Protein Tyrosine Nitration in Cytokine-activated Murine Macrophages

INVolVEMENT OF A PEROXIDASE/NITRITE PATHWAY RATHER THAN PEROXYNITRITE*

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Peroxy nitrite, formed in a rapid reaction of nitric oxide (NO) and superoxide anion radical (O₂•⁻), is thought to mediate protein tyrosine nitration in inflammatory and infectious diseases. However, a recent in vitro study indicated that peroxynitrite exhibits poor nitrat ing efficiency at biologically relevant steady-state concentrations (Pfeiffer, S., Schmidt, K., and Mayer, B. (2000) J. Biol. Chem. 275, 6346–6352). To investigate the molecular mechanism of protein tyrosine nitration in intact cells, murine RAW 264.7 macrophages were activated with immunological stimuli, causing inducible NO synthase expression (interferon-γ in combination with either lipopolysaccharide or zymosan A), followed by the determination of protein-bound 3-nitrotyrosine levels and release of potential triggers of nitration (NO, O₂•⁻H₂O₂, peroxynitrite, and nitrite). Levels of 3-nitrotyrosine started to increase at 16–18 h and exhibited a maximum at 20–24 h post-stimulation. Formation of O₂•⁻ was maximal at 1–5 h and decreased to base line 5 h after stimulation. Release of NO peaked at ~6 and ~9 h after stimulation with interferon-γ/lipopolysaccharide and interferon-γ/zymosan A, respectively, followed by a rapid decline to base line within the next 4 h. NO formation resulted in accumulation of nitrite, which leveled off at about 50 μM 15 h post-stimulation. Significant release of peroxynitrite was detectable only upon treatment of cytokine-activated cells with phorbol 12-myristate-13-acetate, which led to a 2.2-fold increase in dihydroorhodamine oxidation without significantly increasing the levels of 3-nitrotyrosine. Tyrosine nitration was inhibited by azide and catalase and mimicked by incubation of unstimulated cells with nitrite. Together with the striking discrepancy in the time course of NO/O₂•⁻ release versus 3-nitrotyrosine formation, these results suggest that protein tyrosine nitration in activated macrophages is caused by a nitrite-dependent peroxidase reaction rather than peroxynitrite.

The free radical nitric oxide (NO) is produced by constitutive and inducible nitric-oxide synthases and regulates numerous biological processes, including relaxation of blood vessels and neurotransmitter release in the brain. However, overproduction of NO appears to contribute essentially to tissue injury in inflammatory and ischemic conditions (1). One of the mechanisms by which excess NO can injure tissues is by its nearly diffusion-controlled reaction with O₂•⁻ to give peroxynitrite, a potent oxidant thought to be a key mediator of NO-mediated tissue injury in atherosclerosis, congestive heart failure, glutamate excitotoxicity, and other disease states involving inflammatory oxidative stress (2). There are several pieces of evidence implicating peroxynitrite as toxic agent in these pathologies as follows. (i) All of these diseases are associated with increased expression of inducible NO synthase, resulting in sustained formation of NO over relatively long periods of time, (ii) oxidative stress causes increased generation of O₂•⁻, (iii) authentic peroxynitrite triggers tyrosine nitration of a wide variety of proteins known to subserve important cellular functions that are lost upon nitration, and (iv) 3-nitrotyrosine levels have been observed in the injured tissues by both immunohistochemical techniques and quantitative analyses with HPLC and gas chromatography-mass spectrometry (3).

Despite this apparently conclusive link between oxidative tissue injury, peroxynitrite, and tyrosine nitration, direct evidence for peroxynitrite-mediated nitration in vivo is still lacking (4, 5). This is of particular relevance because recent in vitro studies suggest that co-generation of NO and O₂•⁻ an obviously better approximation to the in vivo situation than bolus addition of concentrated peroxynitrite solutions, does not cause significant nitration of free tyrosine (6–10). Although all of those studies, performed in four independent laboratories with a number of different NO/O₂•⁻ generating systems including pulse radiolysis, gave essentially identical results, Sawa et al. (11) recently reported on highly efficient tyrosine nitration by low fluxes of NO/O₂•⁻. The reason for this discrepancy is unclear.

The striking difference between peroxynitrite generated in situ at relatively low fluxes and bolus addition of authentic peroxynitrite appears to be a consequence of the different steady-state concentrations that are achieved with the two experimental protocols; at low (submicromolar) steady-state concentrations, the reaction of peroxynitrite with tyrosine was found to give almost exclusively dityrosine, i.e. the product of tyrosyl radical dimerization, whereas 3-nitrotyrosine is the major product at the fairly high concentrations of peroxynitrite.

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The abbreviations used are: HPLC, high performance liquid chromatography; N-AcTyr, N-acetyl 3-aminotyrosine; DHR, dihydroorhodamine 123; LASP, lipopolysaccharide; MetTAP, manganese (III) tetraakis(4-benzoic acid) porphyrin; L-NNa, N⁺-nitro-L-arginine; NO, nitric oxide; O₂•⁻, superoxide anion radical; PBS, phosphate-buffered saline; PEG-Cat, polyethylene glycol-labeled catalase; PEG-SOD, PEG-labeled superoxide dismutase; PMA, phorbol 12-myristate 13-acetate; TBST, Tris-buffered saline containing Tween 20; Zy, zymosan A; IFN, interferon.
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Tyrosine nitration has been shown to occur upon bolus addition of the authentic compound (8). Recent studies have revealed an alternative mechanism of tyrosine nitration with potential in vivo relevance (4). Heme peroxidases such as myeloperoxidase or eosinophil peroxidase have been shown to utilize H₂O₂ to oxidize nitrite to a reactive nitrogen oxide species that triggers nitration of protein tyrosine residues and other phenolic compounds (12–14). Since inflammatory processes are typically associated with an infiltration of phagocytes, which contain high levels of heme peroxidases, this pathway has to be considered as a possible alternative to peroxynitrite in mediating protein tyrosine nitration in vivo. Intriguingly, the dependence of peroxidase-catalyzed nitration on the local levels of NO and H₂O₂ suggests that the peroxidase pathway operates under exactly the conditions that favor formation of peroxynitrite, i.e., increased formation of both NO and O₂⁻, the reactive precursors of NO₂⁻ and H₂O₂, respectively.

Thus, the experimental evidence currently available does not allow a decision as to which of the two pathways is responsible for tyrosine nitration in vivo. Although several in vitro studies with NO/NO₂-generating systems (see above) argue against peroxynitrite as a mediator of nitration, it should be emphasized that these studies were performed with highly artificial in vitro systems not necessarily reflecting the in vivo situation. As a first approach in addressing this issue, we attempted to clarify the cellular pathways mediating protein tyrosine nitration in cultured macrophages activated with established immunological stimuli. As a model system we used the murine macrophage RAW 264.7 cell line. These cells are known to express high levels of inducible NO synthase and 3-nitrotyrosine-like immunoreactivity in response to immunological challenge with IFN-γ in combination with LPS or zymosan (15). Upon cell activation, we measured several key parameters of the NO/NO₂/peroxynitrite pathway as a function of time and compared the data with protein tyrosine nitration. These experiments revealed a striking discrepancy in the time course of NO formation and nitration and showed that peroxynitrosoinhibitors as well as catalase attenuated the formation of 3-nitrotyrosine, whereas peroxynitrite scavengers had no significant effects. Based on these results we suggest that tyrosine nitration in cytokine-activated macrophages is mediated by a peroxidase/nitrite pathway rather than NO/NO₂-derived peroxynitrite.

EXPERIMENTAL PROCEDURES

Materials—DHR and 3-nitrotyrosine were from Fluka (Vienna, Austria). Recombinant mouse IFN-γ and pronase were from Roche Molecular Biochemicals (Vienna, Austria). MnTBAP was from Alexis (Vienna, Austria). Rabbit anti-human myeloperoxidase antibody was from DAKO (Vienna, Austria). Human myeloperoxidase was from Planta (Salzburg, Austria). Yersinia enterocolitica lipopolysaccharide (clone IA6, mouse monomodal IgG, 100 μg/100 μl) was from Upstate Biotechnology (Lake Placid, NY). Penicillin, amphotericin, and fetal calf serum were from PAA Laboratories GmbH (Linz, Austria). The ECL Western blotting detection system was obtained from Amersham Pharmacia Biotech. Centrifuge tube filters (0.22 μm cellulose acetate) were from Szabo (Austria). The 3-nitrotyrosine antibody (clone 1A6, DAKO (Vienna, Austria). Human myeloperoxidase was from Planta (Salzburg, Austria). The extinction coefficient of 78,800 M⁻¹ cm⁻¹ at 260 nm.

Determination of NO Release—The culture medium was removed, and the cells (one Petri dish for each measurement) were washed with PBS, harvested, centrifuged, and resuspended in 0.5 ml of PBS. NO release was continuously monitored with a Clark-type NO-sensitive electrode (iso-NO, World Precision Instruments, Berlin, Germany) at 37 °C in disposable tubes (19). After 1 min, 5 μl of a 0.1 M solution of l-arginine (final concentration, 1 mM) was injected. NO formation was quantified from the initial release rates obtained after injection of l-arginine using the Macintosh CHART software.

Determination of Nitrite Accumulation—The concentration of nitrite in the cell culture supernatants was determined photometrically with the Griess assay as previously described (18).

Determination of Oxygen Consumption—Cells were cultured in Petri dishes (diameter, 90 mm) at 37 °C and 5% CO₂ in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, penicillin (100 units/ml), amphotericin (1.25 μg/ml), and NaHCO₃ (3.7 g/l) as described (17). Cells were grown to confluence (~5 × 10⁴ cells/dish) and incubated for up to 48 h in the presence of IFN-γ (50 units/ml) and either l-arginine (0.5 μg/ml) or zymosan A (0.5 mg/ml) in fresh phenol red-free Dulbecco’s modified Eagle’s medium. After the time points indicated in the text and graphs, the activated cells were assayed for the following parameters: nitrite accumulation in the culture medium, release of NO, O₂⁻, and H₂O₂, DHR oxidation, and intracellular levels of protein-bound 3-nitrotyrosine.

Determination of Nitric Oxide—Release—Rates of O₂⁻ release were measured as PEG-SOD-inhibitable reduction of acetylated cytochrome c as described (20). At the indicated time points, the cells were washed three times and equilibrated for 30 min in PBS followed by incubation for 3 min with 0.5 μM acetylated cytochrome c for 45 min with and without 150 units of PEG-SOD/ml. The cell supernatants were centrifuged at 1,300 × g for 3 min followed by the determination of the absorbance at 550 nm against PEG-SOD-containing blanks. PEG-SOD inhibited total cytochrome c reduction by ~80%. O₂⁻ release was calculated using an extinction coefficient of 27,700 M⁻¹ cm⁻¹ at 550 nm (21).

Determination of Nitric Oxide—Formation—Formation of NO₂⁻ was measured as horseradish peroxidase-catalyzed oxidation of fluorescent scopoletin as described (22). At the indicated time points, macrophages were washed three times with PBS and incubated for 45 min with an assay mixture containing 10 μM scopoletin, 1 mM NaNO₂, and 10 units/ml horseradish peroxidase in Krebs-Ringer phosphate buffer. Supernatants were centrifuged at 1,300 × g for 5 min followed by determination of the absorbance at 550 nm against blank samples obtained by incubation of the assay mixture without cells as the blank. The method was calibrated with standard solutions of NO₂⁻ adjusted photometrically using an extinction coefficient of 40 M⁻¹ cm⁻¹ at 240 nm.

Determination of DHR Oxidation—Oxidation of DHR was determined as the increase in absorbance at 500 nm against a blank containing 50 μM DHR and 1% DMSO.

Determination of Protein-bound 3-Nitrotyrosine—Protein-bound 3-nitrotyrosine was determined by HPLC with electrochemical detection after derivatization to N-AcAycys Tyr following a protocol described recently (24). The cells were homogenized in 0.5 μM phosphate buffer, pH 7.4, and adjusted to a protein concentration of 16–30 mg of protein/ml. Protein was determined with the Bradford method using bovine serum albumin as a standard (25). Homogenates (0.5 ml) were precipitated with 0.6 ml of HPLC grade acetonitrile, thoroughly vortexed and centrifuged (1000 × g), followed by resuspension of the precipitates in 0.1 M phosphate buffer, pH 7.4, and sonication for ~10 s at 50 watts. This procedure was repeated three times to efficiently wash out non-protein material. The final suspensions were incubated overnight (16–20 h) at 50 °C with 1–2 mg of pronase and 0.5 μM CaCl₂. Subsequently, 350-μl aliquots of the samples were centrifuged (20,000 × g), and an equal volume of 3 μM phosphate buffer, pH 9.6, was added followed by the addition of 25 μl of acetic anhydride. After 10 min of incubation at ambient temperature, ethyl acetate (1 ml) and formic acid (0.2 ml) were added. The samples were thoroughly vortexed for 30 s and then centrifuged at 20,000 × g for 1 min. The ethyl acetate phase was concentrated under a gentle stream of air at ~50 °C. In the case of the phenolic acetyl group, the samples were resuspended in 1 N NaOH (60 μl). After 30 min of incubation at 37 °C, 60 μl of 1 N phosphate buffer, pH 6.5, was added followed by the addition of 0.1 N sodium dithionite (10 μl) to reduce the nitro substituent to the corresponding amine. The samples were incubated for 10 min at ambient temperature, acidified by addition of concentrated hydrochloric acid (25 μl), and centrifuged at...
20,000 × g for 10 min in centrifuge tube filters. Aliquots (100 µl) were injected onto a 250 × 4 cm C18 reversed phase HPLC column (LiChrospher 100 RP-18, 5-µm particle size, Merck) and eluted with 10 mM H3PO4 at 0.7 ml/min. The performance of the column decreased gradually over time. This loss in resolution was overcome by supplementing the mobile phase with up to 2% (v/v) methanol. N-AcATyr was detected electrochemically with an ESA Coulchem II detector. The potentials of the two electrodes were set to −70 mV and +70 mV (versus palladium), respectively. The method was calibrated daily with authentic N-AcATyr (5–500 nM) prepared as described (24). The recovery of authentic 3-nitrotyrosine added to homogenates of resting RAW 264.7 macrophages was 83 ± 1.2%.

**Immunostaining**—To visualize 3-nitrotyrosine formation, RAW 264.7 macrophages were subjected to immunostaining with a monoclonal antibody. The cells were grown to confluence on 1-polylysine-treated cover slides followed by activation with IFN-γ/Zy in phenol red-free Dulbecco’s modified Eagle’s medium for 24 h. After 14 h of activation the test compounds (methionine, 0.25 mM; MnTBAP, 50 µM; KCN, 0.25 mM; NaN3, 0.25 mM, and PEG-CAT, 2000 units/ml) were added following incubation for a further 10 h. As a negative control, non-activated macrophages were incubated under identical conditions for 24 h. For positive control, the cells were treated with authentic peroxynitrite (1 mM) for 1 h. After incubation, the cover slides were rinsed three times with PBS followed by fixation for 1 h (with a solution, pH 7.4, containing NaH2PO4 (6.5 g/liter), Na2HPO4 (4 g/liter), (v/v), 15 ml/liter methanol (15 ml/liter; v/v), and formaldehyde (100 ml of 37% liter, v/v)). Thereafter, cover slides were gently rinsed three times with PBS. The 3-nitrotyrosine antibody was diluted to 10 µg/ml in PBS containing 1% bovine serum albumin (w/v). 50 µl of this solution were carefully applied to each cover slide to cover the entire surface and incubated for 1 h at 37 °C under humidified atmosphere. After gentle rinsing of the cover slides, three times with PBS, 50 µl of biotinylated goat anti-mouse IgG (part of the mouse ExtrAvidin peroxidase staining kit obtained from Sigma diluted 1/20 in PBS containing 1% bovine serum albumin) were applied on the cover slides and incubated for 30 min at 37 °C under a humidified atmosphere. Then, cover slides were again gently rinsed three times with PBS followed by the application of 100 µl of ExtrAvidin peroxidase (10 µg/ml in PBS on each cover slide and incubation for 30 min at 37 °C under humidified atmosphere and rinsing of the slides with PBS. The staining solution was prepared by mixing 0.2 ml of 20 mg of 3-aminio-9-ethylcarbazole in 2.5 ml of dimethylformamide with 3.8 ml of 0.05 M acetate buffer, pH 5.0. Before use, 20 µl of 3% (v/v) H2O2 were added to the staining solution, and 100 µl were applied on the cover slides until the appropriate color development (3–5 min). Reactions were terminated by rinsing the slides gently with distilled water.

**SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting**—Cell homogenates were subjected to SDS-polyacrylamide gel electrophoresis on 12% slab gels and transferred onto nitrocellulose membranes in 25 mM Tris/HCl, pH 6.3, containing 192 mM glycine, 0.02% (w/v) SDS, and 20% (v/v) methanol at 250 mA for 90 min. Unspecific binding sites were saturated by overnight incubation of the membranes at 4 °C in TBST containing 3% (w/v) ovalbumin. Subsequently, the membranes were washed twice for 5 min followed by incubation for 2 h with the anti-myoeloperoxidase antibody diluted 1:500 in TBST containing 3% (w/v) ovalbumin. Subsequently, the membranes were washed twice for 15 min with TBST and incubated for 1 h with horseradish peroxidase-labeled anti-rabbit-IgG antibody that had been diluted 1:5000 in TBST buffer containing 0.3% (w/v) ovalbumin. Finally, the membranes were washed three times for 20 min with TBST buffer and processed with the ECL Western-blotting detection system according to the recommendations of Amersham Pharmacia Biotech.

**Data Analysis and Statistics**—Unless otherwise indicated, release rates are expressed as amounts of product (pmol or pg)/min/mg of total cell protein. Results are the mean values ± S.E. of n experiments as indicated in the figure legends. The statistical significance of the data shown in Fig. 6 was evaluated by analysis of variance using Fisher’s protected least significant difference test.

**RESULTS**

**Time Course of NO and NO2 Formation by Activated Macrophages**—Activation of RAW 264.7 macrophages with either IFN-γ/LPS or IFN-γ/Zy led to a pronounced release of NO accompanied by an accumulation of nitrite in the cell culture media. As shown in Fig. 1A, the maximal rates of NO release were 116.2 ± 15.0 and 90.9 ± 11.5 pmol × min−1 × mg−1 at 7 and 9 h after stimulation with IFN-γ/LPS and IFN-γ/Zy, respectively. Note that with both stimuli, NO release was virtually back to baseline line after 14 h of incubation. The inset in Fig. 1A shows that NO release was markedly increased upon the addition of l-arginine, an observation that agrees well with previous studies reporting on a pronounced dependence of macrophage NO synthesis on extracellular substrate supply (27, 28). NO release was not significantly affected by the addition of SOD (1000 units/ml). The signal rapidly declined to zero upon the addition of the NO scavenger hemoglobin, demonstrating the specificity of the Clark-type NO electrode.

The time course of nitrite accumulation in the cell culture supernatant was virtually identical with both combinations of stimuli (Fig. 1B). Nitrite levels progressively increased from 4 to 15 h of incubation followed by a plateau corresponding to nitrite concentrations of about 50 µM. Conversion of the rates of NO release from macrophages activated with either cytokine combination (Fig. 1A) to accumulating concentrations revealed that the decrease in the rates of NO release is in good accordance with the observed reduction in the rate of nitrite accumulation; based on the nitrite data, the apparent recovery of NO detected with the Clark electrode was −50% (not shown). These results indicate that macrophage NO synthesis ceased after about 15 h of cell activation, presumably due to inducible NO synthase inactivation and/or limiting cofactor supply. Interestingly, the small but significant rightward shift of the time course of NO release from macrophages activated with IFN-γ/Zy- as compared with that from IFN-γ/LPS-stimulated cells...
release were measured 7 h after cell stimulation through activation of protein kinase C (29). This and H₂O₂ formation had already declined close to basal levels. These results with both stimulation with IFN-γ and LPS or IFN-γ/Zy (30, 31) during the first hour of stimulation followed by a steady decline that reached basal rates 4 h after stimulation. As shown in Fig. 2A, release of O₂⁻ from cells stimulated with IFN-γ/LPS was much less pronounced. The maximal rate of 12.8 ± 1.6 pmol min⁻¹ mg⁻¹ observed 2 h post-stimulation had declined to basal rates 4 h after stimulation. As shown in Fig. 2B, the time course of H₂O₂ formation was similar to that of O₂⁻ with both stimulation protocols, but the overall fluxes were ~1000-fold higher (note the different scales in the two y axes of Fig. 2B).

We considered the possibility that the apparent decrease in O₂⁻ formation was a consequence of a rapid reaction of O₂⁻ with NO to yield peroxynitrite and carried out two sets of experiments to test this hypothesis. First, we repeated the experiments shown in Fig. 2A using cells treated with a high concentration of a non-selective NO synthase inhibitor (L-NNa; 1 mM). L-NNa almost completely inhibited nitrite accumulation in the cell culture supernatant (data not shown) but had no effect on the rates of O₂⁻ release measured 7 h after cell stimulation (inset to Fig. 2A). Secondly, we determined the time course of DHR oxidation as a measure for peroxynitrite formation. Neither of the two protocols of macrophage activation (IFN-γ/LPS and IFN-γ/Zy) resulted in a considerable increase in the rates of DHR oxidation (1–3 pmol min⁻¹ mg⁻¹), which was insensitive to L-NNa (data not shown). Together, these results argue against peroxynitrite as a major reactive nitrogen species formed by activated macrophages.

**Evidence against the Involvement of Peroxynitrite in Tyrosine Nitrations**—Protein-bound 3-nitrotyrosine was measured in the cell extracts as the N-acetyl-amino derivative (N-AcATyr). As expected, treatment of macrophages with authentic peroxynitrite (1 mM final) resulted in a pronounced increase in tyrosine nitrination from 19.4 ± 17.3 to 855.9 ± 270.2 pg of N-AcATyr/mg of cellular protein (n = 3 each). Fig. 3 shows that a significant increase in nitrination was also observed upon activation of the macrophages with either IFN-γ/Zy or IFN-γ/LPS. The time course of N-AcATyr formation was similar with both combinations of stimuli, although IFN-γ/Zy led to a 3-fold higher product formation than IFN-γ/LPS (385.3 ± 77.8 and 127.9 ± 8.7 pg × mg⁻¹, respectively). Nitrination occurred with a pronounced lag phase of 6 (IFN-γ/Zy) to 18 h (IFN-γ/LPS), was maximal 24 h post-stimulation, and slowly declined during the next 24 h.

Thus, we observed a pronounced difference in the time course of protein tyrosine nitrination and NO/O₂⁻ formation such that nitration started to increase at a time when the rates of NO/O₂⁻ had already declined back to basal levels. These results argue against peroxynitrite as a mediator of tyrosine nitrination in activated macrophages. However, because of the apparent lack of peroxynitrite formation, as evident from the lack of significant DHR oxidation by activated cells, the data do not exclude that peroxynitrite, if produced, is capable of nitrating cellular proteins. To clarify this issue, we treated IFN-γ/LPS-activated macrophages with PMA, which is known to trigger O₂⁻ formation through activation of protein kinase C (29). This protocol was expected to result in an intracellular co-generation of both NO (through induction of NO synthase) and O₂⁻ (through activation of protein kinase C). Fig. 4A shows that PMA (0.5 μM) led to ~2-fold increase in the rates of DHR oxidation from 2.6 ± 0.1 to 5.4 ± 0.2 pmol min⁻¹ mg⁻¹ after 30 min. This effect of PMA was sensitive to inhibition of NO synthase and protein kinase C with L-NNa (1 mM) and H-7 (30 μM), respectively (Fig. 4B). These data indicated that co-activation of macrophages with IFN-γ/LPS and PMA resulted in formation of peroxynitrite, detectable as increased rates of DHR oxidation. However, as shown in Fig. 4C, this apparent peroxynitrite formation was not accompanied by an increase in the yields of protein nitrination, measured 3.5 h after the addition of PMA, i.e. 9 h after IFN-γ/LPS. Note that the lack of effect of IFN-γ/LPS on nitrination after 9 h of incubation is in accordance with the results shown in Fig. 3.

**Evidence for the Involvement of a Nitrite/Peroxidase Pathway**

We considered the possibility that the apparent decrease in protein tyrosine nitrination after 30 min. This effect of PMA was sensitive to inhibition of NO synthase and protein kinase C with L-NNa (1 mM) and H-7 (30 μM), respectively (Fig. 4B). These data indicated that co-activation of macrophages with IFN-γ/LPS and PMA resulted in formation of peroxynitrite, detectable as increased rates of DHR oxidation. However, as shown in Fig. 4C, this apparent peroxynitrite formation was not accompanied by an increase in the yields of protein nitrination, measured 3.5 h after the addition of PMA, i.e. 9 h after IFN-γ/LPS. Note that the lack of effect of IFN-γ/LPS on nitrination after 9 h of incubation is in accordance with the results shown in Fig. 3.

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**Fig. 2. Formation of O₂⁻ and H₂O₂ by macrophages activated with IFN-γ/LPS or IFN-γ/Zy.** Macrophages were incubated in the presence of IFN-γ/LPS (open symbols) or IFN-γ/Zy (filled symbols) for up to 24 h. The data are the mean values ± S.E. of three experiments.

**Fig. 3. Tyrosine nitrination by macrophages activated with IFN-γ/LPS or IFN-γ/Zy.** Macrophages were incubated in the presence of IFN-γ/LPS (open symbols) or IFN-γ/Zy (filled symbols) for up to 48 h. At the indicated time points, protein-bound 3-nitrotyrosine was determined as N-AcATyr derivative by HPLC and electrochemical detection as described under “Experimental Procedures.” The data are the mean values ± S.E. of four experiments performed in duplicate.
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way in Tyrosine Nitrati on—So far, the results indicated that macrophages activated with typical NO synthase inducers did not produce significant amounts of peroxynitrite unless the NADPH oxidase pathway was additionally stimulated to generate $O_2^-$. The finding that peroxynitrite even when produced did not trigger significant nitration extended our observations made previously with cell-free systems and free tyrosine (7, 8) to protein nitration in intact cells. Based on these data, we speculated that a nitrite/peroxidase pathway (5) rather than peroxynitrite may be responsible for protein tyrosine nitration in activated macrophages.

The known peroxidase pathways of tyrosine nitration do all utilize nitrite as a substrate (5). Thus, assuming the involvement of such a pathway in nitration would imply that induction of macrophage NO synthase with IFN-γ/Zy or IFN-γ/LPS is mimicked by treatment of the cells with nitrite. To address this issue, we measured N-AcATyr levels in non-activated macrophages treated for 5 h with increasing concentrations of nitrite. As shown in Fig. 5A, incubation with 20–100 μM nitrite led to a 3–4-fold increase in nitration. The amount of N-AcATyr in the nitrite-treated cells (100–150 pg × mg⁻¹) was clearly less than in IFN-γ/Zy-activated cells but identical to that measured 24 h after activation of the macrophages with IFN-γ/LPS (see Fig. 3). A time course of nitration in response to 20 μM nitrite is shown in Fig. 5B.

In another set of experiments we studied the effects of peroxynitrite/O$_2^-$ scavengers, peroxidase inhibitors, and catalase. To minimize nonspecific and/or cytotoxic effects such as the described inhibition of NO formation by manganese porphyrins and catalase (30, 31), the compounds were added 14–15 h after IFN-γ/Zy followed by 9–10 h of incubation (24 h total) and subsequent determination of protein-bound 3-nitrotyrosine. To account for this protocol, the results shown in Fig. 6 are expressed as the rates of N-AcATyr formation (pg × h⁻¹ × mg⁻¹) during the 9–10 h of treatment (i.e. from 14–15 to 24 h after the addition of IFN-γ/Zy). Under control conditions, this rate was 15.1 ± 2.38 pg × h⁻¹ × mg⁻¹. NaN$_3$ and KCN (0.25 mM each), the most commonly used inhibitors of all types of heme peroxidases (32), reduced the rate of N-AcATyr formation to 2.90 ± 2.50 and 7.03 ± 4.68 pg × h⁻¹ × mg⁻¹, respectively. The effect of NaN$_3$ was statistically significant ($p < 0.05$). Peroxidase-catalyzed nitration was reported to be strictly dependent on H$_2$O$_2$ (12). Indeed, tyrosine nitration by activated macrophages was completely blocked by incubation of the activated cells with PEG-Cat (−1.23 ± 1.99 pg × h⁻¹ × mg⁻¹). In contrast, tyrosine nitration was not significantly affected by the peroxynitrite/O$_2^-$ scavengers methionine (0.25 mM) and MnTBAP (50 μM) (33, 34).

The results obtained by HPLC determination of N-AcATyr
were corroborated by immunostaining of the cells with a monoclonal 3-nitrotyrosine antibody. As shown in Fig. 7, treatment of macrophages with 1 mM peroxynitrite (panel A) or activation with IFN-γ/Zy for 24 h (panel B) resulted in pronounced staining of the cells. Although removal of H₂O₂ with PEG-Cat or inhibition of peroxidases (KCN, NaN₃) (panels E–G) resulted in a significant reduction of the staining intensity, scavenging of O₂⁻ or peroxynitrite with MnT-BAP or methionine, respectively (panels C and D), was much less effective. The results confirm the quantitative data shown in Fig. 6, suggesting that a peroxidase/nitrite pathway rather than peroxynitrite was responsible for tyrosine nitration in our experiments.

Among several heme peroxidases, myeloperoxidase appeared to be a likely candidate catalyzing nitrite/H₂O₂-dependent tyrosine nitration, but it is unclear whether this enzyme occurs in RAW 264.7 macrophages (35, 36). We attempted to clarify the possible involvement of myeloperoxidase in protein nitration using the fairly selective myeloperoxidase inhibitor 4-amino-benzoic acid hydrazide (32). However, this drug reduced the apparent N-AcATyr levels even below basal levels (data not shown), presumably because of an interference with 3-nitrotyrosine derivatization and/or electrochemical detection of the N-AcATyr derivative. Moreover, several published myeloperoxidase activity assays yielded negative results. To allow a more sensitive detection of this enzyme, we used a selective myeloperoxidase antibody (37, 38) for immunoblotting of macrophage homogenates. As illustrated by a representative blot shown in Fig. 8, the antiserum recognized a protein with an apparent molecular mass of ~57 kDa, which comigrated with the 57-kDa band of purified human myeloperoxidase (lane 4). Similar amounts of the protein were found in non-activated macrophages (lane 1) and in cells activated with IFN-γLPS (lane 2) or IFN-γ/Zy (lane 3). These results suggest that RAW 264.7 macrophages contain small amounts of myeloperoxidase that might contribute to tyrosine nitration upon cytokine activation of the l-arginine/NO pathway in these cells.

DISCUSSION

In the present study we investigated the mechanisms of protein tyrosine nitration in activated RAW 264.7 murine macrophages with a special focus on the potential involvement of peroxynitrite and heme peroxidases. In contrast to an earlier report (39), our data do not support the view that peroxynitrite is a major reactive nitrogen species formed by macrophages activated with either IFN-γ/Zy or IFN-γLPS. With both combinations of stimuli, we observed a pronounced release of NO and accumulation of nitrite in the cell culture supernatant. Although maximal rates of NO release were observed 6–8 h after stimulation, release of O₂⁻ was maximal at much earlier time points, i.e. 1–3 h after stimulation, and then rapidly declined. It was conceivable that the decline in the rates of O₂⁻...
release reflected the rapid reaction of \( \text{O}_2^- \) with NO formed by low levels of induced NO synthase. However, we observed no considerable increase in the rates of DHR oxidation, which remained about 10-fold lower than \( \text{O}_2^- \) release at all time points (1–3 versus 10–25 pmol × min\(^{-1} \) × mg\(^{-1} \)), indicating that peroxynitrite was not a major reactive nitrogen species released from activated macrophages. These results do not exclude that peroxynitrite was formed at low steady-state concentrations inside the cells and rapidly consumed by scavengers, e.g. GSH, ascorbate, or urate, but they argue against the common view that large amounts of peroxynitrite are released from activated macrophages to kill adjacent target cells. In fact, there are a few previous studies suggesting that the killing of pathogens and tumor cells by activated macrophages is mediated by NO rather than peroxynitrite (40–42).

Our data on intracellular protein tyrosine nitration suggest that peroxynitrite, even if produced, does not contribute to nitration in activated macrophages. When cells were treated with either IFN-\( \gamma \)/LPS or IFN-\( \gamma \)/LPS, formation of protein-bound 3-nitrotyrosine was considerably delayed and became significant only when the production of NO/nitrite had virtually ceased, indicating that nitration was not dependent on the presence of active NO synthase and, thus, not mediated by peroxynitrite. This conclusion is further supported by our results obtained with the peroxynitrite scavengers methionine and MnTBAP, both of which had no considerable effects on nitration (cf. Figs. 6 and 7). Co-stimulation of macrophages with IFN-\( \gamma \)/LPS and the phorbol ester PMA, which triggers \( \text{O}_2^- \) release through activation of protein kinase C (29), led to a significant increase in DHR oxidation that was sensitive to the NO synthase and protein kinase C inhibitors L-NNA and H-7, respectively. Thus, the simultaneous generation of NO and \( \text{O}_2^- \) does indeed result in the formation of peroxynitrite that is detectable as DHR oxidation. However, despite this apparent continuous release of peroxynitrite over 4 h, the addition of PMA to IFN-\( \gamma \)/LPS-activated macrophages did not result in increased protein tyrosine nitration even though treating the cells with authentic peroxynitrite led to fairly high levels of protein-bound 3-nitrotyrosine. These data agree well with previous in vitro findings showing that, in contrast to bolus addition of authentic peroxynitrite, the continuous generation of NO/\( \text{O}_2^- \) from various donor systems does not nitrate free tyrosine (6–10). Based on kinetic simulations, we proposed that the poor nitrating efficiency of NO/\( \text{O}_2^- \) may be a consequence of the resulting relatively low peroxynitrite steady-state concentrations, a condition under which dimerization of tyrosyl radicals to form dityrosine out-competes nitration, i.e. the reaction of tyrosyl radicals with NO/\( \text{O}_2^- \) (8). The present results suggest that this conclusion is not confined to test tube chemistry but also holds for the chemical reactivity of the NO/\( \text{O}_2^- \)/tyrosine system in intact cells.

The time course of 3-nitrotyrosine formation indicated that the nitration reaction was dependent on the intracellular levels of nitrite, the stable oxidation product of NO. Indeed, treatment of non-stimulated macrophages with nitrite led to formation of similar amounts of 3-nitrotyrosine as activation of the cells with IFN-\( \gamma \)/LPS. The ~3-fold greater nitrating efficiency of IFN-\( \gamma \)/ZY, despite similar rates of NO formation, suggests that additional factors contribute to tyrosine nitration. Several analytical methods applied previously for the determination of 3-nitrotyrosine in cell and tissue extracts were shown to yield false positive results in the presence of nitrite (4, 5). However, the fairly sophisticated method described by Shigenaga et al. (24) that we used in this study involves rigorous removal of nitrite and showed no increase in the N-AcATyr levels when cell homogenates were treated with 0.1 mM nitrite before sample preparation (data not shown). The nitrite dependence of tyrosine nitration pointed to the involvement of a heme peroxidase as reported previously for nitration in neutrophils (13) and eosinophils (14). This assumption was confirmed by the complete inhibition of 3-nitrotyrosine formation upon removal of \( \text{H}_2\text{O}_2 \) with PEG-CAT and the pronounced inhibitory effect of the heme-site peroxidase inhibitor NaN\(_3\). Together, these data strongly suggest that an as yet unidentified peroxidase catalyzes tyrosine nitration in cytokine-activated RAW 264.7 macrophages. Similar evidence against peroxynitrite as a mediator of tyrosine nitration was recently obtained with primary murine peritoneal macrophages activated in vitro with IFN-\( \gamma \)/LPS as well as macrophages isolated from mice subjected to systemic inflammation by treatment with heat-inactivated Corynebacterium parvum.\(^2\)

The occurrence of heme peroxidases in macrophages is still a controversial issue. It has been shown that myeloperoxidase activity declines rapidly during differentiation of monocytes into macrophages (43), but peroxidase activity was detected in several types of resident tissue macrophages, such as rodent and human alveolar as well as peritoneal macrophages (44–47). Unlike monocytes, which express peroxidase in the primary lysosomes (48), resident macrophages contain peroxidase activity in the rough endoplasmic reticulum and the perinuclear cisterna (48–50). It has been suggested that macrophage peroxidase activity results from acquisition of exogenous peroxidases by vesicular transport or phagocytosis of peroxidase-positive cells (51). However, myeloperoxidase mRNA has been isolated from thioglycollate-elicited mouse peritoneal macrophages, indicating that myeloperoxidase gene expression is inducible in macrophages by selected immunological stimuli (52). This is further indicated by a recent study showing that granulocyte macrophage colony-stimulating factor up-regulates expression of active myeloperoxidase in macrophages residing in atherosclerotic lesions (53). The macrophage cell line RAW 264.7 that we used in this study was established from murine peritoneal macrophages elicited by the intraperitoneal injection of Abelson leukemia virus. Thus, it is conceivable that myeloperoxidase is indeed expressed in this cell line, as suggested by our immunoblot analyses. So far, macrophage heme peroxidases have been only poorly characterized (54), and further work is required to clarify the role of these enzymes in macrophage-dependent nitration and cytotoxicity.

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REFERENCES

1. Mayer, B., and Hemmens, B. (1997) Trends Biochem. Sci. 22, 453–498
2. Beckman, J. S., and Koppenol, W. H. (1996) Am. J. Physiol. Cell Physiol. 40, C1424–C1437
3. Locheropoulos, H. (1998) Arch. Biochem. Biophys. 356, 1–11
4. Halliwell, B., Zhao, K., and Whitman, M. (1999) Free Radical Res. 31, 651–669
5. van der Vliet, A., Eiserich, J. P., Shigenaga, M. K., and Cross, C. E. (1999) Am. J. Respir. Crit. Care Med. 160, 1–9
6. van der Vliet, A., Eiserich, J. P., O’Neill, C. A., Halliwell, B., and Cross, C. E. (1995) Arch. Biochem. Biophys. 319, 341–349
7. Pfeiffer, S., and Mayer, B. (1998) J. Biol. Chem. 273, 27280–27285
8. Pfeiffer, S., Schmidt, K., and Mayer, B. (2000) J. Biol. Chem. 275, 6346–6352
9. Golstein, S., Czapelski, G., Lind, J., and Merenyi, G. (2000) J. Biol. Chem. 275, 3031–3036
10. Hodges, G. R., Marwaha, J., Paul, T., and Ingold, K. U. (2000) Chem. Res. Toxicol. 13, 1287–1293
11. Sawaya, T., Alahari, T., and Maeda, H. (2000) J. Biol. Chem. 275, 32467–32474
12. van der Vliet, A., Eiserich, J. P., Halliwell, B., and Cross, C. E. (1997) J. Biol. Chem. 272, 7617–7625
13. Eiserich, J. P., Hristova, M., Cross, C. E., Jones, A. D., Freeman, B. A., Halliwell, B., and van der Vliet, A. (1998) Nature 391, 395–397
14. Wu, W., Chen, Y., and Hazen, S. L. (1999) J. Biol. Chem. 274, 25933–25944

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INVolvEMENT OF A Peroxidase/Nitrite PATHWAY RATHER THAN 
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