Tyrosine Nitrination by Peroxynitrite Formed from Nitric Oxide and Superoxide Generated by Xanthine Oxidase*

Peroxynitrite (ONOO\textsuperscript{--}) is a potent nitrating and oxidizing agent that is formed by a rapid reaction of nitric oxide (NO) with superoxide anion (O\textsubscript{2}\textsuperscript{−}). It appears to be involved in the pathophysiology of many inflammatory and neurodegenerative diseases. It has recently been reported (Pfeiffer, S., and Mayer, B. (1998) J. Biol. Chem. 273, 27280–27285) that ONOO\textsuperscript{--} generated at neutral pH from NO and O\textsubscript{2}\textsuperscript{−} (NO/O\textsubscript{2}) was substantially less efficient than preformed ONOO\textsuperscript{--} at nitrating tyrosine. Here we re-evaluated tyrosine nitrination by NO/O\textsubscript{2} with a shorter incubation period and a more sensitive electrochemical detection system. Appreciable amounts of nitrotyrosine were produced by ONOO\textsuperscript{--} formed in situ (2.9 μM for 5 min; 10 nM/s) by NO/O\textsubscript{2} flux obtained from propylamine NONOate (CH\textsubscript{3}N[NO-N]NO\textsubscript{−}) (CH\textsubscript{3})\textsubscript{2}NH\textsuperscript{−}CH\textsubscript{3}) and xanthine oxidase using pterin as a substrate in a phosphate buffer (pH 7.0) containing 0.1 mM L-tyrosine. The yield of nitrotyrosine by this NO/O\textsubscript{2} flux was approximately 70% of that produced by the same flux of preformed ONOO\textsuperscript{--} (2.9 μM/s). When hypoxanthine was used as a substrate, tyrosine nitrination by NO/O\textsubscript{2} was largely eliminated because of the inhibitory effect of uric acid produced during the oxidation of hypoxanthine. Tyrosine nitrination caused by NO/O\textsubscript{2} was inhibited by the ONOO\textsuperscript{--} scavenger ebselen and was enhanced 2-fold by NaHCO\textsubscript{3}, as would be expected, because CO\textsubscript{2} promotes tyrosine nitration. The profile of nitrotyrosine and dityrosine formation produced by NO/O\textsubscript{2} flux (2.9 μM/s) was consistent with that produced by preformed ONOO\textsuperscript{--}.

Tyrosine nitrination predominated compared with dityrosine formation caused by a low nanomolar flux of ONOO\textsuperscript{--} at physiological concentrations of free tyrosine (<0.5 mM). In conclusion, our results show that NO generated with O\textsubscript{2}\textsuperscript{−} nitrates tyrosine with a reactivity and efficacy similar to those of chemically synthesized ONOO\textsuperscript{--}, indicating that ONOO\textsuperscript{--} can be a significant source of tyrosine nitrination in physiological and pathological events in vivo.

Nitric oxide (NO)\textsuperscript{1} is a simple inorganic radical exhibiting diverse physiological functions, including the regulation of neu-

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‡ The abbreviations used are: NO, nitric oxide; ONOO\textsuperscript{--}, peroxynitrite; O\textsubscript{2}\textsuperscript{−}, superoxide anion radical; NO\textsubscript{2}\textsuperscript{−}, nitrite; H\textsubscript{2}O\textsubscript{2}, hydrogen peroxide; MS, multiple sclerosis; SOD, Cu/Zn-superoxide dismutase; P-NONOate, propylamine NONOate (CH\textsubscript{3}N[NO-N]NO\textsubscript{−}) (CH\textsubscript{3})\textsubscript{2}NH\textsuperscript{−}CH\textsubscript{3}); PTIO, 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; PTI, 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl; DTPA, diethylentriaminepentaaetetic acid; ESR, electron spin resonance; DCDHF, 2,7-dichlorodihydrofluorescein; HPLC, high performance liquid chromatography.

Pfeiffer et al. corrected their erroneous interpretation of the effect of uric acid (46).
by the long exposure to NO or spermine NONOate as well as ONOO⁻. The long incubation periods and competing reactions could bring about rather complicated and unexpected consequences, causing the apparent lack of tyrosine nitration by NO/O₂. To clarify the significance of ONOO⁻ in tyrosine nitration, we re-evaluated tyrosine nitration caused by NO/Ö while considering the effect of uric acid.

By using a more sensitive and specific 12-electrode electrochemical assay for the HPLC, we could measure nitryrosine formation, using the same NO and O₂ flux as that used by Pfeiffer and Mayer (27) over a 5-min period before substrate depletion and product formation become significant problems. The formation of nitryrosine by cogenration of O₂ and NO was found to be nearly equivalent to that obtained with preformed ONOO⁻.

**Materials—** Tyrosine was obtained from Kyowa Hakko Kogyo Co., Ltd., Osaka, Japan. 3-Nitro-l-tyrosine (nitryrosine), Cu/Zn-superoxide dismutase (SOD), cytochrome c, hypoxanthine, xanthine, pterin (2-amino-4-hydroxypteridine), allipurinol (4-hydroxyppyrrozalo[3,4-d] pyrimidine), and 1-ß-phosphatidylcholine were obtained from Sigma Chemical Co. (St. Louis, MO). Propylamine NONOate (P-NONOate, 3-(2-aminomethyl-2-nitrosoguanidino)-N-methyl-1-propanamine, CH₃N[NO(NO)₂]CH₂NCH₃) 2-(trimethylammonophenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (trimethylammonium-PTIO, P-1,2dimyristoyl-1,2-diexophosphatidylcholine, and diethylenetriaminepentaacetic acid (DTPA) were from Dowd Laboratories (Kumamoto, Japan). 2,7-Dichlorodihydrofluorescein (DCHDF) diacetate was purchased from Molecular Probes, Inc. (Eugene, OR). Xanthine oxidase was obtained from Roche Molecular Biochemicals GmbH (Mannheim, Germany). Xanthine oxidase was desalted by ultrafiltration to remove both ammonium sulfate and stabilizing agents such as salicylate with an ultrafiltration tube (Ultrafree-MC, Millipore Corp., Bedford, MA; cutoff size 30,000 Da). Uricase (8.8 IU/mg protein) was purchased from Oriental Yeast Co., Ltd. (Osaka, Japan). Myeloperoxidase purified from purulent human sputum was obtained from Elastin Products Co., Ltd. (Owensville, MO). Authentic ONOO⁻ was synthesized from acidified NO₃ and H₂O₂ by a quenched-flow method according to the literature (26). Consuming H₂O₂ was then decomposed with manganese dioxide. Ebselen (2-phenyl-1,2-benziselenazol-3(2H)-one) was a kind gift from Daiichi Pharmaceuticals Co., Ltd. (Tokyo, Japan). 3,3’-Dityrosine (dityrosine) was synthesized by oxidizing t-tyrosine with H₂O₂ plus horseradish peroxidase according to an earlier report (31). The dityrosine was purified by HPLC using a reverse phase column (TSKgel ODS-80Ts, 2.1 × 30 cm, Tosoh Co., Ltd., Tokyo, Japan) eluted with a linear gradient of acetonitrile (0–12%) in 0.1% trifluoroacetic acid. The concentration of dityrosine was determined spectrophotometrically using its molar absorption coefficient of 8380 M⁻¹ cm⁻¹ at 351 nm, pH 9.9 (31). All water used was prepared with the Milli-Q Synthesis A10 system (Millipore Corp.) and had a conductivity greater than 18 meqhos. All other reagents were of analytical grade and were used without further purification.

**Tyrosine Modification by NO/O₂ and Authentic ONOO⁻**—To study tyrosine nitration caused by simultaneous generation of NO and O₂, we used P-NONOate, which releases NO with a half-life of 7.6 min at 25°C (32). authentic ONOO⁻ (0.1 m M) was added to the reaction mixture (0.2 ml) containing 0.1 mM sodium phosphate buffer (pH 7.0) containing 0.1 ml effective volume) at room temperature under the following conditions: modulation frequency, 100 kHz; modulation amplitude, 0.05 mT; receiver gain, 500; response time, 0.3 s; sweep time, 5 min; microwave power, 10 mW; and microwave frequency, 9.121 GHz. Each reaction was monitored by wctively obtained 18.9 μM PTIO (entraped in liposomes), 0.1 mM tyrosine, 0.1 mM DTPA, 10 μM P-NONOate, 50 μM pterin, and various concentrations of xanthine oxidase with or without 0.1 mM allopurinol in 0.2 ml of 0.1 M phosphate buffer (pH 7.0). All spectra were recorded at 5 min after mixing and initiating the reaction at room temperature.

The concentration of ONOO⁻ formed from NO/O₂ was also estimated

**EXPERIMENTAL PROCEDURES**

**Generation and Consumption of NO were quantified by electron spin resonance (ESR) spectroscopy using PTIO entrapped in liposomes, which reacts with NO stoichiometrically to give 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl (PTI) (Eq. 2) (33).** Generation and consumption of NO were quantified by electron spin resonance (ESR) spectroscopy using PTIO entrapped in liposomes, which reacts with NO stoichiometrically to give 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl (PTI) (Eq. 2) (33). Generation and consumption of NO were quantified by electron spin resonance (ESR) spectroscopy using PTIO entrapped in liposomes, which reacts with NO stoichiometrically to give 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl (PTI) (Eq. 2) (33). Generation and consumption of NO were quantified by electron spin resonance (ESR) spectroscopy using PTIO entrapped in liposomes, which reacts with NO stoichiometrically to give 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl (PTI) (Eq. 2) (33).
Tyrosine Nitration by Peroxynitrite

Tyrosine Nitration Caused by Authentic ONOO⁻ and NO/O₂⁻—The electrochemical assay for nitrotyrosine used in this study could readily detect 2 nm nitrotyrosine in a 50-μl sample (0.1 pmol), with linear response over the range 0.1 to 1000 nm (data not shown). With this sensitive assay, nitration was observed with as little as 50 nm preformed ONOO⁻ added to 0.1 mM tyrosine in phosphate buffer (pH 7.0) containing 0.1 mM DTPA, 10 μM P-NONOate, 50 μM pterin, and 10 or 15 milliunits/ml xanthine oxidase in a total volume of 1.0 ml of 0.1 mM sodium phosphate buffer (pH 7.0), which was incubated at room temperature in the dark. After 5 min of incubation, nitration of DCDHF was determined by measuring the increase in absorbance at 500 nm. A control reaction sample containing allopurinol (final concentration, 0.8 mM) was used to measure the background rate of DCDHF oxidation. The total amount of ONOO⁻ formed from NO/O₂⁻ was estimated from a standard curve of DCDHF oxidation obtained with authentic ONOO⁻.

Superoxide generation from xanthine oxidase was measured in a separate experiment by using the cytochrome c reduction assay as described previously (16). However, because O₂⁻ production could not be determined by the cytochrome c assay in the presence of NO, in the presence of NO the amount of O₂⁻ produced was corrected by simultaneously quantifying the accumulation of isoxanthopterin. Specifically, when pterin was used as the substrate for xanthine oxidase, formation of isoxanthopterin in 0.1 mM sodium phosphate buffer (pH 7.0) containing 0.1 mM DTPA, 10 μM P-NONOate, and various concentrations of xanthine oxidase was measured fluorometrically, using an excitation wavelength of 345 nm and an emission wavelength of 390 nm (37). A linear correlation between isoxanthopterin formation and O₂⁻ formation from xanthine oxidase was obtained in the absence of NO released from P-NONOate. In addition, isoxanthopterin formation from xanthine oxidase in the presence of an NO flux was not affected by NO released from P-NONOate under our experimental conditions. Therefore, O₂⁻ formation from xanthine oxidase in the presence of an NO flux can be estimated from the amount of isoxanthopterin produced, by using a standard curve of isoxanthopterin versus O₂⁻ generation. In this method, both isoxanthopterin and O₂⁻ were prepared separately using the xanthine oxidase reaction mixture without addition of P-NONOate. Similar experiments were performed with hypoxanthine as a substrate, in which uric acid formation was measured spectrophotometrically by differential absorbance at 290 nm (1.22 × 10⁴ M⁻¹ cm⁻¹ (38)).

Effect of Uric Acid on Tyrosine Nitration—To examine the inhibitory effect of uric acid on tyrosine nitration, the reaction mixture of xanthine oxidase with hypoxanthine was incubated with or without uric acid, which decomposes uric acid to give allantoin and CO₂, and was then subjected to tyrosine nitration by authentic ONOO⁻. Hypoxanthine (50 μM) was incubated with xanthine oxidase (10 milliunits/ml) in 0.1 mM phosphate buffer containing 0.1 mM tyrosine and 0.1 mM DTPA at room temperature. After 5 min of incubation, allopurinol (final concentration, 0.2 mM) was added to the mixture to terminate the enzyme reaction. To the reaction mixture thus obtained was added uric acid (final concentration, 420 milliunits/ml) or 0.1 mM phosphate buffer (as a solvent control for uricase), and the mixture was further incubated at room temperature. After various incubation periods, aliquots of the reaction mixture were exposed to authentic ONOO⁻ (bolus administration at final concentration, 3.0 μM) at room temperature for 1 min, and nitrotyrosine was quantified by HPLC as described above. In addition, tyrosine nitration by both authentic ONOO⁻ and an NO/O₂⁻/myeloperoxidase system was examined in the presence of various concentrations of uric acid. For tyrosine nitration by the NO/O₂⁻/H₂O₂/myeloperoxidase system (39, 40), L-tyrosine (0.1 mM) was incubated at room temperature for 5 min in 0.2 ml of 0.1 mM sodium phosphate buffer (pH 7.0) containing 0.1 mM DTPA, 3 mM NO₂⁻, 3 mM H₂O₂, and 0.17 mM myeloperoxidase with or without catalase and SOD. The reaction was terminated by adding 2 μl of catalase (final concentration, 2600 units/ml). This assay was used to determine the 50% inhibitory concentration (IC₅₀) of uric acid for tyrosine nitration produced by ONOO⁻ and the NO/O₂⁻/H₂O₂/myeloperoxidase system.

RESULTS

Tyrosine Nitration Caused by Authentic ONOO⁻ and NO/O₂⁻—The chemical assay for nitrotyrosine used in this study could readily detect 2 nm nitrotyrosine in a 50-μl sample (0.1 pmol), with linear response over the range 0.1 to 1000 nm (data not shown). With this sensitive assay, nitration was observed with as little as 50 nm preformed ONOO⁻ added to 0.1 mM tyrosine in phosphate buffer (pH 7.0) with 25 mM NaHCO₃ (Fig. 1). Each result on the yield of nitrotyrosine, from 50 to 3000 nm preformed ONOO⁻, was equal to 13 ± 2.3% (mean ± S.D.) based on ONOO⁻. With no addition of NaHCO₃ to the reaction mixture, endogenous bicarbonate was 4.0 mM and CO₂ was 0.6 mM as determined using a CO₂/HCO₃⁻ autoanalyzer (ABL 510; Radiometer Co., Copenhagen, Denmark). Under these conditions, the yield of nitrotyrosine with 3 μM preformed ONOO⁻ was 8.0 ± 0.9% (n = 3; mean ± S.D.). In contrast, no detectable nitrotyrosine was observed with the NO donor P-NONOate at a concentration at or below 10 μM. Therefore, we used 10 μM P-NONOate in subsequent experiments to analyze nitration by the NO/O₂⁻ system.

When O₂⁻ was generated with pterin as the substrate for xanthine oxidase in the presence of P-NONOate, the solution resulted in appreciable tyrosine nitration (Fig. 2A). Nitration was maximal when the flux of O₂⁻ and that of NO were approximately equal. Use of hypoxanthine as the substrate resulted in far less nitration in the NO/O₂⁻ system. Both pterin and isoxanthopterin have no scavenging activity against ONOO⁻. Because the experiments were conducted for 5 min to avoid substrate depletion and product inhibition, we could maintain the steady-state NO/O₂⁻ flux produced from 10 μM P-NONOate (t₁/₂ = 7.6 min) and xanthine oxidase. We observed a linear and dose-dependent increase in O₂⁻ generation with xanthine oxidase system. The amount of O₂⁻ produced with xanthine oxidase plus pterin in 5 min was one-fourth with that produced with xanthine oxidase plus hypoxanthine (Fig. 2B). Superoxide production by xanthine oxidase with either hypoxanthine or pterin as substrate remained linear for the 5-min incubation period (data not shown). These data indicate that no appreciable substrate depletion occurred under our experimental conditions. Moreover, the finding that the nitration yield and level of O₂⁻ generation were inversely related for the two different xanthine oxidase substrates, suggested that inhibition by the hypoxanthine/xanthine oxidase system might account for the lack of tyrosine nitration by ONOO⁻.

Carbon dioxide enhanced tyrosine nitration by both ONOO⁻ and the NO/O₂⁻ system as reported previously (41, 42). Addition of 25 mM NaHCO₃ to the buffer increased the concentration of CO₂ to 1.3 mM from 0.6 mM (endogenous CO₂ concentration). This increased CO₂ concentration resulted in an appreciable increase in tyrosine nitration by both authentic ONOO⁻ and NO/O₂⁻. Specifically, addition of 25 mM NaHCO₃ led to an increase of 1.6 ± 0.1-fold and 2.2 ± 0.2-fold (n = 3; mean ± S.D.) in nitrotyrosine formation due to authentic ONOO⁻ (0.1 μM) and NO/O₂⁻ fluxes (3 milliunits/ml xanthine oxidase plus 50 μM P-NONOate).
hypoxanthine), respectively.

**Estimation of ONOO− Formed from NO/O2−**—The production of ONOO− in the NO/O2− system was estimated by measuring the consumption of NO using PTIO entrapped in liposomes. On the basis of concentration of PTI generated from the reaction of NO/O2− with PTIO, the concentration of NO was calculated. Similarly, the ONOO− generation was estimated as described under “Experimental Procedures.”

To verify that the amount of ONOO− produced by the NO/ xanthine oxidase system was correctly estimated by our ESR assay, we measured ONOO− formation by using the DCDHF oxidation assay (36). The values determined by the DCDHF assay were consistent with those obtained by the ESR-based assay; the amount of ONOO− formed in the reaction of 10 milliunits/ml xanthine oxidase plus 50 μM pterin with 10 μM P-NONOate for 5 min was estimated to be 3.0 ± 0.6 μM (n = 3; mean ± S.D.) by the ESR assay, whereas the value determined by the DCDHF assay was 2.9 ± 0.3 μM, as just described. Similarly, the ONOO− yield in the reaction of 15 milliunits/ml xanthine oxidase plus 50 μM pterin with 10 μM P-NONOate was similar in both DCDHF and ESR assays: 4.3 ± 0.1 μM and 4.0 ± 0.1 μM (n = 3; mean ± S.D.), respectively.

**Yields of Nitrotyrosine and Dityrosine Produced by NO/O2− and Authentic ONOO−**—To determine the efficiency in nitration of the NO/O2− system, 2.9 μM preformed alkaline ONOO− was continuously infused during a 5-min period into the same buffer system containing 0.1 mM tyrosine, with a yield of 171 ± 20 nm nitrotyrosine. The NO/O2− system with pterin using 10 milliunits/ml xanthine oxidase yielded 124 ± 5.3 nm nitrotyrosine, which was 73% of the yield with authentic ONOO− (Table I).

We also compared the yields of nitrotyrosine produced by ONOO− obtained in this study with those of Pfeiffer and Mayer under similar reaction conditions (pH 7.4; tyrosine concentration, 1.0 mM). In general, the yield of tyrosine nitration occurring in the reaction mixture at pH 7.4 was lower than that at pH 7.0. The nitration yield by NO/O2− fluxes produced by our system of P-NONOate/pterin plus xanthine oxidase was much greater than that measured by Pfeiffer and Mayer with sperm-

![Fig. 2. Tyrosine nitration produced by NO and O2− generated simultaneously (A) and O2− generated from xanthine oxidase reaction with different substrates (pterin and hypoxanthine) (B). A, NO was generated from P-NONOate (10 μM), and O2− was generated from xanthine oxidase with either 50 μM hypoxanthine (closed circles) or 50 μM pterin (open circles) as substrate. The NO/O2− flux was produced at room temperature for 5 min in the same buffer containing 0.1 mM L-tyrosine and 25 mM NaHCO3, as described in Fig. 1. Data are means ± S.D. of three experiments. B, hypoxanthine (50 μM, closed circles) or pterin (50 μM, open circles) was incubated with various concentrations of xanthine oxidase in 0.1 M phosphate buffer (pH 7.0) containing 0.1 mM DTPA and 10 μM P-NONOate at room temperature for 5 min. O2− generation was estimated as described under “Experimental Procedures.” Data are means ± S.D. of three experiments (in B, error bars are smaller than the symbols).](https://example.com/fig2.png)

![Fig. 3. Estimation of ONOO− production during NO/O2− flux based on NO consumption. ESR spectra of PTIO in liposomes after reaction with NO/O2− were recorded; the first portion of the spectrum is shown. Concentrations of NO formed were calculated from the peak height of PTI, which was produced by reaction of NO with PTIO. Liposomes were incubated with 10 μM P-NONOate alone (A) or 10 μM P-NONOate together with 50 μM pterin plus 10 milliunits/ml (B) or 15 milliunits/ml (C) xanthine oxidase in 0.1 M sodium phosphate buffer (pH 7.0) containing 0.1 mM tyrosine and 0.1 mM DTPA; incubation proceeded for 5 min at room temperature. D, B plus 0.1 mM allopurinol. Arrows in A indicate v values of the ESR signals derived from PTIO and PTI.](https://example.com/fig3.png)
Tyrosine Nitration by Peroxynitrite

TABLE I
Yield of nitrotyrosine by NO/O2 and authentic ONOO-

| Nitrating agent | Yield* (based on ONOO-) | Conditions | Reference |
|-----------------|-------------------------|------------|-----------|
| NO/O2 (1 mM ONOO-) | 0.07 | Tyr (1 mM)/XO (28 milliunits/ml)/hyp (1 mM)/Sper NONOate (1 mM) for 12 h (pH 7.4) | 27 |
| ONOO- (1 mM) | 3.68 ± 0.17 | Tyr (1 mM)/bolus addition (pH 7.4) | 27 |
| NO/O2 (2.3 µM ONOO-) | 0.5 ± 0.1 | Tyr (1 mM)/XO (6 milliunits/ml)/pterin (50 µM)/P-NONOate (10 µM) for 5 min (pH 7.4) | This work |
| ONOO- (2.3 µM) | 0.6 ± 0.1 | Tyr (1 mM)/constant flux (0.45 µM/min) for 5 min (pH 7.4) | This work |
| NO/O2 (2.9 µM ONOO-) | 4.2 ± 0.2 | Tyr (0.1 mM)/XO (10 milliunits/ml)/pterin (50 µM)/P-NONOate (10 µM) for 5 min (pH 7.0) | This work |
| ONOO- (2.9 µM) | 5.8 ± 0.7 | Tyr (0.1 mM)/constant flux (0.59 µM/min) for 5 min (pH 7.0) | This work |

* Means ± S.D. of three measurements.

ONOO- formed from NO/O2 was estimated by use of the dihydorhodamine oxidation assay (27).

Authentic ONOO was added at bolus to the reaction mixture (27).

ONOO- formed from NO/O2 was estimated by the ESR-based PTIO liposome assay.

Authentic ONOO was introduced into the reaction mixture at constant rates by using a microsyringe pump as described under “Experimental Procedures.”

Fig. 4. Effect of tyrosine concentration on nitration and dimerization of tyrosine (dityrosine formation) caused by authentic ONOO- and NO/O2. Authentic ONOO was introduced continuously into the reaction mixture for 5 min (2.9 µM/5 min) and dityrosine (open circles) and dityrosine (closed circles) was determined by reverse phase HPLC as described under “Experimental Procedures.” Tyrosine (0.1 and 1.0 mM) was also reacted with ONOO- formed in situ (2.9 µM/5 min) produced from NO/O2 flux by P-NONOate (10 µM) and xanthine oxidase (10 milliunits/ml) plus pterin (50 µM) in 0.1 mM sodium phosphate buffer (pH 7.0) containing 0.1 mM DTPA for 5 min. Nitrotyrosine (closed squares) and dityrosine (open squares) were then quantified. Data are means ± S.D. of three experiments.

Fig. 5. Effects of ebselen, SOD, and allopurinol on tyrosine nitration caused by authentic ONOO- and NO/O2 flux. NO and O2 were simultaneously generated to produce NO/O2 by using P-NONOate (10 µM) and xanthine oxidase (5 milliunits/ml) plus hypoxanthine (50 µM). Tyrosine was treated with NO/O2 flux for 5 min or with authentic ONOO- (0.1 µM) in the same manner as in Fig. 1 in the presence or absence of ebselen (0.1 mM), SOD (200 units/ml), or allopurinol (50 µM). Results are expressed as percentage of nitrotyrosine formation compared with the experiment without inhibitors (control). *, below the detection limit. Data are means ± S.D. of three experiments.

Visit this [URL](https://example.com) for more information.

Effect of O2 and ONOO- Scavengers on Tyrosine Nitration—The selenium-containing ONOO- scavenger ebselen (100 µM) (43) inhibited nitration caused by both ONOO- and the NO/O2 system (Fig. 5). Although ebselen appears to have a weak O2 scavenging activity, it did not compete with the reaction of O2 with NO to be evidenced by the ESR study using PTIO liposome (data not shown). Therefore, tyrosine nitration by NO/O2 flux is thought to be caused by ONOO-.

In contrast, allopurinol (50 µM) and SOD (200 units/ml) had no effect on ONOO- mediated nitration but blocked nitration by the NO/O2 system. The concentration of SOD was sufficient to scavenge O2 but too low to substantially enhance tyrosine nitration via ONOO-.

These results indicate that O2 was nec-

necessary for NO to nitrate free tyrosine by forming ONOO- as an intermediate.

To better characterize the limited tyrosine nitration observed with hypoxanthine used as a substrate for xanthine oxidase (Fig. 2), we examined the inhibitory effects of uric acid on tyrosine nitration. Tyrosine nitration caused by 3.0 µM ONOO- was completely inhibited when added to the reaction mixture of 10 milliunits/ml xanthine oxidase plus 50 µM hypoxanthine that was preincubated for 5 min (Fig. 6). Addition of uricase restored nitration by ONOO- in a time-dependent fashion, showing that uric acid was a major inhibitory product of tyrosine nitration formed when hypoxanthine was the substrate.

Nitration-inhibitory Effect of Uric Acid with ONOO- and with the NO2/H2O2/Myeloperoxidase System—We examined the effect of uric acid and other scavengers on tyrosine nitration catalyzed by myeloperoxidase from NO2/H2O2 (Fig. 7), a significant nitrating system proposed by Eiserich et al. (39). Allopurinol, ebselen, SOD, and NaHCO3 did not affect tyrosine nitration catalyzed by myeloperoxidase, whereas uric acid strongly inhibited tyrosine nitration by myeloperoxidase.

modifications produced by a low flux of ONOO- were also observed with different concentrations of tyrosine at pH 7.4 (data not shown).

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Nitration-inhibitory Effect of Uric Acid with ONOO- and with the NO2/H2O2/Myeloperoxidase System—We examined the effect of uric acid and other scavengers on tyrosine nitration catalyzed by myeloperoxidase from NO2/H2O2 (Fig. 7), a significant nitrating system proposed by Eiserich et al. (39). Allopurinol, ebselen, SOD, and NaHCO3 did not affect tyrosine nitration catalyzed by myeloperoxidase, whereas uric acid strongly inhibited tyrosine nitration by myeloperoxidase.
Tyrosine Nitrination by Peroxynitrite

Fig. 6. Effect of uricase on tyrosine nitrination-inhibitory activity obtained in the reaction mixture of xanthine oxidase and hypoxanthine. Xanthine oxidase (10 milliunits/ml) plus hypoxanthine (50 μM) and tyrosine (0.1 mM) were incubated in 0.1 M sodium phosphate buffer (pH 7.0) containing 0.1 mM DTPA for 5 min, after which the reaction was terminated by adding allopurinol (final concentration, 0.2 mM). Uricase (420 milliunits/ml) (closed circles) or vehicle without uricase (open circles) was added to this reaction mixture. After the indicated incubation periods, authentic ONOO⁻ (3.0 μM) was added as a bolus, and nitrotyrosine was assayed. The closed triangle indicates the yield of tyrosine nitration due to authentic ONOO⁻ (3.0 μM) in the absence of xanthine oxidase and hypoxanthine. Data are means ± S.D. of three experiments.

Fig. 7. Effect of various substances on tyrosine nitration caused by the NO2/H2O2/myeloperoxidase system. Tyrosine (0.1 mM) was incubated in the reaction mixture containing NO2⁻ (3 μM), H2O2 (3 μM), and myeloperoxidase (0.17 μM) for 5 min (control). Tyrosine nitrination in this system was also examined in the presence of allopurinol (0.1 mM), ebselein (0.1 mM), SOD (210 units/ml), catalase (2600 units/ml), NaHCO3 (25 mM), or uric acid (50 μM). Data are expressed as percentage of the control values (means ± S.D. of three experiments). *, below the detection limit.

We then compared the efficacy of uric acid for suppression of tyrosine nitrination produced by both ONOO⁻ and the NO2⁻/H2O2/myeloperoxidase system (Fig. 8). The IC50 for tyrosine nitrination by 3 μM ONOO⁻ was 0.3 μM, and 25 mM NaHCO3 weakened the inhibitory effect of uric acid, to an IC50 of 7.0 μM. Tyrosine nitrination by NO2⁻ and H2O2 (3 μM each) with 0.17 μM myeloperoxidase was also sensitive to uric acid (IC50, 3.0 μM), as observed for ONOO⁻. In these tyrosine-nitrating reactions without uric acid, similar levels of nitrotyrosine formation were obtained for ONOO⁻: 241 ± 26 nm (without NaHCO3) and 375 ± 20 nm (with 25 mM NaHCO3), and for the myeloperoxidase system: 322 ± 23 nm.

Fig. 8. Inhibitory effect of uric acid on tyrosine nitrination caused by (A) authentic ONOO⁻ and (B) the NO2⁻/H2O2/myeloperoxidase system. A, tyrosine (0.1 mM) was reacted with 3 μM ONOO⁻ in the presence of various concentrations of uric acid for 1 min. The effect of uric acid was investigated in the presence (closed circles) or absence (open circles) of 25 mM NaHCO3. In the absence of uric acid, the yield of nitrotyrosine by ONOO⁻ with or without 25 mM NaHCO3 was 375 ± 20 nm and 241 ± 26 nm, respectively. B, tyrosine (0.1 mM) was incubated with NO2⁻ (3 μM), H2O2 (3 μM), and myeloperoxidase (0.17 μM) in the presence of indicated concentrations of uric acid for 5 min. The yield of nitrotyrosine caused by the NO2⁻/H2O2/myeloperoxidase system was 322 ± 23 nm for control experiment without uric acid. The IC50 of uric acid on tyrosine nitrination was determined from the plot of uric acid concentration against percentage of inhibition of tyrosine nitrination. Data are means ± S.D. of three experiments.

DISCUSSION

In the present study, we demonstrated that low nanomolar steady-state concentrations of ONOO⁻ formed from NO and O2 (generated via xanthine oxidase) produced significant amounts of nitrotyrosine. Comparative analysis using chemically synthesized alkaline ONOO⁻ versus a steady flux of NO/O2 showed that ONOO⁻ was the most likely molecular species responsible for tyrosine nitrination. More important, the efficacy of tyrosine oxidation to nitrotyrosine as well as dityrosine caused by NO/O2 was similar to that caused by authentic ONOO⁻ over a range of tyrosine concentrations. Furthermore, tyrosine nitrination predominated over dityrosine formation produced by a low nanomolar flux of ONOO⁻ at low concentrations of tyrosine (<0.5 mM).

Our findings disagree with the initial report by Pfeiffer and Mayer that ONOO⁻ formed from NO and O2 does not nitrate tyrosine at physiological pH (27). These opposing results appear to be consequences of different experimental designs as well as failure by Pfeiffer and Mayer to consider substrate (oxygen) depletion and product (uric acid) inhibition (27). They incubated hypoxanthine and xanthine oxidase with a high concentration of spermine NONOate (1 mM) for a long period (3–12 h) to produce a flux of NO and O2. However, accurately maintaining or even measuring production of NO, O2, and ONOO⁻ for such a long incubation is difficult. For example, oxygen consumption as well as autoinactivation during prolonged xanthine oxidase incubation will reduce O2 generation from xanthine oxidase and thereby decrease ONOO⁻ production. In
Tyrosine Nitration by Peroxynitrite

addition, both NO and ONOO⁻ have inhibitory effects on xanthine oxidase activity (29, 30).

To minimize such interference, we used a low concentration of P-NONOate (10 μM) with a much shorter incubation period of 5 min to produce O₂⁻ from xanthine oxidase, which allowed us to observe the strong tyrosine-nitrating potential of NO reacting with O₂⁻ generated by xanthine oxidase plus pterin. Another important factor that severely interfered with tyrosine nitration caused by ONOO⁻ was uric acid produced when hypoxanthine was used as a substrate for xanthine oxidase. In view of all these data, the apparent lack of tyrosine nitration by NO/O²⁻ reported by Pfeiffer and Mayer appears to have resulted from (i) overestimation of ONOO⁻ production from NO and O₂⁻ based on measuring ONOO⁻ formation for only the first 3 min, and (ii) the inhibitory effect of uric acid simultaneously generated with O₂⁻.

Although we observed a linear increase in ONOO⁻ formation with increasing xanthine oxidase concentration in the P-NONOate/pterin/xanthine oxidase system, we found a bell-shaped pattern of nitrotyrosine formation that peaked at similar fluxes of NO and O₂⁻ as shown in Fig. 2A. This impaired nitration efficiency in the presence of excessive O₂⁻ generation may partly be explained by the reaction of tyrosine phenoxy radical (Tyr-O•) with O₂⁻ as recently reported by Goldstein et al. (44). They suggested that nitrotyrosine is formed via a radical reaction of tyrosine with ‘OH and ‘NO₂ that have escaped from a solvent-caged radical pair of ONOOH [ONO−O−OH] (45). During this reaction, Tyr-O• is first formed through the reaction of tyrosine with either ‘OH or ‘NO₂, and then nitrotyrosine is produced by a coupling of radicals Tyr-O• and ‘NO₂. With excessive production of O₂⁻ from xanthine oxidase, however, the steady-state concentration of Tyr-O• is reduced because of quenching with O₂⁻ so that tyrosine nitration may be attenuated. Excess NO also seems to be more inhibitory than O₂⁻ through Tyr-O• quenching by NO (44).

It was recently reported by Pfeiffer et al. that dityrosine formation competes with tyrosine nitration with low concentrations of ONOO⁻, such that dityrosine could be a major product of tyrosine modification by ONOO⁻ (46). However, our present results showed that, although tyrosine nitration is attenuated at tyrosine concentrations above 0.5 mM, very effective nitration occurs even with a low flux of ONOO⁻ (10 nM/s), compared with dityrosine formation, particularly at low tyrosine concentrations (0.1 mM or below) (Fig. 4). The normal range of free L-tyrosine in the human plasma described in the literature is 40–130 μM (47), suggesting that tyrosine nitration will be the dominant tyrosine modification caused by ONOO⁻ at physiological concentrations of free tyrosine or when tyrosine bioavailability is somehow limited.

It is also potentially important that uric acid inhibits tyrosine nitration not only by ONOO⁻ (12, 13, 28) but also by the NO₂⁻/H₂O₂/myeloperoxidase system (39, 40). Uric acid may thus play an important role in modulating tyrosine nitration and the detrimental actions of ONOO⁻ as well in vivo. However, because uric acid is readily oxidized by ONOO⁻, resulting in formation of oxidized metabolites such as allantoin, para-banic acid, urazole, and oxonic acid (48), excessive and prolonged production of ONOO⁻ may decrease the level of uric acid in the microenvironment of the site of ONOO⁻ production, resulting in enhanced susceptibility of free and protein-bound tyrosine to nitration by ONOO⁻ as well as by the NO₂⁻/H₂O₂/myeloperoxidase system.

Uric acid is an end product of purine and nitrogen catabolism in avian and human species; in other animals uric acid is further metabolized to allantoin by uricase. The normal concentration of uric acid in plasma has been reported to be 20–40 μM for mice (13) and 200–300 μM for humans (13, 48). In the presence of such high concentrations of uric acid, tyrosine nitration will be largely inhibited. However, we also found that a physiologically relevant concentration of bicarbonate (25 mM) significantly attenuated the inhibitory effect of uric acid on tyrosine nitration caused by ONOO⁻. Even in the presence of 50 μM uric acid, ONOO⁻ (3 μM) produces a significant amount of nitrotyrosine (41 ± 2.0 nM). This result suggests that in vivo tyrosine nitration by ONOO⁻ is largely due to formation of the more potent nitrating agent ONOO⁻O₂⁻.

It has been long known that uric acid is an important endogenous component of the defense system against free radical tissue injuries because of its antioxidant activity (49). Recently, Hooper et al. (12) and Bagasra et al. (13) reported that uric acid treatment had a remarkable effect on experimental allergic encephalomyelitis (an animal model of MS), in which ONOO⁻ is suggested to be involved in pathogenesis. They also found a significantly lower incidence of MS in hyperuricemic patients (50). Skinner et al. documented that a reaction product of ONOO⁻ and uric acid could act as a nitrovasodilator (48). Therefore, interaction of uric acid with ONOO⁻ would be an important factor in NO-linked pathogenesis of various diseases.

It has been reported that nitration of protein-bound and free tyrosine may modulate cellular functions through inactivation of enzymes such as mitochondrial SOD (18), disruption of assembly of neurofilament L (11, 24), effects on tyrosine phosphorylation-mediated signal transduction (20), and dysfunction of microtubule formation (21). A recent intriguing finding of Kasimaki et al. (51) shows the presence of nitrotyrosine denitrase activity, which repairs protein nitration, in rat tissues, suggesting that a tyrosine nitration-denitration pathway participates in NO- or ONOO⁻-dependent signal transduction, similar to phosphorylation-dephosphorylation systems. These findings indicate that nitrotyrosine formation has great importance not only as a biomarker of reactive nitrogen-mediated tissue injury but also as a means to gain insight into molecular mechanisms of NO-related physiological and pathophysiological phenomena.

In conclusion, our data unambiguously verify that ONOO⁻ generated from NO and O₂⁻ can nitrate tyrosine with similar reactivity and efficacy as chemically synthesized ONOO⁻. Thus, ONOO⁻ formed by the reaction of NO with O₂⁻ may be one of the key intermediates responsible for tyrosine nitration in vivo. Moreover, although uric acid may serve a critical function by modulating tissue injury caused by reactive nitrogen species, it is also possible that ONOO⁻ and NO₂⁻/H₂O₂/myeloperoxidase enhance their tissue-damaging potential by degrading uric acid in vivo.

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