*Proc. Natl. Acad. Sci. USA*
Vol. 94, pp. 3211–3216, April 1997
Medical Sciences

# Inflammation and $NO_x$-induced nitration: Assay for 3-nitrotyrosine by HPLC with electrochemical detection

**(peroxynitrite/nitric oxide synthase/reactive nitrogen oxide species/zymosan)**

MARK K. SHIGENAGA*, HO H. LEE, BENJAMIN C. BLOUNT, STEPHAN CHRISTEN, ERIC T. SHIGENO, HELENA YIP, AND BRUCE N. AMES

Division of Biochemistry and Molecular Biology, University of California, Berkeley, CA 94720

*Contributed by Bruce N. Ames, January 3, 1997*

**ABSTRACT** **The identification of $^{15}N$-labeled 3-nitrotyrosine (NTyr) by gas chromatography/mass spectroscopy in protein hydrolyzates from activated RAW 264.7 macrophages incubated with $^{15}N$-L-arginine confirms that nitric oxide synthase (NOS) is involved in the nitration of protein-bound tyrosine (Tyr). An assay is presented for NTyr that employs HPLC with tandem electrochemical and UV detection. The assay involves enzymatic hydrolysis of protein, acetylation, solvent extraction, *O*-deacetylation, and dithionite reduction to produce an analyte containing *N*-acetyl-3-aminotyrosine, an electrochemically active derivative of NTyr. We estimate the level of protein-bound NTyr in normal rat plasma to be ≈0–1 residues per $10^6$ Tyr with a detection limit of 0.5 per $10^7$ Tyr when ≥100 nmol of Tyr is analyzed and when precautions are taken to limit nitration artifacts. Zymosan-treated RAW 264.7 cells were shown to have an ≈6-fold higher level of protein-bound NTyr compared with control cells and cells treated with $N^G$-monomethyl-L-arginine, an inhibitor of NOS. Intraperitoneal injection of F344 rats with zymosan led to a marked elevation in protein-bound NTyr to ≈13 residues per $10^6$ Tyr, an ≈40-fold elevation compared with plasma protein of untreated rats; cotreatment with $N^G$-monomethyl-L-arginine inhibited the formation of NTyr in plasma protein from blood and peritoneal exudate by 69% and 53%, respectively. This assay offers a highly sensitive and quantitative approach for investigating the role of reactive byproducts of nitric oxide in the many pathological conditions and disease states associated with $NO_x$ exposure such as inflammation and smoking.**

The proposed role for reactive nitrogen oxide species ($NO_x$) in many pathological conditions related to human disease indicates a need for assays that detect the associated molecular modifications. One of these $NO_x$ species, peroxynitrite, which is generated in large part by the rapid reaction of endogenously formed nitric oxide (·NO) and superoxide ($O_2^{\cdot-}$) (1) is proposed, by virtue of its highly reactive nature, to play an important role in the host inflammatory response. Peroxynitrite damages protein (2), lipid (3), and DNA (4, 5) *in vitro* and may also mediate these potentially deleterious effects in various pathological conditions. This reactivity toward cellular constituents may be the basis by which overproduction of ·NO could exert cytotoxic effects (6). Other $NO_x$ and potent nitrating/nitrosating agents such as $\cdot NO_2$ (7), $NO_2Cl$ (8), or $N_2O_3$ (9) may also be relevant in this regard. Previous work has demonstrated that the production of $NO_x$ in rodents assists the body's defenses in killing foreign organisms that might otherwise continue to thrive at the host's expense (10, 11). There also exist many pathological conditions, in which elevated NOS activity is implicated as a contributing factor to cellular injury, including chronic inflammation (12), heart ischemia-reperfusion injury (13, 14), and atherosclerosis (15).

3-Nitrotyrosine (NTyr) has been identified as a stable end product formed upon reaction of free or protein-bound Tyr with $NO_x$ such as peroxynitrite (7, 16), $NO_2^-$ (7, 17, 18), $NO_2^+$ (19), HONO (19), and $NO_2Cl$ (8). Of these, peroxynitrite is postulated, based on its favorable reaction kinetics, to be the most relevant in regard to pathophysiological mechanisms related to many disease states. NTyr has been identified in biological samples using various detection techniques. Polyclonal and monoclonal antibodies raised against peroxynitrite-treated proteins have been used in numerous studies to identify NTyr in tissue sections (15, 20–22). Immunohistochemistry with these antibodies offers a powerful means of localizing NTyr within tissue but is regarded as less quantitative than chromatographic assays. Other quantitative approaches (19, 23–25) have not offered the sensitivity and convenience needed, thus prompting the current investigation.

## MATERIALS AND METHODS

**Materials.** Purchases were obtained as follows: NTyr, 3-aminotyrosine (ATyr), and tetranitromethane from Aldrich; sodium dithionite (DT; sodium hydrosulfite), HPLC-grade acetonitrile, and methanol from Fisher Scientific; [2,3,5,6-$^3H$] L-tyrosine from Amersham; L-arginine·HCl (guanido-$^{15}N_2$, 98%$^+$) from Cambridge Isotope Laboratories (Cambridge, MA); *N*-acetyl-L-tyrosine, L-tyrosine, triethylamine, and gas chromatography/mass spectroscopy (GC-MS) grade acetonitrile (>99%) from Fluka; *N*,*O*-bis(trimethylsilyl)trifluoroacetamide and trifluoroacetic acid from Pierce; $N^G$-monomethyl-L-arginine (NMMA) from Cyclopss Biochem (Salt Lake City); DMEM from Irvine Scientific; undialyzed and dialyzed fetal bovine serum and zymosan A from Sigma; RPMI-SELECT-AMINE and murine recombinant interferon-γ (IFN-γ) from GIBCO/BRL; the transformed murine macrophage cell line RAW 264.7 from the American Type Culture Collection; *Escherichia coli* lipopolysaccharide serotype 055.B5 from Difco; potassium phosphate monobasic and dibasic, extra pure-grade sodium acetate trihydrate, GR-grade 90% formic acid, and phosphoric acid, OmniSolve-grade ethyl acetate, and Instrumental-grade/TRACEPUR HCl from Merck; sodium hydroxide and Baker reagent-grade 5 N volumetric solution from J. T. Baker; AR-grade acetic anhydride from Mallinckrodt; and pronase E from Boehringer Mannheim. MilliQ water free of nitrite and nitrate was used to prepare all buffers, mobile phases, and standard solutions.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.


0027-8424/97/943211-6$2.00/0
PNAS is available online at **http://www.pnas.org**.

Abbreviations: AcATyr, *N*-acetyl 3-aminotyrosine; AcTyr, *N*-acetyltyrosine; ATyr, 3-aminotyrosine; DT, sodium dithionite; EC, electrochemical; IFN-γ, interferon γ; NMMA, $N^G$-monomethyl-L-arginine; NOS, nitric oxide synthase; $NO_x$, reactive nitrogen oxide species; NTyr, 3-nitrotyrosine; GC-MS, gas chromatography/mass spectroscopy.

*To whom reprint requests should be addressed. e-mail: shigen@mendel.berkeley.edu.

**Preparation of Standards.** The synthesis of $^3$H-NTyr was performed by mixing 50 $\mu$Ci (1 Ci = 37 GBq) of high specific activity [2,3,5,6-$^3$H]tyrosine (specific activity = 92 Ci/mmol), 2 $\mu$l of an ethanolic solution of tetranitromethane (840 mM), 20 $\mu$l $H_2O$, and 2 $\mu$l 0.1 M Tris·HCl (pH 8). The mixture was allowed to react for 2 min and purified by HPLC to afford $^3$H-NTyr in 22% yield. *N*-acetyl-NTyr was prepared by acetylating NTyr with acetic anhydride as described below. The identities of the prepared compounds were verified by coelution with authentic standards, UV diode array and mass spectral, and DT-mediated conversion to ATyr. An *N*-acetyl 3-aminotyrosine (AcATyr) standard was prepared by DT-mediated reduction of *N*-acetyl-NTyr and stored without measurable decomposition for 6 months in 0.1 M HCl at −20°C.

**Determination That the Nitro Group Is Derived from the Guanido Group of L-Arginine.** RAW 264.7 macrophages ($3 \times 10^8$) were incubated in 10% dialyzed fetal bovine serum and amino acid defined RPMI (RPMI-SELECT-AMINE) reconstituted with all but $^{14}$N-L-arginine. The defined media was supplemented with 0.5 mM L-arginine·HCl (guanido-$^{15}N_2$) and allowed to exchange with $^{14}$N L-arginine pools for 24 h. The next day, medium was removed, the cells washed twice in PBS, and fresh media of the same composition used on the previous day was added along with lipopolysaccharide (100 ng·ml$^{-1}$) and IFN-$\gamma$ (10 units·ml$^{-1}$). After 7 h, the medium was removed and replaced with Hanks' buffer containing lipopolysaccharide, IFN-$\gamma$, phorbol myristate acetate (100 ng·ml$^{-1}$), BSA (1 mg·ml$^{-1}$), and $^{15}$N L-arginine (0.5 mM) and incubated for an additional 18 h. The culture was centrifuged at 300 × *g* for 5 min at 25°C. The supernatant was then filtered through a Centricon filtration device and the protein retentate washed with PBS to remove residual nitrite and nitrate. This protein was hydrolyzed with 6 M HCl for 24 h at 105°C. The sample was spiked with $^3$H-NTyr and fractionated by C18/OH solid phase extraction (Analytichem International, Harbor City, CA) using increasing amounts of methanol in 0.1 M trifluoroacetic acid to elute the NTyr. The 10% methanol/0.1 M trifluoroacetic acid fraction (42% recovery of $^3$H-Tyr) was concentrated to dryness and derivatized with 100 $\mu$l each of *N*,*O*-bis(trimethylsilyl)trifluoroacetamide and acetonitrile at 100°C for 1 h.

**GC-MS Analysis.** Samples (1 $\mu$l) were delivered by splitless injection using a model 7673 automated liquid sampler linked to a Series II 5890 gas chromatograph/5989A mass spectrometer (Hewlett–Packard) with negative chemical ionization, an injection port temperature of 280°C, a purge activation time of 1 min, and a split vent flow of 50 ml$^{-1}$·min$^{-1}$. Ultra high purity methane (Matheson) was used as the carrier gas at a linear velocity of 55 cm$^{-1}$·sec$^{-1}$. Separations were achieved on a Hewlett–Packard Ultra-2 capillary column (12 m × 0.2 mm internal diameter × 0.33 $\mu$m film thickness) and the following temperature program: 50°C for 1 min followed by a 15°C per min linear ramp to 300°C with a 5-min hold. The GC-MS interface heater and source temperature was set to 300°C and 200°C, respectively.

**Cell Culture.** RAW 264.7 macrophages were cultured at 37°C in 5% $CO_2$ in DMEM supplemented with 10% fetal bovine serum. Cells ($2 \times 10^6$ per well) were incubated in 24-well plates with various combinations of zymosan (0.5 mg·ml$^{-1}$), IFN-$\gamma$ (10 units·ml$^{-1}$) and NMMA (1 mM) for a period of 24 h in a total volume of 2 ml. A portion of the spent media was used for nitrite determination by Griess reactivity and the remainder containing both medium and cells was used for NTyr determinations.

**Zymosan Peritonitis.** All animal experimentation was performed under institutional approval. A stock solution of 50 mg·ml$^{-1}$ zymosan prepared in sterile PBS was heat treated for 30 min at 95°C. Male Fischer 344 rats (180–220 gm) were injected with 250 mg·kg$^{-1}$ of this suspension and killed after 72 h. Some rats also received either 300 or 150 mg·kg$^{-1}$ dose of the NOS inhibitor NMMA immediately following injection with zymosan and further identical doses of this inhibitor on the second and third day. Rats were euthanized at 72 h with ether, and an axial incision was made to recover blood for plasma preparation (700 × *g* for 10 min). Sterile PBS (10 ml) with 20 units·ml$^{-1}$ of sodium heparin was injected i.p. and the peritoneal cavity was massaged for 10 sec to distribute the PBS. The peritoneal exudate was removed with a syringe and transferred to a new Falcon polypropylene tube, which was subsequently centrifuged at 700 × *g* for 10 min at 4°C to pellet the peritoneal exudate cells. The cell-free peritoneal exudate was frozen at −80°C until used.

**Preparation of Protein for Enzymatic Hydrolysis.** Protein suspensions were prepared in 0.1 M NaOAc buffer (pH 7.2) at an initial concentration of 3–5 mg protein per 0.5 ml. This suspension was precipitated with 0.8 ml HPLC grade acetonitrile, vortex mixed for 3 sec, and centrifuged at 700 × *g* for 10 min. The pellet was washed in 0.8 ml of NaOAc buffer, reprecipitated with 0.8 ml acetonitrile, vortex mixed, and centifuged, and the supernatant discarded as described above and washed once again. The sample was resuspended, sonicated (≈6-sec, 40-W burst) to produce a fine suspension of protein, precipitated once again, resuspended in 0.5 ml of 0.1 M NaOAc (pH 7.2), and sonicated once again. The extensive washing procedure was necessary to remove residual nitrite/nitrate and other contaminants. The sample was incubated overnight (12–16 h) at 50°C with 0.6 mg of dialyzed pronase E [≈20% (wt/wt) of the target protein]. Pronase E was prepared by dialysis (Spectra/Por 7; 15 kDa molecular cut-off; Spectrum Laboratories, Houston) against three daily changes of 0.1 M NaOAc (pH 7.2) (500 volume excess). The pronase contributed <2% of the Tyr recovered. The pelletable residue remaining after digestion of plasma protein was mostly nonproteinacious material and contained <2.5% of the total protein. Control experiments with nitrated bovine serum albumin indicated that the rates of enzymatic digestion for protein-bound NTyr and Tyr were essentially the same.

**Derivatization and Extraction.** An equal volume (250 $\mu$l) of 3 M $KH_2PO_4$ (pH 9.6) was added to 50% of the sample followed by 20 $\mu$l acetic anhydride. After allowing the reaction to proceed for 10 min at 25°C, 1 ml of ethyl acetate was added followed by 135 $\mu$l of formic acid. The sample was vortex mixed for 10 sec and centrifuged at 700 × *g* for 1 min. The ethyl acetate phase was transferred to a 2 ml polypropylene microcentrifuge tube and concentrated to dryness under a gentle stream of $N_2$ in a 50°C water bath.

**Deacetylation and Dithionite Reduction.** Selective deacetylation of the phenolic acetate group of Tyr and NTyr was achieved by resuspending the sample in 175 $\mu$l of 0.3 M NaOH and allowing this mixture to react for 30 min at 37°C. After deacetylation, 175 $\mu$l of 1 M $KH_2PO_4$ (pH 6.5) buffer was added to adjust the pH to ≈7–7.5. The nitro substituent was reduced to the corresponding amine by adding 10 $\mu$l of 100 mM DT solution prepared in MilliQ water. After 10 min at 25°C, 35 $\mu$l of concentrated HCl was added to the sample. The samples were transferred to 30-kDa cutoff Millipore Ultrafree MC filters and centrifuged at 7,800 × *g* for 20 min. The filtrate was transferred to polypropylene autoinjector vials and analyzed.

**HPLC-Electrochemical/UV Analysis.** Samples (100 $\mu$l) were delivered by an Hitachi AS4000 autosampler and chromatographed using a Waters model 625 solvent delivery system in combination with an Alltech Associates 5 $\mu$m $C_{18}$ Alltima column (4.6 mm × 25 cm) equipped with a precolumn cartridge assembly at a flow rate of 1 ml·min$^{-1}$. The gradient was as follows: 0–14 min 100% solvent A (1% methanol in 10 mM phosphoric acid, pH 2.56), 14–20 min linear ramp to 100% solvent B (50% methanol in 10 mM phosphoric acid), 20–35 min 100% solvent B, and 35–38 min linear ramp to 100% solvent A. Electrochemical (EC) detection of AcATyr utilized an ESA (Bedford, MA) model 5200

Coulochem detector and model 5011 analytical cell with potentials of electrodes 1 and 2 adjusted to 0.00 and 0.07 V, respectively. *N*-acetyltyrosine (AcTyr) was detected at 280 nm (model 773; Kratos Analytical Instruments). AcATyr and AcTyr elute at ≈11.5 and 23.5 min. Mole quantities were determined against authentic calibrated standards. The calculated mole ratio values AcATyr per $10^6$ AcTyr and NTyr per $10^6$ Tyr are treated as equivalent expressions.

## RESULTS

**Incorporation of $^{15}$N-Labeled Guanido Nitrogen Atom of L-Arginine into NTyr: Detection of Protein-Bound 3-($^{15}NO_2$)-Tyrosine.** Macrophage NOS catalyzes the NADPH-dependent oxidation of L-arginine to L-citrulline and ·NO (26), the latter of which can subsequently be transformed to $NO_x$. To establish L-arginine as the source of the nitro substituent nitrogen atom in NTyr, L-arginine (guanido-$^{15}N_2$) was used as the substrate for the inducible NOS present in RAW 264.7 macrophages.

Table 1 shows the major ions obtained from the negative chemical ionization of the tri-trimethylsilyl (TMS) derivative of authentic NTyr and putative NTyr isolated from protein hydrolyzates of RAW 264.7 macrophages activated with lipopolysaccharide, IFN-γ, and phorbol myristate acetate in the presence of L-arginine·HCl (guanido-$^{15}N_2$). Ionization of authentic tri-TMS-NTyr (M = $m/z$ 441) results in the loss of one *O*-TMS group producing the characteristic ion at $m/z$ 352 $[M - C_3H_9OSi]^-$ or both *O*-TMS groups and decarboxylation to yield the ion $m/z$ 236 $[M - C_7H_{18}O_3Si_2]^-$. By comparing the relative abundances of the major ions obtained from analysis of the sample incubated with $^{15}$N-L-arginine to that of authentic NTyr (Table 1), we observe a marked shift in ion abundance from $m/z$ 352 and $m/z$ 236 to $m/z$ 353 and $m/z$ 237, respectively. This indicates that one of the guanido nitrogens of L-arginine is the source of the nitro group nitrogen atom of NTyr, thereby confirming the role of NOS in the formation of NTyr.

**Electrochemical Detection of AcATyr.** Our attempts to apply mass spectral analysis to the quantitation of NTyr met with difficulty though recent efforts by others have proved successful (see note added in proof). We therefore measured NTyr by HPLC with EC detection, a method with sensitivity comparable to GC-MS with negative chemical ionization (≈20 vs. 5 fmol on-column detection limit, respectively) and ≈$10^3$ fold higher than that of UV detection. EC detection of NTyr required +0.88 V for maximal response, making it difficult to selectively detect this modified amino acid among other components present in protein hydrolyzates from activated macrophage incubates. This led to the development of a method involving the DT-mediated conversion of NTyr to ATyr (27), an EC active compound with a low oxidation potential. The poor retention of ATyr on a $C_{18}$ reversed phase column, however, prompted the development of a derivatization and extraction scheme that enriches for hydrophobic amino acids and improves the chromatography. The method for measuring

Table 1. Activated macrophages catalyze the incorporation of the guanido nitrogen of L-arginine into protein-bound NTyr

| $m/z$ | Authentic NTyr | NTyr from $^{15}$N-L-Arg + Macrophage |
|---|---|---|
| 236 | 91.4 ± 14.1 | 21.5 ± 0.4 |
| 237 | 16.9 ± 2.6 | 89.3 ± 3.9 |
| 238 | 5.3 ± 0.9 | 16.5 ± 1.0 |
| 352 | 100.0 ± 16.3 | 18.1 ± 4.6 |
| 353 | 28.1 ± 4.8 | 100.0 ± 10.4 |
| 354 | 11.9 ± 2.3 | 75.2 ± 5.2 |

Relative ion intensities of the major fragments, $m/z$ 236–238 $[M - C_7H_{18}O_3Si_2]^-$ and $m/z$ 352–354 $[M - C_3H_9OSi]^-$ derived from the corresponding tri-trimethylsilyl derivatives. Values represent the mean ± SD of three determinations.

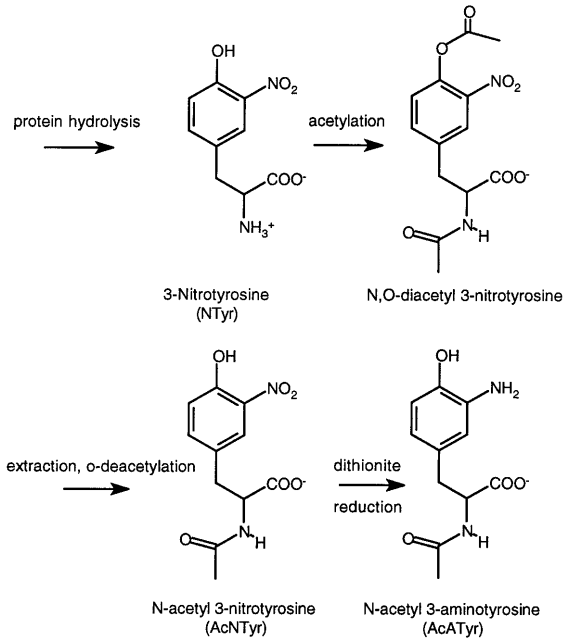


FIG. 1. Conversion of NTyr to AcATyr

NTyr (Fig. 1) involves enzymatic hydrolysis of protein, acetylation, extraction, *O*-deacetylation, and chemical reduction with DT to produce AcATyr. AcATyr has a half potential of +0.037 V with >90% oxidation at 0.070 V. The recovery of NTyr as AcATyr in rat plasma was determined across a broad range of NTyr (225 fmol to 22.5 pmol) that had been spiked into this biological matrix prior to enzymatic digestion and processing. The recovery was estimated to be ≈70–75% with good linearity ($r^2$ = 0.9995). Tyr was shown to be recovered with similar efficiency, thus no correction for recoveries are made in expressing the mole ratios of AcATyr to AcTyr. Enzymatic hydrolysis of tetranitromethane-treated BSA resulted in >95% release of both NTyr and Tyr.

**Acid Artifacts and Nitrotyrosine.** Numerous examples exist for acid-catalyzed nitration of phenolics (28) such as Tyr (19), in the presence of nitrites or nitrates. AcTyr, in the presence of physiological concentrations (10 μM) of nitrite or nitrate under acidic conditions (1 M HCl), forms significant amounts of *N*-acetyl-NTyr, indicating that *ex vivo* nitrosation/nitration of Tyr can result in levels of NTyr that may obscure those formed *in vivo* (Table 2). Addition of 1% phenol to AcTyr samples containing 10 μM sodium nitrate inhibited artifact formation effectively (≈63%), though not completely as did other phenolic compounds and ammonium sulfamate (data not shown).

Table 2. Acid-catalyzed nitrosation/nitration of N-AcTyr

| | AcATyr per $10^6$ AcTyr |
|---|---|
| AcTyr | 0.4 ± 0.1 |
| + HCl | 2.0 ± 0.3† |
| + HCl + $NaNO_2$* | 98.7 ± 10.0‡ |
| + HCl + $NaNO_3$ | 10.3 ± 7.0§ |
| + HCl + $NaNO_3$ + 1% phenol | 3.8 ± 2.3§ |

AcTyr (1 mM) was incubated in 0.1 M NaOAc (pH 7.2) for 30 min on ice. Where indicated, 1 M HCl, sodium nitrite (10 μM), or sodium nitrate (10 μM), and/or 1% phenol were also included in the incubate. Samples were concentrated to dryness, reconstituted in 0.5 M $KH_2PO_4$ buffer (pH 7.0), reduced with DT, and analyzed as described.

*Control experiments indicated that *N*-acetyl-NTyr was the exclusive product of this nitrosation reaction. Values represent the mean ± SD of three determinations.

†$P < 0.0005$; ‡$P < 0.0001$; §$P < 0.05$ compared to AcTyr control; one-tailed Students *t* test.

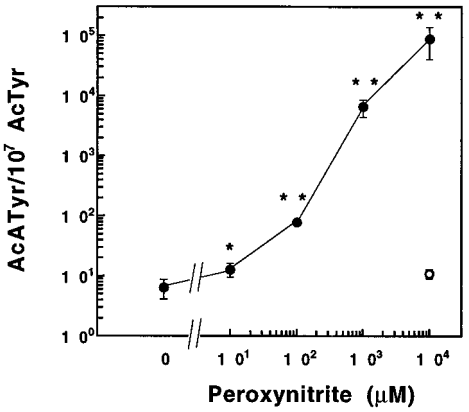


FIG. 2. Treatment of rat plasma with peroxynitrite. Concentration-dependent formation of protein-bound NTyr. Rat plasma (200 μl) was treated with peroxynitrite (●) and processed according to *Materials and Methods*. Rat plasma was also treated with peroxynitrite that was allowed to decompose at pH 7.2 for 15 min before addition (○). Values represent the average of three determinations ± SD. *, $P < 0.05$; **, $P < 0.01$, one-tailed Students *t* test.

**Peroxynitrite-Mediated Nitration of Rat Plasma Protein.** The rigorous control of residual nitrite and nitrate allowed us to examine the nitrating potential of peroxynitrite in a biological matrix. Rat plasma was treated with peroxynitrite and the amount of NTyr (as AcATyr) measured (Fig. 2.). Incremental increases in NTyr were observed with as little as 10 μM peroxynitrite ($P < 0.05$, one-tailed Student's *t* test), despite the presence of 23 μM ascorbate and other plasma antioxidants that were not monitored. The NTyr levels correlated well with the amount of peroxynitrite added to the samples. A marked rise in NTyr formation was observed with the addition of 1 mM peroxynitrite, presumably reflecting consumption of key plasma antioxidants that, in turn, allows for greater nitration efficiency. The addition of 10 mM decomposed peroxynitrite, however, produced NTyr values that were comparable to untreated plasma samples, thus indicating the specificity of peroxynitrite to the NTyr measured, and the rigor of the removal of contaminating traces of nitrite and nitrate. When rat plasma was spiked with 10 μM sodium nitrite and 100 μM sodium nitrate prior to sample processing, no significant elevation in NTyr was observed as the AcATyr per $10^6$ AcTyr ratio of spiked samples (0.05 ± 0.07) versus unspiked samples (0.04 ± 0.04; both sets expressed as mean ± SD, $n = 4$) were comparable. These results again confirm the efficient removal of nitrite and nitrate from the sample. Comparable baseline values (0–1 AcATyr per $10^6$ AcTyr) are observed with human plasma samples from healthy individuals (data not shown).

**Zymosan-Activated RAW 264.7 Macrophage and Protein Nitration.** The activation of RAW 264.7 macrophages with zymosan alone resulted in 5 NTyr per $10^6$ Tyr, which increased further to ≈7 per $10^6$ Tyr when IFN-γ was also present (Table 3, Fig. 3.). Treatment of RAW cells with IFN-γ alone had no significant effect. The marked elevation in NTyr observed with the combination of zymosan and IFN-γ was blocked almost completely with 1 mM NMMA. Nitrite and NTyr levels correlated well.

Table 3. Formation of protein-bound NTyr by zymosan and IFN-γ-treated RAW 264.7 macrophages

| | AcATyr per $10^6$ AcTyr | Nitrite, μM |
|---|---|---|
| Untreated | 1.05 ± 0.46 | 4.6 ± 1.0 |
| Zymosan | 5.13 ± 0.57* | 53.5 ± 1.2 |
| IFN-γ | 1.30 ± 0.49 | 11.1 ± 2.9 |
| Zymosan + IFN-γ | 7.52 ± 0.13*† | 71.0 ± 3.7 |
| Zymosan + IFN-γ + NMMA | 1.30 ± 0.43 | 12.4 ± 0.4 |

Values are expressed as mean ± SD ($n = 4$ determinations for all conditions).
*$P < 0.01$ vs. untreated control; †$P < 0.001$ (zymosan + IFN-γ vs. Zymosan); *P* values calculated by one-way ANOVA, Bonferroni multiple comparison test.

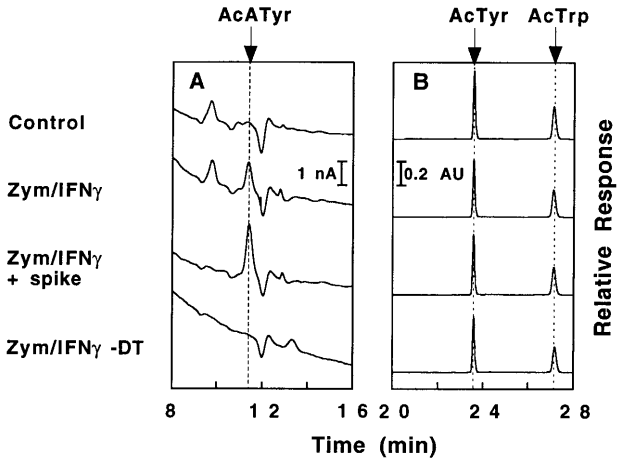


FIG. 3. Representative HPLC chromatograms. AcATyr (*A*, EC) and AcTyr (*B*, UV) were monitored in processed hydrolyzates from RAW 264.7 cultures: spike = 300 fmol authentic AcATyr.

**Zymosan Peritonitis and Protein Nitration.** F344 rats injected i.p. with a single 250 mg·kg$^{-1}$ dose of zymosan exhibited overt signs of systemic illness within the first 2–4 h after injection. Approximately 20% of the zymosan-treated rats died within the first 24–36 h while the surviving animals showed marked weight loss (10–15%) over the first 72-h period after injection. Leakage of plasma protein into the peritoneal cavity was noted in all zymosan-treated animals.

Plasma protein isolated from either blood or peritoneal exudate revealed markedly higher levels of NTyr compared with blood plasma of PBS-treated animals (Table 4). Treatment of rats with zymosan and NMMA was complicated by their low tolerance to this combination of agents at the doses used. Initially, injection of 300 mg·kg$^{-1}$ of NMMA to the zymosan-treated rats resulted in the loss of seven of the eight animals treated, suggesting a possible protective effect or important regulatory role of ·NO in inflammatory injury. Lowering the dose to 150 mg·kg$^{-1}$ increased the survival to two out of a total of five animals. From these three surviving animals, 69% and 53% reduction in plasma protein NTyr was observed from blood and exudate, respectively, compared to zymosan treatment only, thus demonstrating in vivo a relationship between NTyr formation and NOS activity. The marked reduction in NTyr, however, did not correlate with improved vitality. Animals treated with the combination of zymosan and NMMA lost weight, exhibited comparable amounts of plasma protein in the peritoneal cavity, and showed signs of morbidity similar to that of the rats treated with zymosan only.

Table 4. NTyr in plasma protein of blood and peritoneal exudate fluid of zymosan-treated F344 rats

| | | AcATyr per $10^6$ AcTyr | |
|---|---|---|---|
| | *n* | Plasma | Exudate |
| PBS | 6 | 0.37 ± 0.32 | ND |
| Zymosan | 5 | 12.46 ± 3.13* | 14.11 ± 2.33* |
| Zymosan + NMMA | 3 | 3.82 ± 2.88† | 6.69 ± 1.48‡ |

Values are given as mean ± SD. ND, not determined.
*$P < 0.001$ vs. PBS-plasma; †$P < 0.001$ vs. zymosan; ‡$P < 0.01$ vs. zymosan-exudate; *P* values calculated by one-way ANOVA, Bonferonni multiple comparison test.

## DISCUSSION

Evidence implicating $NO_x$ in inflammation, cancer, and many of the degenerative diseases of aging has prompted an assessment of the role of NOS in tyrosine nitration and the development of a sensitive and selective HPLC-EC assay to quantitate NTyr as AcATyr. This assay for NTyr complements the widely used immunohistochemical assay and quantitates the NOS-dependent formation of this molecular lesion in a variety of sample types, thereby potentially extending the range of experiments designed to examine the relationship between ·NO production and the many pathological conditions associated with this enzyme activity.

Rigorous control of residual nitrite and nitrate and the use of mild nonacidic enzymatic hydrolysis reduced the baseline ($\leq$1 NTyr per $10^6$ Tyr) to levels considerably lower than those previously reported (23, 25, 29, 30) and increased the effective sensitivity of NTyr determination from biological samples. Artifactual levels of NTyr ($10^1$–$10^4$ NTyr per $10^6$ Tyr) were seen without this rigorous cleanup.

The yeast cell wall component zymosan is a potent activator of phagocytic cells and has been used in the current study to validate the NTyr assay *in vitro* and *in vivo*. Zymosan- and IFN-γ-treated RAW 264.7 macrophages produce marked increases in NTyr, possibly reflecting the formation of the same $NO_x$ that are responsible for the killing of foreign organisms. Murine peritoneal macrophages, for example, kill *Leishmania major* when challenged with zymosan and IFN-γ, an effect that is inhibited by addition of the NOS inhibitor, *N*-iminoethyl-L-ornithine (31). Thus, production of $NO_x$, that include such potent oxidants and nitrating agents as peroxynitrite or $N_2O_3$, could in large part be responsible for this cytotoxic effect.

The marked elevation in NTyr found with zymosan-treated RAW 264.7 or in zymosan-induced peritonitis illustrates a vigorous response by the host inflammatory machinery to an agent that it recognizes as foreign. This response is important in the host's resistance to the invading pathogen, but can also damage host tissue. For example, zymosan-induced peritonitis, an acute non-pathogen-induced inflammation is characterized by extravasation of plasma protein and infiltration of activated neutrophils and macrophages into the peritoneal cavity. This model of sterile peritonitis exhibits many features of the host response to microbial invasion of tissues, including high output phagocyte-derived synthesis of ·NO, oxidant production and damage, glutathione depletion, hypotension, and distant organ failure (32, 33). When this host inflammatory response is drawn out under chronic conditions (i.e., hepatitis, idiopathic bowel disease), elevated ·NO production is also likely to participate in the process leading to cellular injury. The identification of NTyr can provide the necessary evidence to link NOS activity to these chronic diseases.

The low levels of NTyr detected in treated animals suggests that Tyr may not be a preferred target of $NO_x$. Monitoring Tyr nitration, however, can provide evidence for the existence of endogenously produced $NO_x$. Measuring the nitration of other phenolic species within the body could serve a similar function. Dietary nucleophilic phenolic compounds such as γ-tocopherol (34) has been shown to be a defense "trap" for $NO_x$, and the many polyphenolic compounds in our diet (flavonoids, tea, and wine polyphenolics), may also compete effectively with Tyr for the endogenous $NO_x$ generated during inflammation (35). The reported anti-inflammatory action of these chemicals, may, in part, be rooted in their ability to efficiently trap these species.

We have described in the current study that zymosan and cytokine activated murine macrophages produce $NO_x$ capable of nitrating tyrosine residues in protein. Other cells, including those with macrophage-like function (e.g., including Kupffer cells, alveolar macrophages, microglia, Langerhans cells, and retinal pigment epithelial cells), can also be triggered by cytokines to produce elevated levels of ·NO that may also be accompanied by increased synthesis of $NO_x$ such as peroxynitrite. The increased output of $NO_x$ by these cells may contribute to increased resistance to various pathogens as well as susceptibility to cell injury that can occur during acute and chronic inflammatory conditions. The assay for NTyr could assist in delineating the role of macrophage-derived $NO_x$ in these processes, and serve as a biomarker in animal models and human populations to determine the efficacy of nutritional or pharmacological interventions.

**Note Added in Proof.** The successful application of GC-MS in the analysis of NTyr from low density lipoprotein isolated from human atherosclerotic tissue has recently been described (36).

We thank J. Eiserich, A. Glazer, J. Hibbs, and A. van der Vliet for suggestions and critical reading of this manuscript; T. Hagen, C. Wehr, and J. Lykkesfeldt for help with animals; C. Grunfeld (San Francisco Veterans Administration Medical Center) for the RAW 264.7 macrophages; and A. Fisher for maintaining these cultures. This work was supported by National Cancer Institute Outstanding Investigator Grant CA 39910 and National Institute of Environmental Health Sciences Center Grant ES 01896 (B.N.A.).

1. Huie, R. E. & Padmaja, S. (1993) *Free Radical Res. Commun.* **18,** 195–199.
2. Ischiropoulos, H., Zhu, L. & Beckman, J. S. (1992) *Arch. Biochem. Biophys.* **298,** 446–451.
3. Radi, R., Beckman, J. S., Bush, K. M. & Freeman, B. A. (1991) *Arch. Biochem. Biophys.* **288,** 481–487.
4. Douki, T., Cadet, J. & Ames, B. N. (1996) *Chem. Res. Toxicol.* **9,** 3–7.
5. Yermilov, V., Rubio, J., Becchi, M., Friesen, M. D., Pignatelli, B. & Ohshima, H. (1995) *Carcinogenesis* **16,** 2045–2050.
6. Beckman, J. S., Beckman, T. W., Chen, J., Marshall, P. A. & Freeman, B. A. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 1620–1624.
7. van der Vliet, A., Eiserich, J. P., O'Neill, C. A., Halliwell, B. & Cross, C. E. (1995) *Arch. Biochem. Biophys.* **319,** 341–349.
8. Eiserich, J. P., Cross, C. E., Jones, A. D., Halliwell, B. & van der Vliet, A. (1996) *J. Biol. Chem.* **271,** 19199–19208.
9. Kharitonov, V. G., Sundquist, A. R. & Sharma, V. S. (1995) *J. Biol. Chem.* **270,** 28158–28164.
10. Nathan, C. F. & Hibbs, J. B., Jr. (1991) *Curr. Opin. Immunol.* **3,** 65–70.
11. Hibbs, J. B., Jr., Vavrin, Z. & Taintor, R. R. (1987) *J. Immunol.* **138,** 550–565.
12. Liu, R. H. & Hotchkiss, J. H. (1995) *Mutat. Res.* **339,** 73–89.
13. Matheis, G., Sherman, M. P., Buckberg, G. D., Haybron, D. M., Young, H. H. & Ignarro, L. J. (1992) *Am. J. Physiol.* **262,** H616–H620.
14. Patel, V. C., Yellon, D. M., Singh, K. J., Neild, G. H. & Woolfson, R. G. (1993) *Biochem. Biophys. Res. Commun.* **194,** 234–238.
15. Beckmann, J. S., Ye, Y. Z., Anderson, P. G., Chen, J., Accavitti, M. A., Tarpey, M. M. & White, C. R. (1994) *Biol. Chem. Hoppe-Seyler* **375,** 81–88.
16. Ischiropoulos, H., Zhu, L., Chen, J., Tsai, M., Martin, J. C., Smith, C. D. & Beckman, J. S. (1992) *Arch. Biochem. Biophys.* **298,** 431–437.
17. Prutz, W. A., Monig, H., Butler, J. & Land, E. J. (1985) *Arch. Biochem. Biophys.* **243,** 125–134.
18. Kikugawa, K., Kato, T. & Okamoto, Y. (1994) *Free Radical Biol. Med.* **16,** 373–382.
19. Ohshima, H., Friesen, M., Brouet, I. & Bartsch, H. (1990) *Food Chem. Toxicol.* **28,** 647–652.
20. Miller, M. J., Thompson, J. H., Zhang, X. J., Sadowska-Krowicka, H., Kakkis, J. L., Munshi, U. K., Sandoval, M., Rossi, J. L., Eloby-Childress, S., Beckman, J. S., Ye, Y. Z., Rodi, C. P., Manning, P. T., Currie, M. G. & Clark, D. A. (1995) *Gastroenterology* **109,** 1475–1483.
21. Kooy, N. W., Royall, J. A., Ye, Y. Z., Kelly, D. R. & Beckman, J. S. (1995) *Am. J. Respir. Crit. Care Med.* **151,** 1250–1254.
22. Ischiropoulos, H., Beers, M. F., Ohnishi, S. T., Fisher, D., Garner, S. E. & Thom, S. R. (1996) *J. Clin. Invest.* **97,** 2260–2267.
23. Kaur, H. & Halliwell, B. (1994) *FEBS Lett.* **350,** 9–12.
24. Haddad, I. Y., Ischiropoulos, H., Holm, B. A., Beckman, J. S., Baker, J. R. & Matalon, S. (1993) *Am. J. Physiol.* L555–L564.

25. Schulz, J. B., Matthews, R. T., Muqit, M. M., Browne, S. E. & Beal, M. F. (1995) *J. Neurochem.* **64,** 936–939.
26. Marletta, M. A., Yoon, P. S., Iyengar, R., Leaf, C. D. & Wishnok, J. S. (1988) *Biochemistry* **27,** 8706–8711.
27. Sokolovsky, M., Riordan, J. F. & Vallee, B. L. (1967) *Biochem. Biophys. Res. Commun.* **27,** 20–25.
28. Olah, G. A., Malhotra, R. & Narang, S. C. (1989) *Nitration: Methods and Mechanisms* (VCH, New York).
29. Ischiropoulos, H., al-Mehdi, A. B. & Fisher, A. B. (1995) *Am. J. Physiol.* **269,** L158–L164.
30. Salman-Tabcheh, S., Guerin, M. C. & Torreilles, J. (1995) *Free Radical Biol. Med.* **19,** 695–698.
31. Assreuy, J., Cunha, F. Q., Epperlein, M., Noronha-Dutra, A., O'Donnell, C. A., Liew, F. Y. & Moncada, S. (1994) *Eur. J. Immunol.* **24,** 672–676.
32. Demling, R., Lalonde, C., Youn, Y. K., Daryani, R., Campbell, C. & Knox, J. (1992) *Am. Rev. Respir. Dis.* **146,** 1272–1278.
33. Boughton-Smith, N. K. & Ghelani, A. (1995) *Inflammation Res.* **44,** Suppl 2, S149–S150.
34. Christen, S., Woodall, A. A., Shigenaga, M. K., Southwell-Keely, P. T., Duncan, M. W. & Ames, B. N. (1997) *Proc. Natl. Acad. Sci. U.S.A.* **94**, 3217–3222.
35. Fiala, E. S., Sodum, R. S., Bhattacharya, M. & Li, H. (1996) *Experientia* **52**, 922–926.
36. Leeuwenburgh, C., Hardy, M. M., Hazen, S. L., Wagner, P., Oh-ishi, S., Steinbrecher, U. P. & Heinecke, J. W. (1997) *Biol. Chem.* **272**, 1433–1436.