Apparent hydroxyl radical production by peroxynitrite: Implications for endothelial injury from nitric oxide and superoxide

(endothelium-derived relaxing factor/desferrioxamine/ischemia/superoxide dismutase)

JOSEPH S. BECKMAN*, TANYA W. BECKMAN*, JUN CHEN*, PATRICIA A. MARSHALL*, AND BRUCE A. FREEMAN*

Departments of *Anesthesiology and 1Biochemistry, University of Alabama at Birmingham, Birmingham, AL 35233

Communicated by Irwin Fridovich, December 4, 1989 (received for review October 4, 1989)

ABSTRACT Superoxide dismutase reduces disease and neutrophils, and a half-life of radicals generated by the iron-catalyzed Haber-Weiss reaction may be limited in vivo, suggesting that other reactions may be important in understanding O2·- toxicity to endothelium. We propose that nitric oxide (NO·), a stable free radical, reacts with O2·- in many pathological states to yield secondary cytotoxic species. Recently, endothelium, macrophages, and brain synapto-some preparations have been shown to produce NO· by oxidizing arginine by a calcium-activated NADPH-dependent enzyme (6-9). NO· appears to be a major form of the endothelium-derived relaxing factor (EDRF) (10). Vasodilatory agents such as acetylcholine, ATP, and bradykinin initiate a receptor-mediated influx of Ca2+, triggering the production and extracellular release of NO-, which then activates soluble heme-containing guanylate cyclases to produce cGMP in vascular smooth muscle and platelets. Increased cGMP promotes relaxation in vascular smooth muscle and inhibits platelet aggregation as well as adhesion of platelets to endothelium (11). Macrophages produce NO· as part of their cytotoxic armamentarium (6).

The half-life of EDRF and NO- ranges from 4 to 50 sec (12), which is approximately doubled by SOD (13, 14). NO· does not bind directly to the copper of SOD (15), suggesting that stabilization involves the scavenging of O2·-. Because NO- contains an unpaired electron and is paramagnetic, it rapidly reacts with O2·- to form peroxynitrite anion (ONOO-) in high yield (16). In alkaline solutions, ONOO- is stable but has a pKₐ of 6.6 at 0°C (17) and decays rapidly once protonated.

O2·- + NO- → ONOO- + H+ ⇌ ONOOH

→ HO- + NO₂- → NO₂- + H+. [4]

In the gaseous phase, decomposition of peroxynitrous acid to form HO- and nitrogen dioxide (NO₂-) is important in the formation of smog and acid rain. Although HO- and NO₂- can recombine to form nitric acid, the rate constant for this reaction in solution (3×10⁴ M⁻¹ s⁻¹; refs. 18 and 19) is much slower than most reactions involving HO-. Still, HO- formed by homolytic decomposition of HOONO in aqueous solutions may not escape beyond the solvent cage before reacting with NO₂-, making HO- undetectable. However, peroxynitrite formed by the reaction of H₂O₂ and nitrite at pH 2.0 will hydroxylate benzene rings and polymerize methyl...
methacrylate, reactions characteristic of a free-radical process (20, 21). By measuring oxygen evolution from HO-
oxidation of H₂O₂, a 32% yield of HO- could be detected
during the reaction of H₂O₂ and nitrite at pH 5.0 (18).
Furthermore, oxygen evolution was inhibited by the HO-
scavengers ethanol and benzaldehyde. We have extended these
observations to more physiological conditions and show that
peroxynitrite initiates many reactions currently used to infer
the action of HO-. We propose that the formation of peroxynitrite from O₂⁻ and NO is important in recognizing potential
mechanisms of SOD-inhibitable oxidant injury to endothelium.

METHODS

Peroxynitrite Synthesis. Peroxynitrite was synthesized in a
quenched-flow reactor (22). Solutions of (i) 0.6 M NaNO₂ and
(ii) 0.6 M HCl/0.7 M H₂O₂ were pumped at 26 ml/min into a
tee-junction and mixed in a 3-mm diameter by 2.5-cm glass
tube. The acid-catalyzed reaction of nitrous acid with H₂O₂
to form peroxynitrous acid was quenched by pumping 1.5 M NaOH
at the same rate into a second tee-junction at the end of
the glass tubing. Excess H₂O₂ was removed by passage
over a 1 × 5 cm column filled with 4 g of granular MnO₂. The
solution was frozen at −20°C for as long as a week. Peroxynitrite
tends to form a yellow top layer due to free-base fractionation,
which was scraped for further studies. This top layer typically contained 170–220 mM peroxynitrite as determined by absorbance at 302 nm in 1 M NaOH (ε₃₀₂ =
1670 M⁻¹ cm⁻¹; ref. 23). Interference by other absorbing
compounds (e.g., nitrite) was corrected by subtracting the final absorbance after adding peroxynitrite to 100 mM potas-sium phosphate (pH 7.4).

HO- Assays. The production of HO- from peroxynitrite
decomposition was assayed by the oxidation of DMSO to formaldehyde (24) and deoxyribose to malonidialdehyde
(MDA; ref. 25). All assays were conducted at 37°C and
incubated for 3 min to allow peroxynitrite to fully decompose. Catalase (10 units/ml final concentration; Worthington) was added at the end of each reaction to remove trace
H₂O₂ left after MnO₂ treatment. To assay MDA formed from
deoxyribose, 1 ml of 2.8% trichloroacetic acid and 1.0 ml of
1% thioarbituric acid in 0.1 M NaOH adjusted to pH 3.5
were sequentially added to 1-ml samples, and the mixtures
were then boiled for 10 min. Residual deoxyribose and nitrite
present in the peroxynitrite stock solution caused the appearance of a shoulder overlapping at 532 nm, the absorbance
maximum of the MDA thioarbiturate product. To correct for
this shoulder, absorbances were read at 510, 532, and 560 nm.
The shoulder was assumed to decrease linearly over these
wavelengths and was subtracted by standard matrix methods
(26). Standards of MDA were prepared daily by the hydrolysis
of 100 μM 1,1,3,3-tetramethoxypropane.

DMSO reacts with HO- to form a methyl radical, ultimately yielding 0.5 mol each of formaldehyde and methanol under aerobic conditions. Formaldehyde was assayed by reaction with purpureal (Aldrich) as described by Johansson and Hakan Borg (27).

Peroxynitrite Decomposition. Peroxynitrite decomposition
was followed by absorbance changes at 302 nm every 0.075
s after adding peroxynitrite to a rapidly stirred, temperature-
controlled cuvette. The pH of each reaction was measured
after peroxynitrite addition. The kinetics of peroxynitrite
decomposition were strictly first order over at least three
half-lives as reported (17). The decrease in absorbance at 302 nm was used to calculate apparent first-order rate constants by nonlinear regression (Enzfit; Elsevier BIOSOFT Publishers).

Data Analysis. To estimate the total amount of HO- formed
from peroxynitrite decomposition, a Scatchard-like plot was
used to find the amount of HO- trapped in the presence of
infinite detector concentrations (5). If the rate constant of
HO- reacting with a given concentration of detector D to form
a product P is kₐ and the pooled rate constants of HO-
undergoing all other reactions are summed to give kᵣ, then the
molar concentration of product [P] detected in an assay using
a detector molar concentration [D] may be written as

\[ [P] = \frac{kₐ[D]}{kₐ[D] + kᵣ}, \]

Eq. 5 can be linearized to give the equivalent form of a
Scatchard plot in which

\[ [P]/[D] = -\frac{(kₐ/kᵣ)[P]}{1 + (kₐ/kᵣ)[HO⁻]]. \]

In a plot of [P]/[D] versus [P] for a range of detector concentrations, the x axis intercept equals the HO- concentration that can be trapped at infinite detector concentrations.

The inhibition of apparent HO- production, estimated from
the effect of various scavengers on yield, was calculated as
described by Winterbourn (28). If Fᵢ is the fraction of inhibition from a scavenger i at concentration Sᵢ, then simple competitive kinetics predict

\[ \frac{kₛ_i}{kₐ} = \frac{F_i[D]}{(1 - F_i)[Sᵢ]} \]

where kₛᵢ and kₐ are the respective rate constants for reaction of HO- with scavenger i and detector. The ratio kₛᵢ/kₐ is also the concentration of detector divided by the concentration of scavenger i required to give 50% inhibition of product yield. If scavenging occurs by simple competition, values of Fᵢ[D]/(1 − Fᵢ)[Sᵢ] for each scavenger plotted against the known rate constants of kₛᵢ for reaction of HO- with scavenger Sᵢ will yield a linear relationship of slope 1/kₐ (see Fig. 5).

RESULTS

Peroxynitrite addition to 50 mM deoxyribose or DMSO in 50
mM potassium phosphate (pH 7.4) at 37°C resulted in linear,
peroxynitrite concentration-dependent increases of product
for each detector (Fig. 1). By assuming a stoichiometry of 0.5
mol of product per mol of peroxynitrite added to DMSO, the
slopes calculated from different HO- detection systems in
Fig. 1 corresponded to a yield of 18.8 ± 0.4% formaldehyde for DMSO and 1.53 ± 0.04% MDA for deoxyribose. Product
yields from deoxyribose and DMSO were greater at acid pH
values and were described by the Henderson–Hasselbalch
equation, with the apparent pKₛᵢ being 7.42 ± 0.14 and 7.79 ± 0.08, respectively (Fig. 2).

Peroxynitrite pKₛᵢ and Half-Life. Keith and Powell (17)
determined the pKₛᵢ of peroxynitrite to be 6.6 at 0°C by measuring the rate of peroxynitrite decomposition as a function
of pH:

\[ kᵣ = kₐ[H⁺]^{-1}/[H⁺] + Kₛᵢ], \]

where kᵣ is the apparent rate constant of peroxynitrite
decomposition at a given pH, kₐ is the first-order rate
constant for peroxynitrous acid (ONOOH) and Kₛᵢ is the
ionization constant for peroxynitrous acid. First-order rate
constants for peroxynitrite decomposition measured over a
pH range from 4 to 8.5 (Fig. 3) indicated that the pKₛᵢ was 7.49 ± 0.06 at 37°C. The rate constant kₐ for decay of peroxynitrous acid was 0.654 ± 0.049 s⁻¹, which corresponded to a
half-life of 1.06 s. At pH 7.4, the half-life of peroxynitrite
increased to 1.9 s.
The yield of formaldehyde (●) and MDA (●) from 50 mM DMSO and deoxyribose, respectively, as a function of added peroxynitrite at 37°C. The pH of each buffer (50 mM potassium phosphate) was adjusted to give a final pH of 7.4 after peroxynitrite addition. Total pH changes were <0.15 pH units. Bars indicate standard deviations for three replicates per point.

Maximal Yield of HO·. The yield of formaldehyde from 250 μM peroxynitrite increased hyperbolically with detector concentrations of DMSO at pH 6.0 (Fig. 4). When peroxynitrite was allowed to decompose fully by mixing it with buffer 3 min before DMSO, the trace amounts of formaldehyde produced were the same as controls with DMSO alone. Based upon the estimates from Scatchard-like plots for each of the HO· detection systems, the maximum yield at infinite detector concentrations relative to the amount of peroxynitrite added at pH 6.0 were 24 ± 1% formaldehyde from DMSO and 7.0 ± 0.1% MDA from deoxyribose.

Competitive Inhibition. Classical HO· scavengers resulted in a concentration-dependent inhibition of yield in all three HO· assays, with the mean values of F/[D]/(1 - F)/[S] for each scavenger increasing linearly when plotted against k_S, the known rate constants for scavengers reacting with HO· (Fig. 5). The exceptions were when deoxyribose and DMSO were used as scavengers, both of which deviated strongly from the otherwise linear relationship. The reciprocals of the slopes gave estimates for k_D, the reaction rate of peroxynitrite with the detector, of 1.8 × 10^8 M^−1s^−1 for deoxyribose and 1.3 × 10^9 M^−1s^−1 for DMSO. The k_D for deoxyribose reacting with peroxynitrite agrees fairly well with its reaction rate for HO· (1.8 × 10^9 M^−1s^−1), while the k_D for DMSO is only 20% of its reaction rate with HO· (7 × 10^9 M^−1s^−1). The relatively low k_D for DMSO for peroxynitrite may account for its relatively poor scavenging ability observed in assays using deoxyribose as a detector.

In the panels of Fig. 5, the fitted lines did not intercept the origins. Because the rate constants plotted on the x axis are literature values for reaction with HO·, the nonzero intercepts may be due to a small and relatively constant difference between the reaction of each scavenger with peroxynitrite as compared with HO·

Reaction of Peroxynitrite with Desferrioxamine. To test the potential contribution of transition metals to peroxynitrite-initiated oxidation, the effects of the metal chelators DTPA and desferrioxamine were examined. In the three HO· assays, DTPA (0.1 mM) had no effect on product yield. In contrast, desferrioxamine was a potent, concentration-dependent inhibitor of DMSO oxidation by peroxynitrite, but less so for MDA formation from deoxyribose (Fig. 6). Desferrioxamine bound to equimolar ferric iron did not influence yield in the two detection systems.

**DISCUSSION**

Peroxynitrite decomposition generates a strong oxidant able to initiate many reactions currently used to implicate the action of HO·. Halfpenny and Robinson (20, 21) previously...
demonstrated the hydroxylation and polymerization of phenolic rings, reactions characteristic of HO·, by peroxynitrite at pH 2.0. We have shown herein that peroxynitrite oxidized deoxyribose to MDA and DMSO to formaldehyde with substantial yields under physiological conditions. Furthermore, HO· scavengers were able to reduce the apparent HO· yield in a concentration-dependent fashion in the approximate order of the known rate constants for the scavengers reacting with HO·.

Oxidation of deoxyribose and DMSO by peroxynitrite decreased at higher pH with pKₐ values close to the pKₐ of 7.49 for peroxynitrite decomposition. The pH dependence is curious because the HO· assays were end-point assays incubated for a minimum of eight half-lives of peroxynitrite at pH 9.0. Even at the most alkaline pH tested, sufficient time had lapsed for >99% of the peroxynitrite anion to become protonated and decompose as peroxynitrous acid. Hence, yield of oxidized products from deoxyribose or DMSO should not have been affected by pH. One possible explanation is that peroxynitrous acid decays by two pathways, the first due to direct internal rearrangement of cis-peroxynitrite (29, 30) to give nitrate without forming strong oxidants and the second due to homolytic fission of trans-peroxynitrite to form HO· plus NO₂⁻ (Eq. 9).

\[
\begin{align*}
\text{Internal rearrangement} & \rightarrow \\
\text{Homolytic fission} & \\
\end{align*}
\]

\[
\begin{align*}
\text{O} &= \text{N} \\
\text{O} &\rightarrow \text{H}^+ + \text{NO}_2^- \\
\end{align*}
\]

\[\text{[deactivation]}\]

The transition state for internal rearrangement may have a shorter half-life due to a lower activation energy, making it less likely to be deactivated by the loss of a proton. Alternatively, the vibrational energy necessary for homolytic fission may be dissipated more readily by proton loss. Thus, internal rearrangement would be favored over homolytic cleavage at high pH, resulting in a lower yield of oxidation products.

**FIG. 5.** Inhibition by HO· scavengers of product yield from 10 mM DMSO (a) or 10 mM deoxyribose (b) after addition of 250 μM peroxynitrite; \(k_s\) is the rate constant for the reaction of HO· with scavenger \(i\). To maximize yields, studies with DMSO and deoxyribose as a detector system used 50 mM potassium phosphate (pH 6.0) at 37°C. The scavengers were Tris (a), mannitol (b), ethanol (b), deoxyribose (c), sodium formate (d), benzoic acid (e), salicylic acid (f), DMSO (g), and uric acid (h). Each scavenger was tested over a concentration range of 1–225 mM, and points represent an average of eight different concentrations tested in duplicate and optimized for each scavenger to span the range giving 50% inhibition. Rate constants were those used in ref. 28.

**FIG. 6.** Inhibition of product yield by desferrioxamine of 250 μM peroxynitrite with 10 mM DMSO (a) and deoxyribose (b) in 50 mM potassium phosphate (pH 7.4) and at 37°C.

**Desferrioxamine and the Role of Metal Ions.** The formation of HO-like oxidants from peroxynitrite did not require metal catalysis, as shown by the absence of DTPA inhibition in the three HO-assays. However, peroxynitrite anion can complex with iron and copper (31), making metal-catalyzed reactions possible and in need of further investigation.

Much of the evidence for a role of iron in free-radical-mediated injury in vivo derives from the reduction of injury by desferrioxamine. In our experiments, desferrioxamine was a strong inhibitor of peroxynitrite oxidation, which was due to a direct, concentration-dependent reaction between desferrioxamine and peroxynitrite rather than by metal chelation. Desferrioxamine contains three hydroxamic acids (=NOH) adjacent to carbonyl groups, which appears to be the functional groups involved in peroxynitrite decomposition, since these sites are occupied in ferric desferrioxamine, which was not inhibitory. The potent inhibition of peroxynitrite-mediated oxidation by low concentrations of desferrioxamine suggests that scavenging of peroxynitrite may be an alternative antioxidant mechanism of desferrioxamine in addition to its role in iron chelation.

The present study has focused upon the apparent formation of HO· from peroxynitrite decomposition, but an equal amount of NO₂⁻ is also expected to be formed. NO₂⁻ contains an unpaired electron, making it reactive with many free radicals including intermediates produced by HO· attack on detector molecules (32). These potential secondary reactions will reduce apparent HO· yield noncompetitively with respect to the detector, so that Scatchard-like plots can only give a minimal estimate of apparent HO· yield.

NO₂⁻ is a highly toxic and potent oxidant (\(E_m = 0.9\) V), capable of initiating fatty acid oxidation (33, 34) and nitrosylation of aromatic amino acids (35). The possibility of nitrogen-based products being produced in vivo during endothelial injury presents a testable conjecture of our hypothesis. In support of this possibility, a nitrogen-centered radical has been detected during reperfusion of an ischemic isolated rat heart preparation (36).

**Implications for Endothelial Oxidant Injury.** Peroxynitrite may have considerably greater toxicity than HO· generated extracellularly; it is formed by a diffusion-limited reaction between O₂⁻ and NO, it has a 1.9-s half-life at pH 7.4 that permits diffusion over several cell diameters, it decomposes to generate a potent oxidant similar to HO· in reactivity, and it may cross cell membranes through anion channels as has been demonstrated for O₂⁻ (37).

Many pathophysiological processes including reperfusion of ischemic tissue, acute inflammation, and sepsis might initiate events that stimulate NO· production. For example, ischemic endothelium will accumulate Ca²⁺, but will be...
unable to support NO synthesis without oxygen (38). Reperfusion may stimulate rapid NO synthesis by providing the oxygen needed to produce NO, since the intracellular messenger, Ca²⁺, and other substrates—arginine and NADPH—would already be made available by the ischemic insult.

Rates of NO production have not been measured under pathological conditions and will be an important test of the hypotheses advanced here. However, it is possible to calculate that substantial amounts of NO may be produced in small vessels by assuming rates of production equivalent to that of rabbit aorta. Rabbit aortic endothelium can produce 40 pmol of NO min⁻¹·cm⁻² of luminal surface when stimulated by ATP (7). In a 50-μm-diameter vessel, the surface area of endothelium enclosing a 1-liter volume of blood is 2 × 10⁵ cm². Then, the intraluminal rate of NO production could theoretically reach 8 μM min⁻¹. Ca²⁺ entry into endothelium is also known to increase O₂⁻ production (39). Since the rate of peroxynitrite formation depends upon the product of O₂⁻ and NO⁻ concentrations, it will increase 100-fold for every 10-fold increase in O₂⁻ and NO⁻ concentration. Thus, relatively small increases in rates of O₂⁻ and NO⁻ production may greatly increase rates of peroxynitrite formation to potentially cytotoxic levels.

One of the most puzzling effects of SOD is to understand how it can protect ischemic tissue in experimental models when injected into the circulation just prior to reperfusion. Injury to the endothelium is a major consequence of ischemia/reperfusion injury, causing edema formation due to the loss of barrier function and favoring platelet adhesion to endothelium. The protective action of SOD may in part be due to preventing the decomposition of NO by scavenging O₂⁻, which would help maintain normal vasodilation and inhibit thrombosis. We propose that SOD also protects endothelium in vivo by preventing the formation of peroxynitrite, which is toxic due to its decomposition to form potent, cytotoxic oxidants. On the other hand, the simultaneous generation of O₂⁻ and NO⁻ by macrophages and activated neutrophils (6, 40) presents an additional mechanism mediating the cytotoxic action of these cell types.

We thank Drs. Irwin Fridovich and James N. Siedow (Duke University, Durham, NC) for their advice, and Kelly Blair and John Morgan for their technical assistance. This work was supported by grants NS 24338 (to J.S.B.) and NS 24275 (to B.A.F.) from the National Institutes of Health and grants from the Alabama Affiliate of the American Heart Association (to J.S.B.).