Peroxynitrite Oxidation of Sulfhydryls

THE CYTOTOXIC POTENTIAL OF SUPEROXIDE AND NITRIC OXIDE*

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Peroxynitrite anion (ONOO⁻) is a potent oxidant that mediates oxidation of both nonprotein and protein sulfhydryls. Endothelial cells, macrophages, and neutrophils can generate superoxide as well as nitric oxide, leading to the production of peroxynitrite anion in vivo. Apparent second order rate constants were 5,900 M⁻¹s⁻¹ and 2,600-2,800 M⁻¹s⁻¹ for the reaction of peroxynitrite anion with free cysteine and the single thiol of albumin, respectively, at pH 7.4 and 37 °C. These rate constants are 3 orders of magnitude greater than the corresponding rate constants for the reaction of hydrogen peroxide with sulfhydryls at pH 7.4. Unlike hydrogen peroxide, which oxidizes thiolate anion, peroxynitrite anion reacts preferentially with the undisassociated form of the thiol group. Peroxynitrite oxidizes cysteine to cystine and the bovine serum albumin thiol group to an arsenite nonreducible product, suggesting oxidation beyond sulfenic acid. Peroxynitrous acid was a less effective thiol-oxidizing agent than its anion, with oxidation presumably mediated by the decomposition products, hydroxyl radical and nitrogen dioxide. The reactive peroxynitrite anion may exert cytotoxic effects in part by oxidizing tissue sulfhydryls.

Endothelial cells produce and release nitric oxide (NO) as well as superoxide (O₂⁻) (1, 2). Nitric oxide appears to be a major form of the endothelial-derived relaxing factor that is enzymatically synthesized via arginine oxidation by a cal- logy, with intracellular sources of O₂ including mitochondrial, endoplasmic reticular, and nuclear membrane electron transport processes and soluble proteins such as hemoglobin, aldehyde oxidase, and xanthine oxidase (6). Superoxide production can be enhanced by pathological processes including hyperoxia (7), xenobiotic metabolism (8), and ische- mia/reperfusion (10). When generated in excess, O₂⁻ is a key mediator of cell injury (11). Superoxide is implicated as a toxic species in free radical-mediated cytotoxicity when superoxide dismutase limits cell injury. For example, augmentation of intracellular superoxide dismutase prevents oxygen-induced damage in endothelial cells (12). However, O₂⁻ chemical reactivity is limited, compared to other free radicals (13). Whereas O₂⁻ can react with critical cellular targets to directly exert toxicity (14), enzymatic and spontaneous dismutation can be effective competing reactions that may limit direct toxic effects of O₂⁻ (14, 15). While other partially reduced oxygen species such as hydrogen peroxide (H₂O₂) and hydroxyl radical (·OH) are cytotoxic, hydroxyl radical is frequently proposed as an ultimate cytotoxic agent due to its high reactivity (16). Hydroxyl radical can be generated through an O₂⁻-driven Fenton reaction, in which O₂⁻ plays the role of a reducing agent (17).

O₂⁻ + Fe²⁺ → O₂ + Fe³⁺ (1)

H₂O₂ + Fe²⁺ → ·OH + OH⁻ + Fe³⁺ (2)

Our current understanding of oxygen-mediated toxicity via the Haber-Weiss and Fenton reactions does not always give a consistent explanation for protective effects lent by superoxide dismutase. It has been suggested that O₂⁻ serves as a reductant for Fe³⁺ (Equation 1) and that superoxide dismutase blunts ·OH production by preventing Fe³⁺ reduction of H₂O₂ (17). Other investigators have shown that ·OH formation can be supported by other biological reductants of Fe³⁺. Ascorbate or glutathione are present at much higher concentrations than O₂⁻ in cells and can alternatively serve to reduce Fe³⁺ under physiological conditions (18). Thus, other reactions than those leading to ·OH formation may be important in understanding mechanisms of O₂⁻-dependent toxicity and the protective role of superoxide dismutase.

An important biological reaction of O₂⁻ may be with endo- thelial-derived NO, a free radical species. Macrophages and neutrophils are also capable of production and release of both O₂⁻ and ·NO (19, 20). The reaction between these two radical species is diffusion-limited in gaseous phase and is extremely rapid in aqueous phase, yielding peroxynitrite (ONOO⁻) (Ref. 21). Potentially toxic levels of peroxynitrite can be achieved in tissues under conditions when ·NO and O₂⁻ production are stimulated, because a 100-fold increase in the rate of peroxynitrite formation should occur for every 10-fold increase in ·NO and O₂⁻ concentration.

Peroxynitrite has a pKa of 6.8 at 37 °C. Peroxynitrous acid is unstable with a half-life of under 1.0 s (22), decomposing to give a species with hydroxyl radical-like reactivity, according to the following reaction.

H⁺ + O₂⁻ + ·NO → ONOOH → ·OH + NO₂ (3)
Peroxy nitrite acid was revealed as a highly reactive species generating a strong oxidant capable of oxidizing deoxyribose and dimethyl sulfoxide with high yields at acidic pH (22). Nitrogen dioxide is the other product of peroxynitrite decomposition and is also a strong oxidant with significant cytotoxic potential (23–25).

We investigated the reactions of peroxynitrite towards protein and nonprotein sulfhydryl groups and compared this with H₂O₂-mediated thiol oxidation. Sulfhydryls are common targets for free radicals, with sulfhydryl oxidation being a key mechanism of free radical-mediated toxicity at the molecular level (26). Thios are critical to the active site of many enzymes and for maintaining the native conformation of proteins (27). Herein, we report that peroxynitrite anion oxidizes sulfhydryls about 10⁴ times faster than does H₂O₂ at 37 °C and pH 7.4 and propose that this reaction may be an important mechanism of oxygen radical-mediated toxicity.

EXPERIMENTAL PROCEDURES

Materials—Fatty acid-free BSA¹ (fraction V), cysteine, sodium borohydride (NaBH₄), potassium cyanide (KC₅N), sodium arsenite (NaAsO₃), DTT (cCHOCH₂SH,₂), DTNB, pCMPS, N-ethylmaleimide (NEM), and DTPA were obtained from Sigma. Hydrogen peroxide was obtained from Fluka (Switzerland), catalase from Worthington, desferrioxamine from Ciba Geigy (Switzerland), and 2-mercaptoethanol from Fisher. All other reagents were of analytical grade.

Albumin Preparation—Bovine serum albumin was dissolved in 1.52 mM in 0.01 M potassium phosphate, pH 7.4, and dialyzed overnight in 10 mM 2-mercaptoethanol in the same buffer to reduce sulfhydryl oxidation during purification and storage. After 36-h dialysis with six changes of 0.01 M potassium phosphate, BSA typically had a sulfhydryl to albumin molar ratio of 0.6–0.7, in accordance with previous analyses of fresh plasma albumin (28). The thiol group of mercaptoethanol-treated BSA was chemically modified in three ways; 1.4 mM NaBH₄, or NEM, or HgCl₂ treatment left no residual sulfhydryls whereas H₂O₂ treatment gave a final SH/BSA ratio of 0.16. Protein was quantitated with a bicinchoninic acid assay (29).

Peroxy nitrite Synthesis—Peroxy nitrite was synthesized in a quenched flow reactor (30). Solutions of (a) 0.5 M NaNO₂ and (b) 0.6 M Na₂SO₃ were pumpeated at 26 mL/min into a 3-nm-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 T-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 Mg(OH)₂. The solution was frozen at −50 °C for as long as a week.

Peroxy nitrite forms a yellow top layer due to freezation fractionation, which was retained for further studies. This top layer typically contained 170–220 mM peroxynitrite as determined by absorbance at 302 nm (ε₃₀₂ = 1.670 M⁻¹ cm⁻¹; Ref. 31).

Sulfur Chemistry—Sulfhydryls were quantitated using DTNB at 412 nm (ε₄₁₂ = 1.36 × 10⁴ M⁻¹ cm⁻¹; Ref. 32). A 10 mM DTNB stock solution was prepared, and reactions were performed in 0.05 M potassium phosphate, 2 mM EDTA, pH 9.0, for 60 min at room temperature. In some experiments, pCMPS was used for determination of sulfhydryl content; pCMPS was prepared as a 2 mM stock solution, and sulfhydryl content was detected in 0.05 M Tris-HCl, pH 7.4, for 10 min at room temperature (ε₂₃₇ = 7,470 M⁻¹ cm⁻¹ for cysteine). Final DTNB and pCMPS concentrations used were 0.20 mM, always in excess over assay sulfhydryl concentration.

Sulfur-reducing agents, including sodium borohydride, sodium arsenite, potassium cyanide, and DTNB were used for reductive recovery of oxidized thiol groups. Oxidized DTT was measured by its absorbance at 283 nm (ε₂₈₃ = 273 M⁻¹ cm⁻¹; Ref. 33). The percentage of sulfhydryls recovered after oxidation by peroxynitrite or H₂O₂ was calculated from the ratio between the "recovered" thiol in the presence of the reductants versus the initial amount of oxidized thiol.

\[ % \text{ recovery} = \frac{S_1 - S_2}{S_1} \times 100 \]  

In Equation 4, \( S_1 \) and \( S_2 \) represent initial and final thiol concentrations, and \( S_1 \) is the thiol concentration after addition of reductant to oxidized samples.

Kinetic Experiments—Peroxynitrite decomposition in the presence of sulfhydryls was studied by stopped flow absorbance spectroscopy at 302 nm (Hi-Tech Instruments). The kinetics of peroxynitrite decomposition were fitted to a pseudo-first-order reaction equation by nonlinear regression. The nitrogen-containing species derived from peroxynitrite reaction (i.e. nitrite and nitrate) absorb at 340 nm and did not interfere with the 302 nm measurements. A typical run consisted of 500 points collected over 0.1–10 s, so that at least 99% of peroxynitrite disappeared.

Thiol oxidation by H₂O₂ was determined by taking timed aliquots for sulfhydryl content analysis.

RESULTS

Peroxynitrite Reaction with Sulfhydryls—In the presence of a 20–100-fold excess of cysteine, the disappearance of peroxynitrite followed pseudo-first-order kinetics when monitored at 302 nm by stopped flow spectroscopy (Fig. 1). Under these conditions, the spontaneous decomposition of peroxynitrite appeared as a small non-zero intercept (Fig. 1b). Cysteine and cystine do not absorb significantly at 302 nm, thus not interfering with peroxynitrite quantitation. The pH dependence of the second-order rate constant (Fig. 2) suggests that cysteine reacts rapidly with peroxynitrite anion. The reaction can be described by

\[ k' = k \left( \frac{K_c}{[H^+] + [H^+] + [H^+]} \right) \]  

where \( k' \) is the apparent rate constant at a given pH, \( k \) is the second-order rate constant of cysteine reaction with peroxynitrite anion, \( K_c \) is the dissociation constant of peroxynitrite and \( K_{cysteine} \) is the dissociation constant of cysteine to the thiolate anion. The best fit of the data in Fig. 2 gives \( k = 5,900 \pm 160 M^{-1} \cdot s^{-1} \) with \( pK_{cysteine} = 6.6 \) and \( pK_{cysteine} = 8.3 \) The \( pK_{cysteine} \) of peroxynitrite at 37 °C is 6.8.¹ The \( pK_{cysteine} \) of 7.5 at 37 °C reported previously for peroxynitrite (22) was in error, due to instrument problems. The apparent \( pK_{cysteine} \) of 8.3 corresponds to the ionization of cysteine.

The overall apparent rate constant for BSA reacting with peroxynitrite was 5,020 ± 480 M⁻¹ · s⁻¹ (mean ± S.D., \( n = 7 \)) at pH 7.4, which includes reaction of peroxynitrite with all

¹ The abbreviations used are: BSA, bovine serum albumin; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); pCMPS, p-chloromercuriphenylsulfonic acid; DTPA, diethylenetriamnepentacetic acid; DTT, dithiothreitol; NEM, N-ethylmaleimide.

² R. Radi, J. S. Beckman, K. M. Bush, and B. A. Freeman, unpublished data.
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The rate constant of BSA sulfhydryl reaction with peroxynitrite was also estimated by reaction of BSA with limiting amounts of peroxynitrite and assessment of residual BSA sulfhydryl content (Fig. 3). Under these conditions, the rate of peroxynitrite decomposition may be written as

$$\frac{d[ONOO^-]}{dt} = -k_{sp}[SH][ONOO^-] - k_1[ONOO^-]$$  (7)

where [SH] is the concentration of the sulfhydryl compound, $k_{sp}$ is the second-order rate constant between peroxynitrite and SH, and $k_1$ is the sum of all peroxynitrite decomposition processes, which include spontaneous decomposition and other reactions with BSA. Thus, $k_1$ implicitly depends upon BSA concentration. Because only a small amount of SH is consumed in this reaction, the integral of Equation 7 can be approximated by

$$[ONOO^-] = [ONOO^-]_o e^{-k_{sp}[SH]o+k_1t}$$

where [SH]$o$ and [ONOO]$^-o$ are the initial concentrations of SH and peroxynitrite. The rate of SH oxidation is expressed as follows.

$$\frac{d[SH]}{dt} = -k_{sp}[SH][ONOO^-]$$

If Equation 7 is divided by [SH], it may be integrated to give Equation 10.

$$\int_0^t \frac{d[SH]}{[SH]} = \ln \left[ \frac{[SH]}{[SH]_o} \right] = -k_{sp} \int_0^t [ONOO^-]dt$$

Substituting Equation 8 into 10 gives Equation 11.

$$\ln \left[ \frac{[SH]}{[SH]_o} \right] = -k_{sp} \ln \left[ \frac{[ONOO^-]}{[ONOO^-]_o} \right] e^{-k_{sp}[SH]o+k_1t}$$

As time increases to infinity, Equation 11 reduces to Equation 12.

$$\frac{\ln [SH]}{[SH]_o} = \frac{-k_{sp}}{k_{sp}[SH]o + k_1}$$

The left-hand expression can be determined as the slope from Fig. 3, which was $-1,490 \text{ M}^{-1} \text{s}^{-1}$ for 0.340 mM BSA sulfhydryl.

Fig. 1. Peroxynitrite anion decomposition in the presence of cysteine. a, time course of peroxynitrite decomposition. Peroxynitrite (0.60 mM) was added to a reaction medium containing 0, 2.5, 5.0, 15, or 22.5 mM cysteine, in 50 mM potassium phosphate, pH 7.5, at 37 °C. $P_0$ is the initial peroxynitrite concentration, and $P$ is its concentration at a given time. b, pseudo-first-order rate constants for peroxynitrite decomposition obtained from Fig. 1a are shown as a function of cysteine concentration. An apparent second-order rate constant was determined from the slope of the plot. Each data point represents an average of three to eight determinations.

Fig. 2. Rate constants for the reaction of peroxynitrite with cysteine as a function of pH. Peroxynitrite (0.60 mM) was added to 37.5 mM cysteine in 50 mM potassium phosphate at different pH values at 37 °C. Apparent second-order rate constants were obtained as described in the legend to Fig. 1. Data have been corrected for spontaneous decomposition of peroxynitrite.

Fig. 3. BSA sulfhydryl oxidation by peroxynitrite. Reactions were started by addition of peroxynitrite to 0.34 mM BSA sulfhydryl ([BSA] = 0.52 mM, SH/BSA = 0.65) in the presence (○) or absence (□) of 10 mM DTPA in 0.05 M potassium phosphate, pH 7.2. Reactions were incubated for 5 min at 37 °C and immediately assayed for sulfhydryl content. $S_o$ is the initial sulfhydryl concentration, and $S$ is its final concentration after reaction with peroxynitrite. Data are the mean of two different analyses from a representative experiment.
The term $k_d$ was taken to be $0.911 \pm 0.059$ by averaging the three estimates of $0.898 \pm 0.089$, $0.861 \pm 0.036$, and $0.977 \pm 0.107$ s$^{-1}$ from stopped flow measurements of peroxy nitrite disappearance at 302 nm using the same concentration of BSA after sulfhydryl reaction with H$_2$O$_2$, NEM, or HgCl$_2$. Substituting these values and rearranging Equation 14 to solve $k_{sp}$ gave a rate constant of $2.800 \pm 180$ M$^{-1}$s$^{-1}$. Thus, two separate methods for approximating the rate constant of peroxy nitrite reaction with the BSA sulfhydryl gives estimates that are about 50% of values observed with free cysteine.

Effects of Metal Chelators—Desferrioxamine was a concentration-dependent inhibitor of dimethyl sulfoxide and deoxyribose oxidation by peroxy nitrous acid (22). In contrast, the metal chelator DTPA had no effect on the extent of sulfhydryl oxidation by peroxy nitrite (Fig. 3). Desferrioxamine tested in a broad concentration range (25–500 μM) at pH 7.3 and 8.0 also had no significant influence on sulfhydryl oxidation (data not shown).

Oxidative Yield and pH—Sulfhydryl oxidation of BSA or cysteine by peroxy nitrite occurred at acidic pH, but was greater at alkaline pH, having a 50% oxidation yield at pH 6.84 ± 0.09 (Fig. 4, ○) for BSA and pH 7.02 ± 0.06 (Fig. 4, ▲) for cysteine at 37 °C. Under our experimental conditions, the extent of sulfhydryl oxidation was about 3–4 times greater for cysteine than for BSA. Cysteine, in a 10-fold excess over peroxy nitrite (2.5–0.25 mM) at pH 9.0 (thus making spontaneous decomposition insignificant), was oxidized in a ratio of 2.2 ± 0.1 (mean ± S.D., n = 3) mol of cysteine/mol of peroxy nitrite.

Reaction with H$_2$O$_2$—The reaction of H$_2$O$_2$ with BSA-SH or cysteine obeyed a second-order rate law ($r > 0.99$), giving apparent rate constants of 1.14 ± 0.03 M$^{-1}$s$^{-1}$ for BSA-SH and 4.69 ± 0.06 M$^{-1}$s$^{-1}$ for cysteine at 37 °C and pH 7.4 (Fig. 5). Reaction rates were greater at alkaline pH (Fig. 6). Fitting the results to the Henderson-Hasselbalch equation gave $pK_a$ values of 8.00 ± 0.03 for the reaction of H$_2$O$_2$ with BSA-SH and 8.04 ± 0.07 for the reaction of H$_2$O$_2$ with cysteine. Since H$_2$O$_2$ has a $pK_a$ of 11.8 (34), the pH dependence of sulfhydryl oxidation was more closely related to the dissociation of the thiol group to the thiolate anion (35).
cysteine reacted 2-4 times faster with H₂O₂ compared with the reaction of BSA-SH with H₂O₂. During cysteine oxidation to cystine, it has been assumed by us and others (35) that the rate-limiting reaction is the formation of the cysteine sulfenic acid intermediate.

**UV Difference Spectroscopy and pK₀ Determination of the BSA Sulfhydryl—**Ultrasound difference spectroscopic analysis of BSA at pH 8.0 versus pH 5.0 absorbs at 230-240 nm, indicative of thiolate anion (Fig. 7). At pH < 7.0, oxidized BSA absorbed more strongly than native BSA, whereas at pH > 9.0 the absolute value of the spectroscopic difference decreases. Thus, other substituent groups or conformational changes of BSA are also involved in 230-240 nm absorbance. We determined an ε₉₅₀ = 5,340 M⁻¹ cm⁻¹ for the thiolate anion of BSA, similar to previously reported extinction coefficients for other protein and nonprotein thiol anion species (36).

Titration of BSA above pH 7.5 increased absorbance of the thiolate anion, maximal at pH 8.0-8.5 (Fig. 7). The 50% increase of absorbance at pH 7.86, close to the pK₀ of the reaction of BSA-SH with H₂O₂, supports the role of BSA thiol in the sulfhydryl species reactive with ONOO⁻.

**Thiol Oxidation State—**Arsenite, cyanide, and diethiothreitol-mediated reduction of oxidized thiols permitted determination of the reversibility of oxidation processes and aided in identification of thiol oxidation states (Fig. 8). Arsenite reduces sulfenic acid (RSOH) to sulfhydryl (RSH) but does not reduce disulfides (RSSR; Ref. 37). Cyanide and DTT reduce disulfides to thiols with different reaction stoichiometries.

RSH + CN⁻ → R⁻ + SCN⁻

RSH + (CHOH-CH₂-SH)₂ → 2RSSR

One mole of cyanide and DTT recovers 1 and 2 mol of RSH from disulfide, respectively. Under our assay conditions, NaBH₄-reduced BSA had a sulfhydryl to BSA ratio of 1.0 in control samples, suggesting total reduction of the single cysteine sulfur of BSA not participating in intramolecular disulfide formation. Higher NaBH₄ concentrations (=25-fold greater) were used by others for reduction of constitutive disulfides of BSA (38).

**Peroxynitrite-mediated BSA-SH oxidation gave a product**

**Fig. 7. Spectroscopic assessment of BSA thiolate anion.** UV difference spectra were recorded with 20 μM BSA concentration at 25°C with a 0.5-nm bandwidth and 1-cm path length. Hydrogen peroxide-oxidized BSA (SH/BSA = 0.16, upper curve) was dissolved in 0.1 M potassium phosphate, pH 8.5. Nonoxidized BSA (SH/BSA = 0.71, lower curve) was dissolved in 0.1 M potassium phosphate, pH 8.5. For spectroscopic reference, a nonoxidized BSA solution was dissolved in 0.1 M potassium phosphate, pH 5.0. An 11.6 μM SH concentration decrease for H₂O₂-oxidized BSA was accompanied by a decrease of 0.0619 absorbance units at 237 nm. The inset shows the spectroscopic titration of BSA-SH, with data representing the absorption differences at 237 nm between nonoxidized and oxidized BSA at different pH values.

**DISCUSSION**

Peroxynitrite-mediated oxidation of both nonprotein and protein sulphydryls. The higher yields of sulphydryl oxidation at alkaline pH indicate that peroxynitrite anion, rather than peroxynitrous acid, was the primary oxidizing species. In the acidic pH range, some oxidation still occurred, most resistant to arsenite reduction, whereas NaBH₄ reduction of oxidized BSA thiol was 75% (Table I). In contrast, 52% of H₂O₂-mediated BSA-SH oxidation was reduced by arsenite, suggesting a significant amount of sulfenic acid formation (Table I). Sodium borohydride similarly reduced 90% of the H₂O₂-oxidized BSA sulphydryls (Table I). Electrophoretic analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed that neither peroxynitrite nor H₂O₂ resulted in disulfide-mediated dimerization or peptide cleavage of BSA at pH 7.4 (data not shown). Intramolecular disulfide formation is not possible since BSA has only one thiol.

Peroxynitrite-dependent cysteine oxidation was 90% cyanide- and DTT-reducible, with arsenite having no effect. Hydrogen peroxide-mediated cysteine oxidation was 64% cyanide-reducible and 48% DTT-reducible; arsenite had no significant effect (Tables II and III). Cysteine thiols were measured using pCMPS, to avoid artifact from cyanide-dependent-DTNB reduction. Similar results were obtained when DTNB was used in place of pCMPS for quantitating arsenite reduction of cysteine oxidation products.
likely due to decomposition of peroxynitrous acid. This process would produce strong oxidants with reactivity like OH· and nitrogen dioxide that can attack sulphydryls. The pH dependence of the reaction rates of peroxynitrite anion towards thiols (Fig. 2) indicates that peroxynitrite anion reacts preferentially with the undissociated form of the thiol group (SH). At pH values above the pH values of the thiol, oxidation yields are still high (Fig. 4) because the spontaneous decomposition of peroxynitrite is slower than at acidic pH. Thiolate anion species are usually more susceptible to oxidation (37), but this would not be expected for peroxynitrite anion due to electrostatic repulsion between the two negatively charged species.

When H₂O₂ was the oxidant, the pH dependence of sulphydryl oxidation followed thiolate anion dissociation (H₂O₂ pKₐ = 10.8). The pH values of the cysteine sulphydryl at 25 °C is 8.3–8.5 (39), and the heat of ionization of SH groups is about 7 cal/mole. Thus, the pKₐ should decrease by 0.2 when temperature increases from 25 to 37 °C. Thus, a pKₐ for cysteine of 8.10–8.30 at 37 °C is close to the pKₐ value of 8.04 reported herein, confirming that the thiolate anion was the reactive sulfur species in H₂O₂-mediated oxidation reactions.

The sulphydryl of BSA had a pKₐ of 7.86–8.00, according to both spectroscopic and oxidation kinetics data. The pKₐ of protein sulphydryls may differ significantly from the pKₐ of free cysteine sulphydryls, depending upon the sulphydryl microenvironment (27). Nevertheless, the pKₐ of BSA-SH was similar to cysteine. The reactivity of peroxynitrite and H₂O₂ towards BSA-SH was probably less than that of cysteine due to steric restrictions.

Peroxynitrite is more reactive with sulphydryls than H₂O₂. According to the effective second-order rate constants, we observed that peroxynitrite reacted about 2,600 times faster towards BSA-SH and about 1,200 times faster towards cysteine than did H₂O₂ at 37 °C, pH 7.4 (Figs. 1 and 5). The rate constant determined for the reaction of H₂O₂ with cysteine agrees with that previously reported, ranging between 10 and 13 M⁻¹·s⁻¹ at 25 °C (35).

Sulphydryl oxidation analysis after reaction of H₂O₂ and peroxynitrite showed different products. Electrophoretic analysis showed that intermolecular disulfide bond formation by BSA after peroxynitrite or H₂O₂ oxidation did not occur. Steric restrictions due to the size of the albumin molecule or the location of the single albumin thiol preclude disulfide formation, only occurring with denaturing conditions (40). Sterically isolated SH groups can be oxidized beyond the disulfide, with at least 52% of the BSA-SH oxidized to sulfenic acid (RSOH) by H₂O₂, and the remainder of the sulphydryl groups further oxidized to possibly sulfenic (RSO₂H) or sulfonic (RSO₃⁻) acid (Table I). In agreement with our observations, the BSA-SH is oxidized by iodoine or thiocyanogen to sulfenyl iodide or sulfenyl thiocyanate, respectively, oxidation states equivalent to sulfenic acid (37). Under stronger H₂O₂-mediated oxidizing conditions, no sulfenic acid was detectable (41). Peroxynitrite did not oxidize BSA-SH to sulfenic acid, but a high proportion of peroxynitrite-oxidized sulphydryls were borohydride-reducible. Apart from the eventual formation of sulfonic or sulfenic acid after peroxynitrite oxidation, other products of the type RSNO₂ may have been formed, many of which are unstable in H₂O. For example, the oxidation of glutathione by tetranitromethane (C(NO₂)₄) formed a sulfenic acid derivative, with sulfenyl nitrate formed (RSNO₂) as a transient intermediate (42). Such an intermediate could be stabilized in an appropriate protein microenvironment.

Cysteine was oxidized with an excess of either H₂O₂ or peroxynitrite to predominantly the disulfide form, with no formation of sulfenic acid. The main product of H₂O₂ oxidation of cysteine beyond the disulfide is cysteic (sulfonic) acid (37). Peroxynitrite gave a >90% yield of disulfide from cysteine.
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teine (Table III), similar to that observed for the oxidation of thiols by - NO and NO₂ (25).

Transition metals did not have a significant role in peroxynitrite-mediated sulphydryl oxidation, since neither DTPA nor desferrioxamine modified oxidation yields (Fig. 3). Desferrioxamine, but not DTPA, is a strong inhibitor of peroxynitrite and thiol-mediated oxidation of sulfhydryls and dimethyl sulfoxide in a manner unrelated to its iron-chelating ability (22). No significant effect of desferrioxamine was detected in our system, supporting the concept that desferrioxamine reacts preferentially with peroxynitrite anion rather than with peroxynitrite.

Peroxynitrite-mediated oxidation of sulphydryls has significant cytotoxic potential. First, oxidation of low molecular weight sulphydryls (i.e. cysteine and glutathione) leads to depletion of one of the most important intra- and extracellular scavenging mechanisms serving as a defense against free radical-mediated damage. This increases the possibility of alteration and destruction of critical macromolecules such as DNA, enzymes, structural proteins, structural polysaccharides, and membrane phospholipids. Second, oxidation of protein sulphydryls (i.e. containing thiol-containing amino acids (i.e. cysteine and glutathione) leads to almost diffusion-controlled rates.

The bolus concentrations of peroxynitrite used in the present study are relatively high, compared to steady-state concentrations of peroxynitrite that might occur in vivo. Nevertheless, if one considers dosage in terms of time X concentration, bolus addition of 100 μM peroxynitrite (decaying only by proton-catalyzed decomposition) would be equivalent to a physiologically relevant steady-state concentration of 2.8 μM for 1 min.

Superoxide-derived peroxynitrite is highly reactive toward sulphydryls, unlike superoxide-derived H₂O₂. These differential reactivities of peroxynitrite and H₂O₂ may well extend to other biochemical targets and provide new understanding of mechanisms of O₂⁻-mediated toxicity.

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