the oxidation of dihydrorhodamine (DHR) from normally undetectable levels to approximately 15 \textmu M, suggesting the formation of a potent oxidant. Superoxide dismutase, but not catalase, inhibited this oxidative reaction, suggesting that O\textsubscript{2}\textsuperscript{-} and not hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) interacts with NO to generate a potent oxidizing agent. Excess production of either radical virtually eliminated the oxidation of DHR. In the presence of 5 \textmu M Fe\textsuperscript{3+}-EDTA to insure optimum O\textsubscript{2}\textsuperscript{-}-driven Fenton chemistry, NO enhanced modestly HX/xanthine oxidase-induced oxidation of DHR. As expected, both superoxide dismutase and catalase inhibited this Fe-catalyzed oxidation reaction. Excess NO production with respect to O\textsubscript{2}\textsuperscript{-} flux produced only modest inhibition (33\%) of DHR oxidation. In a separate series of studies, we found that equimolar fluxes of O\textsubscript{2}\textsuperscript{-} and NO in the absence of iron only modestly enhanced hydroxylation of benzoic acid from undetectable levels to 0.6 \textmu M 2-hydroxybenzoate. In the presence of 5 \textmu M Fe\textsuperscript{3+}-EDTA, HX/xanthine oxidase-mediated hydroxylation of benzoic acid increased dramatically from undetectable levels to 4.5 \textmu M of the hydroxylated product. Superoxide dismutase and catalase were both effective at inhibiting this classic O\textsubscript{2}\textsuperscript{-}-driven Fenton reaction. Interestingly, NO inhibited this iron-catalyzed hydroxylation reaction in a concentration-dependent manner such that fluxes of NO approximating those of O\textsubscript{2}\textsuperscript{-} and H\textsubscript{2}O\textsubscript{2} virtually abolished the hydroxylation of benzoic acid. We conclude that in the absence of iron, equimolar fluxes of NO and O\textsubscript{2}\textsuperscript{-} interact to yield potent oxidants such as ONOO\textsuperscript{-}, ONOOH, which oxidize organic compounds. Excess production of either radical remarkably inhibits these oxidative reactions. In the presence of low molecular weight redox-active iron complexes, NO may enhance or inhibit O\textsubscript{2}\textsuperscript{-}-dependent oxidation and hydroxylation reactions depending upon their relative fluxes.

It is becoming increasingly apparent that certain types of inflammatory tissue injury are mediated by reactive metabolites of oxygen and nitrogen. For example, it has been demonstrated that administration of superoxide dismutase is effective at attenuating the tissue injury observed in experimental models of arthritis, chronic gut inflammation, and immune complex-induced pulmonary injury (1–3). Furthermore, models of joint, bowel, and lung inflammation have been shown to be associated with enhanced production of nitrogen oxides derived from the free radical nitric oxide (NO) (4–6). Indeed, recent studies have demonstrated that inhibition of NO synthase also provides substantial protection against the inflammatory tissue injury observed in these models of acute and chronic inflammation (4–6). These data suggest that both superoxide (O\textsubscript{2}\textsuperscript{-}) and NO are important mediators of inflammation-induced tissue injury and dysfunction. The mechanisms by which O\textsubscript{2}\textsuperscript{-} and NO may either separately or in tandem mediate tissue injury during inflammation remain the subject of active debate.

Recent chemical studies have demonstrated that O\textsubscript{2}\textsuperscript{-} and NO rapidly interact via a radical-radical reaction at a diffusion-limited rate (k = 6.7 \times 10\textsuperscript{6} M \textsuperscript{-1} s \textsuperscript{-1}) to generate the potent oxidant peroxynitrite (ONOO\textsuperscript{-}) (7). Beckman and co-workers (8) have suggested that the interaction between these two free radicals to yield ONOO\textsuperscript{-} and its conjugate acid, peroxynitrous acid (ONOOH), enhances dramatically the toxicity of either O\textsubscript{2}\textsuperscript{-} or NO alone. Indeed, it has been demonstrated in vitro using preformed or chemically synthesized ONOO\textsuperscript{-}/ONOOH that these oxidants are capable of directly oxidizing carbohydrates (8), sulfhydryls (9), lipids (10, 11), and DNA bases (12) as well as mediating bacteriocidal and endothelial cell toxicity (13, 14). It has also been demonstrated that the simultaneous production of NO and O\textsubscript{2}\textsuperscript{-} by macrophages may result in the formation of ONOO\textsuperscript{-}/ONOOH (15). However, a series of recent reports demonstrate that NO may actually inhibit O\textsubscript{2}\textsuperscript{-}-dependent, iron (or hemoprotein)-catalyzed lipid peroxidation in vitro (16–20).

This apparent “antioxidant” activity of NO has prompted some investigators to suggest that the interaction between O\textsubscript{2}\textsuperscript{-} and NO is an important detoxification pathway of potentially injurious O\textsubscript{2}\textsuperscript{-}-derived reactive oxygen metabolites and thus may actually represent an endogenous anti-inflammatory pathway.
Indeed there is increasing evidence to suggest that NO may protect cells and tissue against reactive oxygen metabolite-mediated oxidative damage (16–20). Wink et al. (17) have shown that exogenous NO protects Chinese hamster lung fibroblasts, rat H4 hepatoma cells, and rat mesenchymal dopaminergic cells against reactive oxygen metabolite-induced cell injury. Assreuy et al. (16) recently demonstrated that although generation of NO by activated macrophages is cytotoxic to Leishmania major, simultaneous generation of NO and O$_2^*$ or addition of authentic ONOO$^-$ failed to induce any microbial activity. Finally, several investigators have demonstrated that exogenous administration of NO inhibits ischemia-induced microvascular dysfunction produced by O$_2^*$-dependent adherence and emigration of neutrophils in the post capillary venules in vivo (21–23).

The reasons for these apparent discrepant results are not clear. However, recent evidence by Rubbo et al. (10) suggests that the relative fluxes of the two free radicals may be an important determinant as to whether NO enhances or inhibits O$_2^*$-dependent, iron-catalyzed lipid peroxidation (10). In these studies the effects of NO were assessed only in the presence of ferric iron. A recent preliminary study from our laboratory suggested that the effects of NO on O$_2^*$-dependent oxidative reactions may be quite different depending upon whether redox-active transition metals are present or absent (24). Therefore, the objectives of the present study were to: (a) systematically quantify the oxidizing and hydroxylating activity of NO and O$_2^*$ in the absence or the presence of redox-active iron and (b) characterize these reactions using different fluxes of each radical. The physiological significance of our findings is discussed.

MATERIALS AND METHODS

Chemicals—Hypoxanthine (HX),$^1$ benzoic acid (BA), 2-hydroxybenzoic acid (HB), dimethyl formamide, spermine, diethylentriaminepentaacetic acid, and cytochrome c (horse heart) were purchased from Sigma. Potassium superoxide (K$_2$O$_2$) was obtained from Pfaltz and Bauer, Inc. (Waterbury, CT). Xanthine oxidase derived from bovine milk was supplied by Calbiochem, and rhodamine 123 (RH) and dihydrorhodamine 123 (DHR) were purchased from Molecular Probes, Inc. (Eugene, OR). Spermine/NO adduct (Sp/NO) was a generous gift from Dr. Larry Keefer (National Cancer Institute, Frederick, MD). Human recombinant copper-zinc superoxide dismutase was obtained from Kabi-Pharmacia AB (Uppsala, Sweden), and catalase was purchased from Boehringer Mannheim. The tetramethylammonium salt of ONOO$^-$ was synthesized according to the method of Bohle et al. (25).

Briefly, in a dry box [Me$_4$N][ONOO$^-$] (60.57 mmol) was transferred into a 100-ml side arm Schlenk flask and capped up with clean septum. The Schlenk flask was then brought out of the dry box, purged with dry nitrogen, and cooled to ~78°C in a dry ice/ethanol bath. Anhydrous ammonia (~30 ml) is then condensed into the system while keeping the flask at positive pressure of nitrogen. A gas mixture of dry nitrogen and nitric oxide, prepared by reduction of NaNO$_2$ (250 mg, 3.62 mmol) by a 250-mesh nitrogen purged diethylformamide, and cytochrome (Eugene, OR). The spermine/NO adduct (Sp/NO) was a generous gift from Dr. Bauer, Inc. (Waterbury, CT). Xanthine oxidase derived from bovine serum, or ONOO$^-$ or ONOO$^-$ is capable of oxidizing DHR to RH. Neither O$_2^*$, H$_2$O$_2$, nor NO (in the presence or absence of O$_2$) oxidized substantial amounts of DHR in the time course of our experiments. 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. A 5 ml stock solution of DHR was prepared fresh each day by appropriate dilution of the 25 mM stock in dimethyl formamide. 500-μl reaction volumes containing 20 μM potassium phosphate buffer (pH 7.4), 0.15 m NaCl, catalase (15 μg/ml), 0.05 m DHR, 0.5 m HX, and various concentrations of xanthine oxidase (0–10 μl/min) or Sp/NO (0–20 μm) were incubated at 37°C for 15 min at 37°C and addition of catalase insured that H$_2$O$_2$ did not accumulate to levels that could participate in the O$_2^*$-driven Fenton reaction. For some experiments, catalase (15 μg/ml) or superoxide dismutase (100 μg/ml) was included in the reaction volumes, whereas in other experiments catalase and superoxide dismutase were omitted and 5 μM Fe$^{2+}$-EDTA was included. The omission of catalase and the addition of 5 μM Fe$^{2+}$-EDTA is a strategy to inhibit Fenton chemistry and OH$^-$ production. Following the 30-min incubation period, reactions were terminated by dilution with 1.0 ml of cold phosphate-buffered saline (pH 7.4). Rhodamine formation was quantified using fluorescence spectroscopy in which an excitation wavelength of 500 nm and an emission wavelength of 536 nm were used. The concentration of RH was then calculated using regression values (i.e., slope and intercept values) obtained from plots using RH standards.

Hydroxylating activity of the various systems described above was quantified by measuring the hydroxylation of HB to BA. At pH 7.4, the principal products of BA hydroxylation are the monohydroxylated derivatives, 2-, 3-, and 4-hydroxybenzoate, with 2-hydroxybenzoic acid being the principal product (26). Hydroxylation of BA was quantified using fluorescencespectroscopy in which an excitation wavelength of 340 nm and an emission detected at 410 nm (i.e. for the three purified derivatives at equimolar concentrations and physiological pH (32)). 500-μl reaction volumes containing 20 μM potassium phosphate buffer (pH 7.4) and 0.15 m NaCl, 1.0 m BA, 0.5 m HX, and various concentrations of xanthine oxidase (20–100 μl/min) were incubated at 37°C for 5 min. For some experiments, catalase (20 μg/ml) or superoxide dismutase (100 μg/ml) were included in the reaction volumes, whereas in other experiments catalase and superoxide dismutase were omitted and 5 μM Fe$^{2+}$-EDTA was included. The omission of catalase and the addition of 5 μM Fe$^{2+}$-EDTA is a strategy to inhibit Fenton chemistry and OH$^-$ production. Following the 30-min incubation period, reactions were terminated by dilution with 0.5 ml of cold phosphate-buffered saline (pH 7.4). Production of HB was quantified by measuring the fluorescence obtained with excitation and emission wavelengths of 290 and 410 nm, respectively. The concentration of HB was determined using HB standards. All fluorescence measurements and spec-
Interaction between Peroxynitrite and Excess Superoxide or Nitric Oxide—The ability of excess NO to interact with chemically synthesized ONOO$^-\text{H}$ was assessed using NO generated from Sp/NO and the tetramethylammonium salt of ONOO$^-\text{H}$. Briefly, NO formation (1.0 nmol/min) was continuously monitored using electrochemical detection (WPI NO electrode) in the absence or the presence of a bolus addition of a small aliquot of ONOO$^-\text{H}$ (11 nmol) in a 2-ml reaction volume containing 50 mM phosphate buffer. The rapid disappearance of the NO signal signified the interaction between NO and ONOO$^-\text{H}$. The interaction between excess $O_2^-$ and ONOO$^-\text{H}$ was determined by mixing a 10-fold molar excess of potassium superoxide (KO$_2$) prepared in ice-cold 0.1 N NaOH containing 0.1 mM diethylenetriaminepentaacetic acid with an alkaline solution of the ONOO$^-\text{H}$ salt prepared as described above. A small aliquot alkaline O$_2$/ONOO$^-\text{H}$ was immediately (<1 min) added to a 1-ml reaction volume containing 50 mM potassium phosphate buffer (pH 7.4) and 50 $\mu$M DHR. Oxidation of DHR was compared with that when the same amount of alkaline ONOO$^-\text{H}$ was added. A decrease in the oxidation of DHR was used as evidence to suggest that O$_2^-$ decomposes ONOO$^-\text{H}$.

RESULTS

Oxidation of Dihydrorhodamine in the Absence of Iron—Oxidation of DHR to rhodamine 123 by different fluxes of NO in the presence of a constant flux of O$_2^-$ of 1.0 nmol/min is presented in Fig. 1. In the presence of catalase and the absence of exogenous iron, the production of RH increased sharply from normally undetectable amounts in the absence of NO to a maximum concentration of $\sim 15 \mu$M at fluxes of NO approximately equal to that of O$_2^-$ (1.0 nmol/min). Increasing NO fluxes above 1.0 nmol/min produced a decrease in DHR oxidation such that when the flux of NO approached 4–5 nmol/min, there was a 90% reduction in RH formation. The addition of 200 $\mu$M spermine to reaction volumes producing fluxes of superoxide and NO of approximately 1.0 nmol/min did not inhibit rhodamine production. Subsequent studies revealed that addition of ONOO$^-\text{H}$ to a solution of NO generated by the decomposition of Sp/NO transiently reduced the NO signal, suggesting an interaction between ONOO$^-\text{H}$ and excess NO (Fig. 2). In a similar series of experiments performed in the presence of catalase and the absence of exogenously added Fe$^{3+}$ and a constant flux of NO of 1.0 nmol/min, we found that increasing fluxes of O$_2^-$ resulted in the production of RH in a pattern similar to that produced above such that RH oxidation was maximal when fluxes of NO and O$_2^-$ were approximately equal at 1.0 nmol/min for each (Fig. 3). Moreover, the yield of RH decreased substan-
inhibited RH production by more than 75%. The initial rate of RH production in the presence of 1.0 nmol/min NO and 1.0 nmol O2/min was almost 10-fold higher when compared with the rate achieved with a O2 flux of 10 nmol/min (data not shown).

Oxidation of DHR in the Presence of Fe3+—Oxidation of DHR by different fluxes of NO in the presence of trace amounts of Fe3+ was achieved by adding 1.0 μM Fe3+ or Fe3+EDTA to 1.0 nmol/min of DHR in a 1.0-ml reaction volume containing 50 mM potassium phosphate buffer (pH 7.4) at 37°C and incubated for 30 min. RH production was quantified as described under “Materials and Methods.”

The addition of catalase or superoxide dismutase to solutions containing Fe3+EDTA and HX/xanthine oxidase attenuated RH production by >90% (Fig. 7). The generation of NO in Fe3+EDTA solutions in the absence of O2 and H2O2 resulted in production of very small amounts RH (<20 μM), whereas the simultaneous production of O2/H2O2 and NO in the presence of Fe3+EDTA oxidized approximately 13 μM DHR. Hydroxylation of Benzoic Acid in the Absence of Iron—Hydroxylation of BA to yield HB with increasing fluxes of NO in the presence of a continuous flux of O2 (1.0 nmol/min) is shown in Fig. 8A. In the presence of catalase and the absence of exogenous Fe3+EDTA, we found that HB increased sharply from normally undetectable amounts in the absence of NO to a maximum of 650 nM (650 pmol/ml) at fluxes of NO and O2 of 1.0 nmol/min each. Continued increases in NO flux above 1.0 nmol NO/min attenuated hydroxylation with complete inhibition of hydroxylation occurring at a flux of NO that was four to five times that of O2. Using similar experimental conditions we found that increasing the flux of O2 in the presence of continuous NO generation (1.0 nmol/min) caused a comparable rise in benzoate hydroxylation such that maximum hydroxylation occurred when fluxes of each radical were 1.0 nmol/min (Fig. 8B). As the flux of O2 increased with respect to NO production, we observed a 50% inhibition of BA hydroxylation at fluxes of O2 that were five times that of NO. Complete inhibition was achieved when the flux of O2 was 10 nmol/min or 10 times greater than that of NO (data not shown). It should be noted that the absolute amounts of HB (600–700 nM) produced by equimolar fluxes of O2 and NO were in fact very small and were
Fig. 9 demonstrates that neither O$_2$ nor NO alone were capable of mediating significant hydroxylation in the absence of Fe$^{3+}$-EDTA. Equimolar fluxes (1.0 nmol/min) of each radical produced more than 510 nM HB, which was inhibited by 60% by the addition of superoxide dismutase (0.1 mg/ml), suggesting that O$_2$ and NO interact to yield an oxidant with only modest hydroxylating activity.

Hydroxylation of Benzoic Acid in the Presence of Fe$^{3+}$-EDTA—Fig. 10 illustrates BA hydroxylation with increasing amounts of NO in the presence of Fe$^{3+}$-EDTA and a continuous flux of O$_2$ and H$_2$O$_2$ of 1.0 nmol/min for each oxidant. Unlike the hydroxylation reaction in the absence of iron and H$_2$O$_2$, production of HB increased dramatically in the absence of NO such that hydroxylation increased more than 6-fold in the absence of NO. We found that NO inhibited the O$_2$/H$_2$O$_2$-dependent iron-catalyzed hydroxylation reaction in a concentration-dependent manner such that as little as 4 nmol/min of NO virtually eliminated the hydroxylation reaction (Fig. 10A). Neither spermine nor NO$_2$ (data not shown) significantly affected BA hydroxylation. When the NO flux was held constant at 1.0 nmol/min and O$_2$ and H$_2$O$_2$ production were increased, we found a concentration-dependent increase in hydroxylation of BA such that maximum hydroxylation was obtained at fluxes of O$_2$ and H$_2$O$_2$ of approximately 2.0 nmol/min (Fig. 10B). Little inhibition of HB formation was observed even when the O$_2$ and H$_2$O$_2$ fluxes were increased to 5.0 nmol/min (Fig. 10B).

DISCUSSION

Much of the vascular and tissue injury observed in certain models of inflammation have been shown to be inhibited by either superoxide dismutase or NO synthase inhibitors, suggesting that both O$_2$ and NO are important mediators of tissue injury and dysfunction (1–6). Because neither O$_2$ nor NO are particularly potent oxidants or cytotoxins, it has been suggested that O$_2$ and NO may combine to produce the potent cytotoxic oxidants ONOO$^-$ and ONOOH (8). Indeed, this hypothesis has generated tremendous interest because it has provided a biochemical rationale to account for the remarkable but perplexing protective effects of intravenous administration of L-arginine analogs (NO synthase inhibitors) or superoxide dismutase in these pathophysiologic models of tissue injury and inflammation (1–6). Numerous studies have been published describing the physicochemical and cytotoxic properties of chemically synthesized ONOO$^-$ (8–14). However, there is a paucity of information quantitatively characterizing the interaction between O$_2$ and NO under physiologic conditions. Thus, we have attempted to systematically quantify the interaction between NO and O$_2$ in the absence or the presence of redox-active iron.

Data obtained in the present study demonstrate that in the
NO and \( \text{O}_2 \)-dependent Oxidation and Hydroxylation Reactions

Figure 10. A, the effect of increasing NO flux on \( \text{O}_2/\text{H}_2\text{O}_2 \)-dependent hydroxylation of benzoate in the presence of iron. Assays were performed as described under "Materials and Methods." All samples contained 5 \( \mu \text{M} \) Fe \( ^{3+} \)-EDTA, and \( \text{O}_2/\text{H}_2\text{O}_2 \) fluxes were held constant at \( -1.0 \) nmol/min in 20 mM potassium phosphate buffer (pH 7.4) at \( 37^\circ \text{C} \). B, the effect of increasing \( \text{O}_2/\text{H}_2\text{O}_2 \) fluxes on \( \text{O}_2/\text{H}_2\text{O}_2 \) and NO-dependent hydroxylation of benzoate in the presence of iron. Assays were performed as described under "Materials and Methods." All samples contained 5 \( \mu \text{M} \) Fe \( ^{3+} \)-EDTA, and the NO flux was constant at \( -1.0 \) nmol/min.

absence of iron-catalyzed reactions, simultaneous generation of equimolar fluxes of \( \text{O}_2 \) and NO synergize to yield an oxidant or oxidants capable of oxidizing DHR to RH (Figs. 1 and 3). Because catalase was present throughout these experiments and because superoxide dismutase decreased RH production by 90\%, we propose that \( \text{O}_2^\cdot \) but not \( \text{H}_2\text{O}_2 \) nor \( \text{OH}^- \) interacts with NO to yield the oxidant or oxidants (Fig. 5). These data also confirm a previous report (31) that found that neither \( \text{O}_2/\text{H}_2\text{O}_2 \) nor NO per se is capable of oxidizing substantial amounts of DHR in the absence of redox active metals such as iron or hemoproteins. Only oxidants such as those derived from Fenton-type reactions, ferryl hemoproteins, or ONOO\(^-\)/ONOONH are potent enough oxidizing agents to oxidize DHR. Indeed, decomposition of peroxynitrous acid to nitrate has been suggested to proceed via a rate-limiting isomerization reaction that yields a potent oxidizing agent capable of hydroxylation of organic substrates (8). Thus, we also assessed the ability of \( \text{O}_2/\text{H}_2\text{O}_2 \) and NO to interact (in the absence of iron and \( \text{H}_2\text{O}_2 \)) to hydroxylate BA. We found a similar pattern of hydroxylation of BA as observed for DHR oxidation in that equimolar fluxes (1.0 nmol/min) of \( \text{O}_2/\text{NO} \) appeared to synergize to hydroxylate BA to HB (Fig. 8, A and B), although the magnitude of this hydroxylation reaction was rather small (<15\% that with iron and \( \text{H}_2\text{O}_2 \) present). Although we have not definitely identified ONOO\(^-\)/ONOONH as the oxidants produced in this system, we expect that this would be the likely reaction pathway because of the rapid interaction between \( \text{O}_2^\cdot \) and NO and because of the lack of alternative explanations for the production of equally potent oxidants.

We speculate that the decreased production of RH or HB in the absence of iron and \( \text{H}_2\text{O}_2 \) but in the presence of either excess NO or \( \text{O}_2^\cdot \) may be accounted for on the basis of either secondary chemical interactions occurring directly between NO or \( \text{O}_2^\cdot \) and ONOOH. It may also be due to the interaction between NO or \( \text{O}_2^\cdot \) with free radical intermediates of DHR or BA to yield adducts with diminished fluorescence. The latter possibility does not appear to be a major pathway because we did not observe dramatic inhibition of DHR oxidation by excess NO in the iron-containing system (Fig. 6A) nor did nitrosation of HB by NO-derived nitrosating agents attenuate its fluorescence (data not shown). The former hypothesis appears to be the more viable explanation.

Although the direct reaction of ONOOH with either NO or \( \text{O}_2^\cdot \) has not been definitively demonstrated, it has been suggested to be thermodynamically possible (33, 34). Koppenol et al. (34) have calculated Gibbs free energies (\( \Delta G \)) of \( -36 \) and \( -27 \) at neutral pH and 25 \( ^\circ \text{C} \).

\[
\text{ONOOH} + \text{O}_2^\cdot + \text{H}^+ \rightarrow \text{NO}_2 + \text{O}_2 + \text{H}_2\text{O}
\]

\[
\text{ONOOH} + \text{NO} \rightarrow \text{NO}_2 + \text{NO}
\]

Although reaction rates are not forthcoming from calculated thermodynamic values, the possibility of the interaction of ONOOH with NO or \( \text{O}_2^\cdot \) is at least indicated. Therefore, competing reactions involving excess NO or \( \text{O}_2^\cdot \) with ONOOH could be a possible mechanism for the decreased DHR oxidation and BA hydroxylation in the absence of iron-catalyzed reactions. Albeit, our data indicate that excess NO or \( \text{O}_2^\cdot \) may be acting (at least in our system) as modulators of the pro-oxidant characteristics of ONOO\(^-\)/ONOONH, and by extension, we suggest that under similar conditions in vivo, excess NO or \( \text{O}_2^\cdot \) may act as an endogenous modulator of ONOOH-mediated tissue damage. On the other hand, depending on the ratio of fluxes of \( \text{O}_2^\cdot \) to NO, oxidation and hydroxylation reactions may be either enhanced or inhibited in the absence of iron (Figs. 1, 3, and 10).

Under conditions of limiting \( \text{O}_2^\cdot \) flux, excess NO will instead be auto-oxidized in the presence of molecular oxygen-producing nitrogen oxides (e.g. \( \text{NO}_2 \), \( \text{N}_2\text{O}_3 \), or \( \text{N}_2\text{O}_2 \) that are not potent oxidizing or hydroxylating agents but are potent \( \text{N}^-\)nitrosating agents (35). We have recently demonstrated that \( \text{O}_2^\cdot \) will effectively inhibit NO-mediated \( \text{N}^-\)nitrosation reactions (36), and contrary to a recent report (37), we detected no significant change in xanthine oxidase activity (measured via urate production) in the presence of 200 \( \mu \text{M} \) Sp/NO, which produces a flux of \( -4.0 \) nmol NO/min (data not shown). Furthermore, the production of hydrogen peroxide from xanthine oxidase was not inhibited by the presence of 200 \( \mu \text{M} \) Sp/NO or 1 \( \text{mM} \) DEA/NO (data not shown). On the other hand, increased production of \( \text{O}_2^\cdot \) at higher xanthine oxidase concentrations was concomitant with increased urate production (a potent free radical scavenger). Whereas urate-mediated inhibition cannot be totally discounted, it apparently was not a significant factor at \( \text{O}_2^\cdot \) fluxes below 1.0 nmol/min, as indicated by the similarity in the shape of curves shown in Figs. 1 and 3. Moreover, total elimination of possible urate interference was achieved with the use of pterin (500 \( \mu \text{M} \)) as substrate in place of HX, yet the data were virtually identical to those shown in Fig. 3 (data not shown).

When the same oxidation and hydroxylation experiments were performed in the presence of \( \text{O}_2^\cdot \), \( \text{H}_2\text{O}_2 \), and 5 \( \mu \text{M} \) Fe \( ^{3+} \)-EDTA, qualitatively different results were obtained. Generation of \( \text{O}_2^\cdot \) and \( \text{H}_2\text{O}_2 \) in the presence of Fe \( ^{3+} \)-EDTA but not NO stimulated oxidation of DHR producing approximately 15 \( \mu \text{M} \)
RH compared with the formation of 20 μM RH in the presence of NO (1.0 nmol/min; Fig. 6A). These data suggest that as the ratio of NO/O₂⁻ increased from 0 to 2, oxidation of DHR increased by approximately 30% (Fig. 6A). As the ratio was increased further to 4.5, RH production was reduced by 40%. These data are reminiscent of those reported by Rubbo et al. (10) using xanthine oxidase-dependent iron-catalyzed lipid peroxidation. As expected, generation of O₂⁻ and H₂O₂ (1.0 nmol/min each) in the presence of iron increased production of HB tremendously (i.e., -4000 nM) (Fig. 10A). Remarkably, the addition of NO to this iron-catalyzed hydroxylation system dramatically inhibited hydroxylation of BA such that equimolar fluxes of NO inhibited hydroxylation by 80% (Fig. 10A). Kanner et al. (38) recently suggested that NO may modulate iron-mediated oxidative reactions by forming nitrosyl complexes with ferrous iron or by the direct interaction of NO with H₂O₂. The sequence of reactions involving NO and iron may proceed as follows:

\[
\text{Fe}^3\text{-EDTA} + \text{O}_2 \rightarrow \text{Fe}^2\text{-EDTA} + \text{O}_2
\]

\[
\text{Fe}^2\text{-EDTA} + \text{NO} \rightarrow \text{NO-Fe}^2\text{-EDTA}
\]

\[
\text{NO-Fe}^2\text{-EDTA} + \text{H}_2\text{O}_2 \rightarrow \text{NO-Fe}^2\text{-EDTA} + \text{HNO}_2 + \text{OH}^-
\]

The efficiency of such interactions could explain the results in Fig. 10A. Indeed it is well known that NO binds under physiological conditions with ferrous heme containing compounds (e.g. hemoglobin and myoglobin), and moreover these reactions are the chemical basis of current methodology used for NO detection (39). An alternative and more likely explanation for this dramatic inhibitory effect of NO may be that NO shunts O₂⁻ away from iron-catalyzed OH⁻ formation by the Fenton reaction and toward the formation of an oxidant (e.g. ONOO⁻ / ONOOH) with only weak hydroxylating activity.

Our data confirm and extend the results recently reported by Rubbo et al. (10), who demonstrated that increasing fluxes of NO with respect to O₂⁻ and H₂O₂ modestly stimulated iron-catalyzed lipid peroxidation followed by inhibition when fluxes of NO exceed those of O₂⁻. Furthermore, these same investigators demonstrated that NO could partially inhibit ONOO⁻-induced lipid peroxidation (10). Interestingly, in our studies in the presence of iron, conditions under which total inhibition of hydroxylation occurred resulted in only a modest 33% inhibition of DHR oxidation (Figs. 6 and 10). Suggesting that NO is shunting O₂⁻ away from iron-catalyzed OH⁻ formation and toward the formation of a potent oxidant with weak hydroxylating activity.

Two major physiological implications arise from our present study. (a) The role of ONOO⁻ at sites where both O₂⁻ and NO are produced (e.g. inflammatory foci) is dependent upon the relative fluxes of NO and O₂⁻ in the extracellular space. Our data suggest that excess production of one radical over the other may act as an endogenous modulator of ONOO⁻ formation such that the steady state levels of this potent cytotoxic oxidant never accumulates above a certain amount. Indeed, the spontaneous acid-catalyzed decomposition of another potent oxidant, hypothiocyanous acid, is an example of autocatalytic regulation of oxidation formation (40). (b) NO may enhance or inhibit oxidation and hydroxylation reactions depending upon the absence or the presence of low molecular weight, redox-active metals such as iron. Normally, there is little low molecular weight iron (e.g. amino acid, carbohydrate, or nucleotide chelates of iron) present in most cells and tissue, with the vast majority of this metal sequestered in its redox-inactive, protein-bound forms such as ferritin- or transferrin-bound iron. However, it is known that certain reductants (e.g. ascorbate and O₂⁻) are very effective in releasing iron from ferritin by reducing ferritin-bound Fe³⁺ to Fe²⁺, which is no longer capable of being bound by the protein (41, 42). In addition to ferritin, there is also a small but significant pool of low molecular weight iron chelate (e.g. non-protein-bound iron) located within cells. Studies by Deighton and Hider (43) have identified this low molecular weight iron chelate as a glutamate-iron complex (molecular weight of 1000–1500) that can easily exchange its iron with other more potent chelators.

Important consequences of the oxidant/hydroxylation-dependent flux of NO and O₂⁻ are temporal and spatial considerations. To achieve the maximum oxidant resulting from NO/O₂⁻, the site orientation and timing of the formation of these two species is crucial. The timing of the superoxide production relative to the NO can be distinctly different in vivo and have a limited overlap under some immunological and pathophysiological conditions. For instance, superoxide formation of neutrophils reaches a flux 10 times higher than that of NO within the first few minutes after treatment with phorbol ester (36). However, the flux of superoxide formed quickly subsides within an hour, whereas the NO production continues for several hours. The time overlap in which the flux of these two radicals is one to one is for a very limited time; therefore the amount of peroxynitrite formed is small. Conversely, cytokine-stimulated alveolar macrophage are thought to generate both NO and O₂⁻ at the same sustained rate for long period of time, implying that the oxidant formed may be intentionally held high in this specific cell line (15). Yet, RAW macrophages appear to generate solely nitrosating agents via the NO/O₂⁻ reaction without the presence of superoxide (35). This switching between oxidation, hydroxylation, and nitrosation appears to be well orchestrated in the immune response to pathogens and appears to be critical in host defense. Although NO and superoxide can be generated from the same cell type and cytokine influence, kinetic considerations must be carefully examined to determine the reactive intermediates involved.

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