Peroxynitrite is one of the biological oxidants whose addition to cells has been shown to either activate signaling pathways or lead to cell injury, depending on cell type and oxidant concentration. The intermediacy of free radicals in these processes has been directly demonstrated only during the interaction of peroxynitrite with erythrocytes, a particular cell type, due to its high hemoglobin content. Here, we demonstrate that the addition of peroxynitrite to a macrophage cell line (J774) led to the production of glutathionyl and protein-tyrosyl radicals. The glutathionyl radical was characterized by EPR spin-trapping experiments with 5,5-dimethyl-1-pyrroline-N-oxide. Protein-tyrosyl radical formation was suggested by direct EPR spectroscopy and confirmed by EPR spin-trapping experiments with 3,5-dibromo-4-nitrosobenzenesulfonic acid and Western blot analysis of nitrosated proteins in treated macrophages. Time dependence studies of free radical formation indicate that intracellular glutathione and unidentified proteins are the initial peroxynitrite targets in macrophages and that their derived radicals trigger radical chain reactions. The results are likely to be relevant to the understanding of the bioregulatory and biodamaging effects of peroxynitrite.

**Peroxynitrite** (ONOO⁻ + ONOOH),¹ which is formed by the fast reaction between nitric oxide and superoxide anion, has been receiving increasing attention as a mediator of human diseases and as a toxin against invading microorganisms (1–5). The compound is a strong oxidant that is able to oxidize and nitrate a variety of biotargets by mechanisms that are presently being elucidated (6–16). Peroxynitrite-mediated oxidations are either bimolecular, first-order on peroxynitrite and target concentration or unimolecular, first order on peroxynitrite and independent of target concentration. Bimolecular processes can result in product yield either around stoichiometry and above, as is the case for thiol oxidation (17), or around 35%, as is the case for carbon dioxide oxidation (10, 14–16). Presently, most investigators accept that product yields around 30% are characteristic of peroxynitrite-mediated free radical processes. Indeed, it has been established that peroxynitrite protonation (pKₐ = 6.6) leads to its fast decomposition (k = 0.17 s⁻¹ and τ₁ = 4.1 s at pH 7.4, 25 °C) to yield ~70% nitrate and 30% hydroxyl radical and nitrogen dioxide (Reactions 1 and 2) (7–9, 11–14). These radicals are the species responsible for peroxynitrite-mediated unimolecular oxidations.

\[
\text{ONOO}^- + \text{H}^+ \rightleftharpoons \text{ONOOH}
\]

\[
\text{ONOOH} \rightarrow 0.7 \text{NO}_3^- + 0.7 \text{H}^+ + 0.3 \text`\text{NO}_2^- + 0.3 \text`\text{OH}
\]

**REACTIONS 1 AND 2**

In biological systems, the half-life of peroxynitrite is expected to be much lower because of its reactions with biotargets, particularly carbon dioxide, hemoproteins, and thiol-containing compounds, all of which react fast with the oxidant in bimolecular reactions (2, 18, 19). The reaction with the biologically ubiquitous carbon dioxide is particularly fast (k = 2.6 × 10¹⁴ M⁻¹ s⁻¹ at pH 7.4, 25 °C) and produces ~65% nitrate and 35% carbonate radical anion and nitrogen dioxide (Reaction 3) (10, 14–16, 18, 19). Relevantly, this reaction diverts biotarget oxidation by peroxynitrite at neutral pH values from two- to one-electron mechanisms (20).

\[
\text{ONOO}^- + \text{CO}_2 \rightarrow [\text{ONOOCO}_2^-] \rightarrow \text{0.65 NO}_3^- + 0.65 \text{CO}_2 + 0.35 \text`\text{NO}_2^- + 0.35 \text`\text{CO}_3^-
\]

**REACTION 3**

Peroxynitrite is capable of diffusing across biological membranes (21–24), a property that allows the interaction of extracellularly generated peroxynitrite with intracellular targets. There are many studies demonstrating that the addition of exogenous peroxynitrite to cells and cell cultures triggers oxidative events that either activate signaling pathways (see, for instance, Refs. 25–28) or lead to cell injury (see, for instance, Refs. 29 and 30), depending on cell type and oxidant concentration. The intermediacy of free radicals in these processes has been directly demonstrated only during the interaction of peroxynitrite with red blood cells, a particular cell type, because of its high hemoglobin content (31). Accordingly, a long lived hemoglobin-tyrosyl radical has been detected by EPR in incubations of peroxynitrite with erythrocytes (31). Free radical formation during the interaction of peroxynitrite with other cell types has not been examined. Here, we demonstrate that

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† The abbreviations used are: peroxynitrite, the sum of peroxynitrite anion (ONOO⁻, oxoperoxonitrate (−1)) and peroxynitric acid (ONOOH, hydrogen oxoperoxonitrate) unless specified; DBNBS, 3,5-dibromo-4-nitrosobenzenesulfonic acid; DMPO, 5,5-dimethyl-1-pyrroline-N-oxide; PBS, phosphate-buffered saline.
peroxy nitrite addition to a macrophage cell line (J774) led to the EPR detection of glutathionyl and protein-tyrosyl radicals.

EXPERIMENTAL PROCEDURES

Chemicals—All reagents were purchased from Sigma, Merck, or Fisher and were analytical grade or better. Peroxynitrite was synthesized from sodium nitrite (0.6 M) and hydrogen peroxide (0.65 M) in a quenched-flow reactor. To eliminate excess hydrogen peroxide, the peroxy nitrite solution was treated with manganese dioxide. Synthesized peroxy nitrite contained low levels of contaminating hydrogen peroxide (<1%) and nitrite (10–30%) that were determined as previously described (32) by the titanyl method and by absorbance measurements at 354 nm (ε = 24.6 M\(^{-1}\) cm\(^{-1}\)), respectively. The concentration of peroxy nitrite stock solutions was determined spectrophotometrically at 302 nm using an extinction coefficient of 1.67 × 10\(^{4}\) M\(^{-1}\) cm\(^{-1}\). Concentrations of CO\(_2\) were calculated from the added HCO\(_3\)\(^-\) (1%).

Cell Cultures—The mouse macrophage cell line J774 (33) was routinely grown in Dulbecco’s modified Eagle’s medium supplemented with 100 to remove contaminant metal ions. All solutions were prepared with distilled water purified with a Millipore Milli-Q system.

Cell suspensions in modified PBS (1×10\(^8\) cells/ml) in modified PBS (0.14 M NaCl, 50 mM Na\(_2\)HPO\(_4\), 0.4 mM KH\(_2\)PO\(_4\), 0.39 mM MgCl\(_2\), and 0.9 mM CaCl\(_2\)) and then treated with spin traps and peroxy nitrite at room temperature (25 ± 2 °C). In some experiments, cell cultures were pretreated for 24 h with 100 mM BSO before being collected (34).

Cell Viability—Cell suspensions in modified PBS (1×10\(^6\) cells/ml) were treated with peroxynitrite and corresponding controls. After a 10-min incubation at room temperature (25 ± 2 °C), they were diluted 1:1 (v/v) with a trypsin blue solution, and cell viability was determined from hemocytometer counts after nucleus staining. The percentage of lysed cells was determined by comparing the total number of viable plus nonviable cells with the cell number in the initial suspension.

Soluble Thiols—Total soluble thiol (protein and nonprotein) was measured (35) to prevent protein precipitation at low pH values (36), which produces nitric oxide from nitrite leading to thiol depletion as confirmed by control experiments. Acid precipitation was used only to determine total nonprotein thiol (mostly GSH) in untreated cell suspensions. Detergents were not used because of the high cell density and the difficulties in solubilizing cell nuclei. Cell suspensions before and after treatment with peroxynitrite were mixed 1:2:1 (v/v/v) with 1 M phosphate buffer, pH 7.4, and 5 mM DTNB. After 20 min, the suspensions were centrifuged at 18,000 rpm for 5 min. Total soluble thiol in the supernatant was determined by absorbance measurements at 412 nm (ε = 13.6 × 10\(^3\) M\(^{-1}\) cm\(^{-1}\)) (35, 36).

RESULTS

Detection of Glutathionyl Radical—The addition of 1 mM peroxynitrite to a macrophage suspension (1 × 10\(^6\) cells/ml) in modified PBS containing 50 mM DMPO led to the early (1-min incubation) detection of an EPR composite spectrum of two radical adducts (Fig. 1A). These adducts were identified by computer simulation of the experimental spectrum as the...
Table I

Effects of peroxynitrite on macrophage viability and soluble thiol depletion

| Macrophage treatment | Viability | Soluble thiol (nmol/10⁶ cells) |
|----------------------|-----------|-------------------------------|
| PBS                  | 92        | 6.9 ± 0.5                     |
| 1 mM decomposed ONOO⁻ | NM        | 6.5 ± 0.6                     |
| 0.25 mM ONOO⁻       | 57        | 5.1 ± 0.6                     |
| 0.5 mM ONOO⁻        | 78        | 4.6 ± 0.5                     |
| 1.0 mM ONOO⁻        | 85        | 3.4 ± 0.2                     |

Fig. 1. Representative EPR spectra of DMPO adducts obtained in macrophage suspensions (J774), cell pellet and lysed cells after peroxynitrite addition. The spectra were obtained 7 min after the addition of 1 mM peroxynitrite to macrophage (1 × 10⁶ cells/ml) suspensions in modified PBS, pH 7.4, at room temperature. A, macrophage suspension; B, resuspended pellet of peroxynitrite-treated macrophages; C, macrophage lysed before peroxynitrite addition. Instrumental conditions were as follows: microwave power, 20 milliwatts; time constant, 327.7 ms; scan rate, 0.3 G/s; modulation amplitude, 5 G; gain, 3.99 × 10⁶.

DMPO/GSH (α_N = 15.2 G; α_H = 15.9 G) and DMPO/OH (α_N = 14.9 G; α_H = 14.9 G) (38) radical adducts in relative yields of 76 and 24%, respectively (Fig. 1D). These relative yields varied some in different experiments, but the DMPO/GSH radical adduct was always the main spectrum component. The EPR signal of the DMPO/GSH adduct was detectable with peroxynitrite concentrations of ≥0.25 mM and cell numbers of ≥5 × 10⁶ cells/ml, but the signals were less intense than those shown in Fig. 1 (data not shown). The initial EPR signal decayed fast, and after 7–10 min it was dominated by the DMPO/GSH spectrum (Fig. 1, E and H). No EPR signal was detected in the absence of macrophages (data not shown) or upon the addition of previously decomposed peroxynitrite to macrophages (Fig. 1, C and G). Depletion of macrophage GSH levels (>80%) by pretreatment with 100 μM BSO for 24 h (34) led to a much less intense EPR signal that was dominated by the DMPO/OH radical adduct spectrum (Fig. 1, B and F).

The above results suggest that intracellular glutathione is an important peroxynitrite target in macrophages. Accordingly, peroxynitrite led to a concentration-dependent depletion of macrophage soluble thiol but had marginal effects upon cell viability in the time scale of the experiments (Table I). Total soluble thiol (protein and nonprotein) was measured to prevent protein precipitation at low pH values, which produces nitric oxide from nitrite (39), leading to thiol depletion as confirmed by control experiments. Nitrite is a peroxynitrite decomposition product at neutral pH values and a contaminant of peroxynitrite synthesis (40, 41). In these control experiments, total nonprotein thiol content of macrophages (mostly reduced glutathione) was determined to be 4.5 ± 0.6 nmol/10⁶ cells. Cell viability and cell lysis were marginally affected by peroxynitrite, but some cell lysis (−20–30%, depending on incubation time) occurred in modified PBS (Table I). Cell lysis was minimized by the addition of 5 mM glucose or by the use of Dulbecco’s modified Eagle’s medium, but these media alone treated with peroxynitrite produced radicals (data not shown) and could not be used in the EPR experiments. Consequently, it became important to obtain further evidence that peroxynitrite was reacting with intracellular GSH under our experimental conditions. Cell suspensions were then treated with peroxynitrite, centrifuged, resuspended in PBS, and examined by EPR. As shown in Fig. 2, the EPR signal detected in the resuspended pellet 7 min after treatment (Fig. 2B) was similar to the one detectable in the whole suspension (Fig. 2A), indicating that most of the radical adducts were produced inside the cells. When the cells were first lysed by 30-min incubation with 1 mM phosphate buffer and then treated with peroxynitrite, they showed an intense spectrum dominated by the DMPO/OH adduct (Fig. 2C). This result is similar to those previously reported in incubations of glutathione solutions with peroxynitrite in the presence of DMPO (6). In this case, DMPO/OH is produced by trapping of the hydroxyl radical produced during spontaneous peroxynitrite decomposition and by the decay of the DMPO-OOH adduct. The latter is produced from the superoxide anion resulting from a chain reaction whereby the radicals produced from peroxynitrite (hydroxyl radical and nitrogen dioxide) oxidize glutathione to the glutathionyl radical (Reactions 2, 4, and 5) (6). The latter reacts with excess glutathione, producing the glutathione disulfide radi-
Free Radicals in Peroxynitrite-treated Macrophages

Fig. 4. Representative EPR spectra of DBNBS adducts obtained during the interaction of macrophages (J774) with peroxynitrite. The spectra were obtained after the addition of 1 mM peroxynitrite to a macrophage (1 × 10^6 cells/ml) suspension in modified PBS containing 20 mM DBNBS, pH 7.4, at room temperature. A, control incubation after 1 min; B, same as A in the presence of 1 mM CO_2; C, macrophages treated with DBNBS after 1 min; D, same as A after 7-min incubation; E, same as B after 7-min incubation; F, same as C after 7-min incubation. The composite spectrum of A is labeled to show its components: immobile DBNBS/tyrosyl-protein (2g_\text{||} = 58.60 G) (●), and isotropic DBNBS/tyrosyl-protein (g_\text{||} = 13.51 G) (○) radical adducts. D, the appearance of hyperfine structures is labeled as ×. Instrumental conditions were as follows: microwave power, 20 milliwatts; time constant, 327.7 ms; scan rate, 0.3 G/s; modulation amplitude, 2.5 G; gain, 1.0 × 10^6.

Fig. 5. Representative Western blot analysis of tyrosine nitration in macrophages treated with peroxynitrite. Protein extracts isolated from macrophage suspensions (1 × 10^6 cells/ml) treated with peroxynitrite were subjected to nitrotyrosine immunoprecipitation followed by nitrotyrosine Western blot analysis with an anti-nitrotyrosine antibody as described under “Experimental Procedures.” Lane 1, bovine serum albumin (negative control); lane 2, nitrated bovine serum albumin (positive control); lane 3, molecular weight marker; lane 4, macrophages treated with PBS; lane 5, macrophages treated with 1.0 mM decomposed peroxynitrite (reverse addition); lane 6, macrophages treated with 0.5 mM peroxynitrite; lane 7, macrophages treated with 1.0 mM peroxynitrite. The strong band at 54 kDa corresponds to the antibody used in the immunoprecipitation.

Indeed, we have previously demonstrated that superoxide dismutase inhibits by about 40% the yield of the DMPO-OH adduct produced in incubations of glutathione solutions with peroxynitrite (6). In the case of macrophage suspensions where most glutathione is inside the cells, the dominant adduct was always DMPO/SG (Figs. 1 and 2), and its yield was little affected by the presence of superoxide dismutase (data not shown). Moreover, the presence of 1 mM carbon dioxide that reacts fast with peroxynitrite, greatly diminishing its half-life (from 4.1 to 0.027 s at pH 7.4, 25 °C) and its opportunity to permeate cell membranes, strongly inhibited the DMPO/SG radical adduct yield (data not shown). It should be emphasized that CO_2 increases thyl radical production from the oxidation of thiol solutions by peroxynitrite at neutral pH values due to the formation of carbonate radical anion and nitrogen dioxide (Reaction 3) (15, 20). Peroxynitrite and nitrogen dioxide can permeate biological membranes, but the carbonate radical anion cannot (23, 24). In agreement, exogenous CO_2 inhibited DMPO/SG adduct yield by about 40% (data not shown). In this case, DMPO/OH is likely to be produced by the reaction of the impermeable carbonate radical anion with extracellular DMPO (42).

Detection of Protein-tyrosyl Radical(s)—The addition of 1 mM peroxynitrite to a macrophage suspension (1 × 10^6 cells/ml) in PBS in the absence of DMPO did not produce EPR signals detectable with a modulation amplitude of 1 G, which is the instrumental parameter usually employed for the detection of spin trap radical adducts. Using modulation amplitudes of 5 G and over, it was possible to clearly detect a one-line EPR signal in the absence of DMPO (Fig. 3). No signal was detected in the absence of peroxynitrite or macrophages or in reverse addition experiments (data not shown). There are very few biomolecule-derived radicals that can be detected by direct EPR in aerobic solutions at room temperature. Among the exceptions are protein-bound tyrosyl (31, 43) and protein-bound semiquinone radicals (44). The latter has EPR parameters close to those of the one-line signal detected in peroxynitrite-treated macrophages (g = 2.005; line width = 7.4 G) (Fig. 3A). Most of reported protein-tyrosyl radicals have g values around 2.004 and line widths higher than 20 G (45), although one-line protein signals of about 10 G have been attributed to protein-tyrosyl radicals by parallel spin trapping experiments with nitroso compounds (31). Likewise, in the presence of 20 mM DNBS, the one-line signal detected in macrophages treated with peroxynitrite was
replaced by an EPR signal that is apparently composed of those of an immobilized (2α_{DBNBS} = 58.60 G) and a relatively isotropic radical adduct signal (α_{N} = 13.51 G) (Fig. 4A). Both of these adducts have EPR parameters that are consistent with DBNBS/tyrosyl radical adducts (31, 43). The presence of two radical adducts was also suggested by the fact that the mobile signal decreased with incubation time, whereas the immobilized signal increased (Fig. 4D). This increase indicates the occurrence of radical chain reactions (also see below), because the added peroxynitrite should have decomposed in less than 1 min (t_{1/2} = 4.1 s in the absence of cells). The presence of two major radical adducts was also evidenced by the effects of CO_{2} clearly inhibiting the isotropic signal intensity and having a minor influence on the immobilized radical adduct (Fig. 4D). Of note, CO_{2} also decreased the yield of the one-line signal detected by direct EPR (Fig. 3). The DBNBS/tyrosyl radical adducts were not detectable in control experiments, although other, less intense, EPR signals were observed. Incubation of DBNBS with macrophages led to the detection of a highly immobilized signal that results from DBNBS addition to biomolecules such as lipids and proteins (Fig. 4, C and F) (46). However, this signal intensity was too low to become a considerable contribution to the spectra obtained in the presence of peroxynitrite on the time scale of our experiments (Fig. 4). The addition of peroxynitrite to DBNBS alone produced an isotropic radical adduct (α_{N} = 12.60 G) (data not shown) that has been previously attributed to the DBNBS/OH radical adduct (47).

The above results (Figs. 3 and 4) demonstrate that, together with glutathione (Figs. 1 and 2), protein-tyrosine residues are important peroxynitrite targets in macrophages. The EPR results, however, cannot provide information on the proteins that are oxidized. Both the immobilized and isotropic signals (Fig. 4) may be due to tyrosine residues from different proteins, to different tyrosine residues of the same protein, or even to the same tyrosine residue of one protein in different conformations (45). Under our experimental conditions, at least three macrophage proteins (~76, 69, and 32 kDa) are likely to be oxidized by peroxynitrite to tyrosyl radicals (24, 48) because they were shown to be nitrated by nitrotyrosine Western blot analysis (Fig. 5). It is not possible to exclude minor amounts of DBNBS-lipid radical adducts in the spectra shown in Fig. 4, A and B. This possibility appears unlikely, however, because reported DBNBS/lipid radical adducts show some hyperfine structure (46, 49) in contrast with the spectra shown in Fig. 4, A and B. Hyperfine structures start to appear in the spectra scanned 7 min after peroxynitrite addition (Fig. 4, D and E; labeled as X), suggesting that lipid oxidation may be a secondary event resulting from free radical chain reactions triggered by peroxynitrite.

**DISCUSSION**

Our results demonstrate that the addition of peroxynitrite to a macrophage suspension promotes the oxidation of intracellular glutathione and proteins with the production of glutathionyl and protein-tyrosyl radicals, respectively (Figs. 1–5; Table 1). The glutathionyl radical was identified by EPR spin-trapping experiments (Figs. 1 and 2), whereas production of protein-tyrosyl radicals was suggested by direct EPR spectroscopy (Fig. 3) and confirmed by EPR spin-trapping experiments (Fig. 4) and Western blot analysis of nitrated proteins in treated macrophages (Fig. 5). Time dependence studies of free radical production indicate that glutathione (Fig. 1, A and E) and unidentified proteins (Figs. 3 and 4, A and D) are likely to be the initial peroxynitrite targets in macrophages. The radicals produced from them trigger radical chain reactions because the yield of the immobilized DBNBS/tyrosyl-protein radical adduct (labeled as * in Fig. 4A) keeps increasing after complete peroxynitrite decomposition (Fig. 4D). This late process appears to be accompanied by some lipid oxidation, because hyperfine structures characteristic of DBNBS/lipid radical adducts (labeled as X in Fig. 4D) (46, 49) appear in spectra scanned 7 min after peroxynitrite addition to macrophages. Taken together, the results confirm and extend previous studies demonstrating that peroxynitrous acid and nitrogen dioxide produced from it (Reactions 1 and 2) readily permeate biological membranes, whereas the carbonate radical anion produced from peroxynitrite in the presence of CO_{2} does not (21–24). Indeed, CO_{2} inhibited the yield of glutathionyl (data not shown) and protein-tyrosyl radicals (Figs. 3 and 4) produced upon the addition of peroxynitrite to macrophages as expected from the quick reaction between peroxynitrite and CO_{2} that greatly decreases the half-life of the oxidant (Reaction 3). Conversely, the inhibitory effects of CO_{2} confirm that most of the oxidant is reacting with intracellular macrophage targets, despite some cell lysis observed under our experimental conditions (Table 1). A completely different situation is expected to occur when peroxynitrite production and reaction with CO_{2} occur intracellularly, particularly at neutral pH values. Under these conditions, CO_{2} should increase glutathionyl (20) and protein-tyrosyl radical (48) production from peroxynitrite, as has been demonstrated to occur in homogenous solutions. Therefore, our results indicate that oxidation of biotargets to the corresponding radicals is likely to be an important result of the physiological production of peroxynitrite in most cell types.

The above conclusion could be questioned, because high cell densities and high bolus peroxynitrite concentrations were used to detect free radicals by EPR during the interaction of peroxynitrite with macrophages (this work). These conditions are certainly not physiological but should reflect what occurs physiologically, because cellular thiol oxidation (28, 30, 35, 50) and protein nitrination (see, for instance, Ref. 3) are recurrent events associated with treatment of cells with low peroxynitrite concentrations or fluxes. These processes should occur through the intermediacy of radicals, but the unequivocal EPR detection of the latter requires more drastic conditions due to the intrinsic characteristics of free radicals and EPR spectroscopy (6, 7, 15, 20, 31–46). Despite its drawbacks, EPR spectroscopy can provide unique mechanistic clues due to the characterization of important targets and their derived radicals. It is important to note that free radicals and oxidants are presently viewed as playing roles in cell homeostasis and not only in cell injury. Lack or excess of biological oxidants is likely to be deleterious, but transient rises in their concentrations trigger reoxid-sensitive signaling pathways (51, 52). Peroxynitrite is one of the biological oxidants whose addition to cells and cell cultures has been shown to either activate signaling pathways (25–27) or lead to cell injury (28–30, 50), depending on cell type and oxidant concentration. Both of these cellular processes have been associated with protein-thiol oxidation, which in general is controlled by the intracellular concentration of low molecular weight thiols such as glutathione in the case of mammalian cells (51–54). In this context, our results demonstrating peroxynitrite-mediated oxidation of intracellular glutathione to glutathionyl radicals may be relevant to the understanding of the bioregulatory and biodamaging roles of the oxidant.

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EPR Detection of Glutathionyl and Protein-tyrosyl Radicals during the Interaction of Peroxynitrite with Macrophages (J774)

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