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Peroxynitrite Protects Raw 264.7 Macrophage from Lipopolysaccharide/Interferon-γ-Induced Cell Death

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Peroxynitrite, formed by the interaction of superoxide with nitric oxide, has previously been implicated mostly as a cytotoxic agent. In contrast, its physiological and, possibly, beneficial effects are largely unknown. We have previously shown [*Journal of Biological Chemistry, 1997,* 272, *7253*] that RAW 264.7 macrophages can be selected to be resistant toward lipopolysaccharide (LPS)/interferon-γ (IFN-γ)-induced cytotoxicity. Resistant cells produced comparable amount of nitric oxide, but showed increased formation of superoxide, which might lead to increased production of peroxynitrite. We utilized this well characterized cell model to seek evidence that peroxynitrite might cause protection of RAW cells from cytokine toxicity. Exogenous peroxynitrite (30-50 μM), applied to RAW cells before cytokine stimulation, dramatically reduced LPS/IFN-γ toxicity. Measurement of cell viability after overnight incubation with a mixture of LPS (10 μg/ml) and IFN-γ (100 U/ml), showed that pretreatment with 40 μM peroxynitrite completely reverted LPS/IFN-γ cytotoxicity. Differently, pretreatment of RAW cells with peroxynitrite (10-60 μM) did not prevent cytotoxicity induced by the nitric oxide-donors S-Nitroso-L-glutathione (0.2-1 mM), or spermine NONOate (0.2-2 mM), and by Actimomycin D (0.5-1 μg/ml), suggesting that the protective effect is specific for the LPS/IFN-γ pathway. These results were confirmed through extensive controlled studies aimed to optimize cell exposure to peroxynitrite, and showed that peroxynitrite protects macrophages from cytokine-induced cytotoxicity.

The considerable research that in recent years has focused on the biochemical and molecular functions of nitric oxide (NO) has shown that NO mediates a number of physiological responses, but can also be cytotoxic and contribute to cell injury in a variety of disease states (1). It has been suggested that the toxic effects of NO are a result of the formation of peroxynitrite generated via the interaction between NO and superoxide (2).

Peroxynitrite is a highly cytotoxic compound that can result in profound cellular injury and cell death (3-6). It is formed during endotoxemia and contributes to the cellular injury associated with endotoxic shock (7,8). Also, peroxynitrite can cause protein fragmentation via oxidative stress (9), and may inactivate important regulatory proteins through tyrosine nitration (10,11) or thiol group oxidation (12). However, in contrast with its cytotoxic effects, others have found that peroxynitrite can mediate physiological processes that may be beneficial and can result in cellular protection. For example, peroxynitrite inhibits leukocyte-endothelial cell interaction and exerts cytoprotective effects in myocardial ischemia-reperfusion injury (13). Peroxynitrite also produces vascular relaxation in isolated dog and human coronary arteries (14,15), relaxes pulmonary arteries in vitro (16), and inhibits platelet aggregation (17).

It is well known that activation of macrophage by lipopolysaccharide (LPS) and interferon-γ (IFN-γ) is coupled to the production of large quantities of nitric oxide and superoxide, and that endogenously generated or exogenously applied NO, both ultimately result in macrophage cell death (18,19). The macrophage-like cell line RAW 264.7, through the combined use of LPS (10 μg/ml) and IFN-γ (100 U/ml) applied for 16-24 hrs, results in extensive cell death (20). We previously observed that repetitive treatment of RAW cells with LPS/IFN-γ, followed by subculturing of viable cells, selects resistant macrophages (21). The resistant cells produce comparable amounts of nitrite/nitrate in response to agonist treatment, but release twice the amount of superoxide compared to native. An intriguing possibility is that increased superoxide generation, and, perhaps, increased peroxynitrite formation, might channel NO toxicity through pathways that allow cellular defense mechanisms to cope with an otherwise lethal insult.

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In this study, we utilized the cellular model of cytokine-induced toxicity in RAW 264.7 cells to investigate the possibility that peroxynitrite, in physiological systems, may activate cellular protective mechanisms. We applied exogenous peroxynitrite at sub-cytotoxic concentrations to RAW 264.7 cell cultures to confer resistance. Our results showed, indeed, that peroxynitrite activated intracellular defense mechanisms which protected RAW cells from cytokine-induced NO toxicity.

MATERIALS AND METHODS

Cell culture. RAW 264.7 cells were obtained from American Type Culture Collection (Rockville, MD) and maintained in RPMI-1640 media (GIBCO BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, Utah), 100 units/ml penicillin and 100 μg/ml streptomycin (both from GIBCO). Cells were maintained in a humidified 5% CO_2 incubator at 37° C and routinely counted and subcultured to maintain low population density. Before each experiment, cells were serum-starved (0.5% FBS-containing RPMI) for 24 hrs. Cell viability was consistently >95% as determined by trypan blue exclusion.

Synthesis of peroxynitrite. Peroxynitrite was synthesized as previously described (22). Briefly, an ice-cold solution of 0.6 M HCl, 0.7 M H_2O_2 (10 ml) was added to a stirred, cooled (4° C) solution of 0.6 M $NaNO_2$ (10 ml), followed immediately by addition of 1.5 M NaOH (20 ml). Excess H_2O_2 was removed by addition of MnO_2. The mixture was shaken for 30 min at 4° C and then filtered 3 times in order to remove MnO_2. This solution was frozen at −20° C for up to 1 week. Peroxynitrite forms a yellow top layer due to freeze fractionation. The top layer typically contained 50-150 mM peroxynitrite as determined by UV-absorbance spectroscopy at 302 nm in 1 N NaOH (ϵ 302 nm = 1670 M^{-1} cm^{-1}). Fresh peroxynitrite was prepared weekly, aliquoted, and stored at −20°C. Aliquots were discarded after single use.

Exposure of RAW 264.7 cells to peroxynitrite. In most experiments, adherent RAW 264.7 cells were gently scraped, centrifuged, washed once with Dulbecco's phosphate-buffered saline (D-PBS; pH 7.4 without Ca^{++} and Mg^{++}, from GIBCO) and resuspended in 5 ml of experimental buffer or media (2×10^6 cells/ml). Various stock solutions of peroxynitrite were freshly prepared in 0.7 N NaOH to achieve the required concentrations, and 25 μl of each stock was added to separate cell suspensions. Cell suspensions were then immediately and briefly vortexed, and incubated for 10 min at 37° C. Control and vehicle incubations were carried out by exposing cell to 25 μl of D-PBS and 0.7 N NaOH, respectively. To ascertain that the observed effect was actually due to peroxynitrite and not secondary to its decomposition products or to trace residual of synthetic reagents, a cell sample was resuspended in 5 ml of buffer in which the highest concentration of peroxynitrite had been added 10 minutes prior to the treatment (i.e. to ensure comparable decomposition of peroxynitrite). After incubation, the cell samples were pelleted by centrifugation to wash out any possible products from the decomposition of peroxynitrite, resuspended in culture medium, and maintained in a culture incubator for the additional time required for each experiment.

Treatment of cells with lipopolysaccharide and interferon-γ. After exposure to peroxynitrite, cells were washed once with D-PBS and then resuspended in 5 ml of RPMI media (0.5% FBS). Aliquots (200×10^3 cells/well) of cell samples were then plated in 96 well culture plates (Microtest III Falcon, from Becton-Dickinson, Lincoln Park, New Jersey), and 100 μl of media (0.5% FBS) containing a combination of LPS (Sigma, St. Louis, MO) and mouse IFN-γ (Boerhinger Mannheim, Indianapolis, IN) was added to each well to a final concentration of 10 μg/ml and 100 units/ml respectively. Cell viability (MTS assay) was measured after overnight incubation.

Treatment of cells with NO-donors or actinomycin D. Actinomycin D was obtained from Sigma. The NO donors utilized in this study were spermine-NONOate (Cayman Chemicals, Ann Arbor, MI), and S-Nitroso-L-glutathione (GSNO). GSNO was freshly synthesized each time before use, as previously detailed (20). Briefly, glutathione was dissolved in 0.625 N HCl at 0° C, to a final concentration of 625 mM. $NaNO_2$ was added at an equimolar concentration and the mixture was stirred at 0° C for 40 min. After the addition of 2.5 volumes of acetone, stirring was continued for another 20 min, followed by filtration of the precipitate. GSNO was washed once with 80% acetone, two times with 100% acetone, and finally three times with diethyl ether and dried under vacuum. GSNO was characterized by high performance liquid chromatographic analysis and UV-vis spectroscopy.

Cells were prepared as described above for cytokine treatment, resuspended in media, and spermine-NONOate or GSNO or Actinomycin D was added to reach the required final concentration. Cell viability (MTS assay) was measured at different times (6-18 hrs).

Measurement of cell viability after experimental treatment. Cell viability after experimental treatment was measured with the CellTiter 96 Aqueous Assay (Promega, Madison, WI), following the manufacturer protocol. Briefly, at the end of the experimental procedure, a combined solution of the tetrazolium compound MTS and of the electron coupling reagent phenazine methosulfate (PMS), was added to each well of the 96 well assay plate containing the samples. After 2-4 hours incubation at 37° C in a humidified 5% CO_2 atmosphere, the absorbance due to the soluble formazan produced by viable cell metabolism was measured at 490 nm (ref. 630 nm) using an ELISA plate reader. Each sample was assayed in quadruplicate.

At the time of MTS/PMS addition, a cell number standard curve was created by plating (in series) a known number of viable cells obtained from the same batch used for the experiment. The mean absorbance for each standard was plotted versus cell number and the number of viable cells was determined by interpolation.

Statistical analysis. All experiments were performed in duplicates, and each experiment was repeated at least three times. Data are reported as means ± SD values. Data were compared by ANOVA using post-hoc analysis with Fisher's corrected t test. $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

To determine whether peroxynitrite can trigger cellular signals which might translate into a protective effect, we tested peroxynitrite in a well characterized model of NO-induced cell toxicity. That is, we treated RAW 264.7 macrophage with sublethal concentration of peroxynitrite before stimulation with LPS/IFN-γ.

Correct handling technique is critical in order to assess cellular effects of peroxynitrite. Previous reports have shown that peroxynitrite is highly cytotoxic (4,23-26). Likewise in our system, peroxynitrite (60-500 μM) induced extensive and rapid (1 hr) cell death of RAW 264.7 cells (Fig. 1). The NaOH vehicle or peroxynitrite decomposition products had no significant effect on cell viability, excluding any possible pH effect, or indirect effects to the cells resulting from interaction of peroxynitrite with components of cell medium. Quantitative experiments using MTS assay showed that peroxyni-

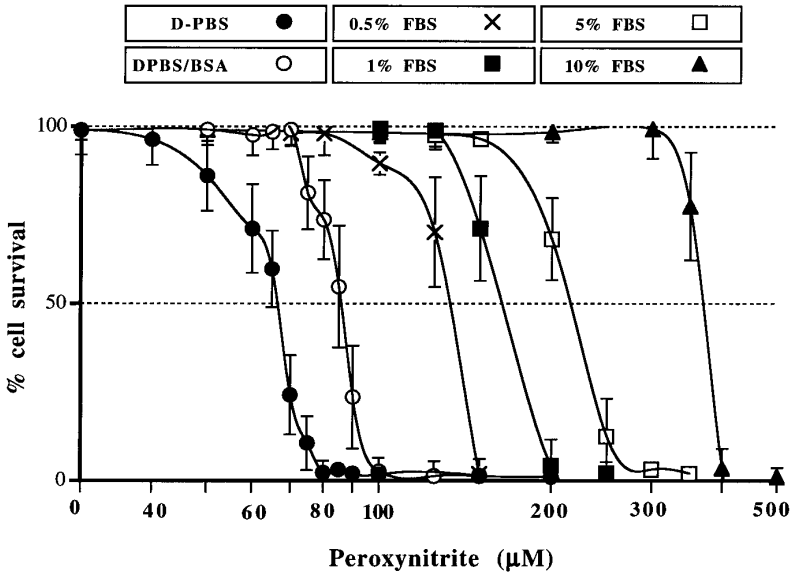

FIG. 1. Dose dependence of peroxynitrite toxicity in RAW 264.7 cells. Serum-starved RAW cells were gently scraped, washed, counted and resuspended in D-PBS alone or supplemented with 1% bovine serum albumin (BSA), or in RPMI media supplemented with 0.5%, 1%, 5%, or 10% FBS, respectively. After 10 min of peroxynitrite exposure, the cells were washed, resuspended in 0.5% FBS-RPMI media, and aliquots were plated in 96 well plates, as detailed in the methods section. Cell viability was measured after 1 hr by MTS assay. The values shown are percentage of viability (cell survival) compared to the number of viable cells of the respective control sample (vehicle only) in each condition. In any case, the controls (vehicle, buffer and decomposed peroxynitrite) were also checked for viability (>95%) by trypan blue exclusion. Results are means ± SD from at least three different experiments.

trite toxicity on RAW cells was dose-dependent with a TC_{50} ranging from 65 to 400 μM depending on the buffer in which the cells were suspended for peroxynitrite treatment. When cells were suspended in complete media (RPMI media containing 10% FBS) a bolus of 500 μM peroxynitrite was necessary to reach 100% cytotoxicity, while a much lower concentration (80 μM) was 100% cytotoxic for cells suspended in D-PBS free of proteins. Peroxynitrite is a highly reactive compound which decomposes rapidly at pH 7.4 (27). The extensive reaction of peroxynitrite with buffer/media components, especially proteins, might account for the requirement of higher cytotoxic concentrations in more complex media or buffer. Most interestingly, in our hands the technique used for stimulation was critical to detect the effects of peroxynitrite. When stimulation was carried out on adherent monolayers in 100 mm dishes, cell death was limited to the cells immediately adjacent to the site where the peroxynitrite was injected, even at the higher (100-500 μM) concentrations (data not shown). For the experiments discussed in this paper, RAW cells were gently scraped, resuspended in media or buffer, and a drop of peroxynitrite stock solution or vehicle was deposited on the tube wall, followed immediately by gentle vortexing. This technique resulted in very consistent and reproducible results. On the contrary, the simple pipetting of peroxynitrite solution into cell suspensions or in dishes led to inconsistent and erratic results. Therefore, the very limited half-life of peroxynitrite, and its high reactivity with many buffer components at pH 7.4, makes the use of authentic peroxynitrite to stimulate cells arduous, emphasizing the importance of the handling techniques used to obtain reliable results. In this study, the accurate assessment of the conditions used to expose cells to peroxynitrite excluded possible artifactual results and allowed us to treat RAW 264.7 cells with the highest sub-cytotoxic concentrations (up to 50 μM in D-PBS) in order to detect effects that otherwise would be missed.

Sub-cytotoxic concentrations of peroxynitrite protect RAW 264.7 macrophage from cytokine-induced toxicity. As shown in Fig. 2, concentrations of peroxynitrite ranging from 30 to 50 μM (in D-PBS), briefly applied to the cells before cytokine stimulation, dramatically protected RAW cells against LPS/IFN-γ-induced toxicity. The measurement of cell viability after overnight incubation with cytokines, showed that pretreatment of cells with 40 μM peroxynitrite completely reverted LPS/IFN-γ cytotoxicity, while lower concentrations were less effective in a dose-dependent fashion.

In contrast with overwhelming number of studies reporting peroxynitrite cytotoxicity, it has been observed that nanomolar concentrations of peroxynitrite may exert protective physiological effects resulting in

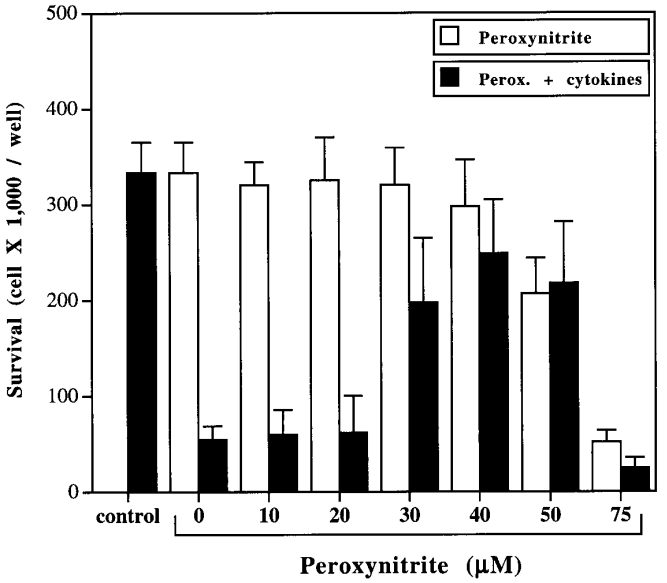

FIG. 2. Effects of peroxynitrite pre-treatment on LPS/IFN-γ-induced cytotoxicity in RAW 264.7 cells. Serum-starved RAW cells were scraped, washed, and resuspended in D-PBS for peroxynitrite treatment. After washing away the peroxynitrite decomposition products, LPS and IFN-γ were added to a final concentration of 10 μg/ml and 100 units/ml respectively. Cell viability (MTS assay) was measured after overnight incubation. The values are number of viable cells / well. Solid white bars indicate samples pre-treated with peroxynitrite, but not stimulated with cytokines. Black bars represent samples pre-treated with peroxynitrite and subsequently stimulated with LPS and IFN-γ. "Control" indicate control samples which received neither peroxynitrite nor cytokine treatment. It is noticeable that cell number in the most viable samples is much higher than the 200,000 cells/well plated for each sample (see details in the methods section). This is because surviving cells rapidly proliferate during the overnight incubation, even in serum-depleted media. Results shown are means ± SD for at least three different experiments.

vascular relaxation (14-16), inhibition of platelet aggregation (17,28), and attenuated PMNs accumulation in the postischemic heart (13). On this line, our study provides important new evidence that peroxynitrite may exert a direct cytoprotective effect at the cellular level. However, the "protective" concentrations (30-40 μM) may appear to be outside the range of physiological relevance. This is an extremely important point since it is highly unlikely that peroxynitrite can be formed or accumulate in vivo at concentrations greater than sub-micromolar. Peroxynitrite forms from the equimolar interaction of NO with superoxide and, as shown recently by Miles et al. (29), the formation rate of NO in vivo (normally 1-20 nM), even if highly increased during diseases states, is not sufficient to produce peroxynitrite at concentrations higher than the nanomolar range. However, because we delivered peroxynitrite as a bolus, the "effective exposure" of cells to peroxynitrite is likely to be much lower. As outlined by Beckman et al. (30), the effects of a species under investigation depends on both the concentration as well as the time of exposure. Indicative net exposures to peroxynitrite can be derived from the pseudo-first-order kinetics of peroxynitrite decomposition. Using a typical physiological decay rate of 0.64/sec, a bolus peroxynitrite concentration of 40 μM is equivalent to the exposure to a steady-state concentration of 0.1 μM for 10 minutes. Moreover, distance traveled through the buffer to cells may result in further lowering the effective exposure based on decaying peroxynitrite. We attempted to maximize efficient exposure to peroxynitrite by using a protein-free buffer (D-PBS) and rapidly vortexing the cellular suspension, as detailed above. Unfortunately, there is no way to measure the real amount of peroxynitrite that reacts with cells in any specific condition, but we can expect that micromolar bolus additions of peroxynitrite to cell culture is equivalent to generating sub-micromolar concentrations in situ.

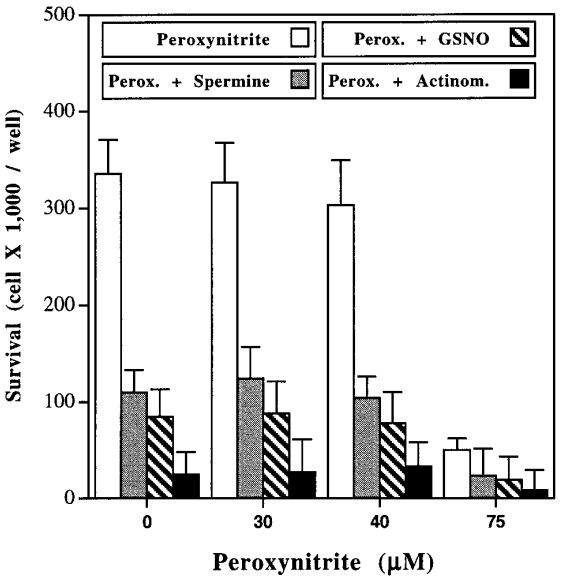

FIG. 3. Effects of peroxynitrite pre-treatment on cytotoxicity induced by Spermine-NONOate, GSNO, or Actinomycin D. Serum-starved RAW cells were scraped, washed, and resuspended in D-PBS for peroxynitrite treatment. After washing away the peroxynitrite decomposition products the cytotoxic agents were separately added. Cell viability was measured by MTS assay. Values are number of viable cells / well. Extensive experiments were performed using variable concentrations (0.2-2 mM spermine-NONOate, 0.2-1 mM GSNO, 0.5-1 μg/ml Actinomycin D) and variable incubation times (6-18 hrs). In all cases, the degree of cytotoxicity induced by spermine-NONOate, GSNO or Actinomycin D, was not altered in any way by peroxynitrite pre-treatment. As a representative result, the effect of peroxynitrite pre-treatment on cytotoxicity induced by overnight incubation of RAW cells with 1 mM spermine-NONOate, or 500 μM GSNO, or 0.5 μg/ml Actinomocyn D, is shown. White bars represent samples which received only peroxynitrite treatment. Patterned bars represent samples exposed to cytotoxic agent after peroxynitrite treatment. Results are means ± SD for at least three different experiments.

Effect of peroxynitrite treatment on cell death induced by other cytotoxic agents. Pretreatment of RAW 264.7 cells with peroxynitrite did not prevent cytotoxicity induced by NO-donors such as GSNO and spermine-NONOate, or by the completely different cytotoxic agent Actimomycin D (Fig. 3). This suggests that the protective effect of peroxynitrite may be quite specific for the LPS/IFN-γ pathway. Several mechanisms of peroxynitrite reaction could be involved. Peroxynitrite reacts at particularly fast rates with key targets, including thiol-containing enzymes (12), iron/sulfur centers (31,32), and zinc fingers (33). Peroxynitrite can also mediate DNA strand breakage (4,34). One important reaction of peroxynitrite is catalyzed by transition metals, including the metal centers of SOD. Transition metals catalyze the heterolytic cleavage of peroxynitrite to produce hydroxyl anion plus nitronium ion (NO^{++}) (35,36). NO^{++} is a powerful nitrating agent that modifies protein tyrosine residues to form nitrotyrosine that may inhibit phosphorylation and, presumably, alter the functional property of the affected proteins (11,37). Recently, it has been proposed that the intracellular reaction of SOD with peroxynitrite may direct a more selective nitration of tyrosines on certain proteins, making the SOD-peroxynitrite complex critically important in selecting the cellular target of peroxynitrite reaction among the great number of alternative substrates (1). It is tempting to predict the selective vulnerability of key tyrosine kinase targets in the LPS/IFN-γ system. However, any putative mechanism for peroxynitrite protective effect remains to be critically approached and investigated.

Conclusions. Our data suggest that peroxynitrite, at sub-micromolar concentrations, which are those most likely to be formed in vivo, can generate a signal, or induce structural changes, within macrophage, which temporarily or permanently commits those cells to altered signal transduction. This may have relevance in the pathophysiology of inflammation, in which both peroxynitrite formation and macrophage activation have been described. It is conceivable that macrophage activation be strictly controlled and balanced in order to result in toxicity to foreign agents while not damaging the same macrophage which initiated it. Peroxynitrite produced in the inflammatory tissue, or by the same macrophage, might be the cell mediator which commits the cells to be more resistant toward cytokine toxicity. This study shows that peroxynitrite protects macrophage from cytokine-induced cytotoxicity, and supports the concept that peroxynitrite can exert physiological and modulatory functions in vivo.

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