Dityrosine Formation Outcompetes Tyrosine Nitration at Low Steady-state Concentrations of Peroxynitrite

IMPLICATIONS FOR TYROSINE MODIFICATION BY NITRIC OXIDE/SUPEROXIDE IN VIVO*

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Formation of peroxynitrite from NO and O2 is considered an important trigger for cellular tyrosine nitration under pathophysiological conditions. However, this view has been questioned by a recent report indicating that NO and O2 generated simultaneously from (Z)-1-[N-[3-aminopropyl]-N-[4-(3-aminopropylammonio)butyl]-amino]diazene-1-ium-1,2-dioolate (SPER/NO) and hypoxanthine/xanthine oxidase, respectively, exhibit much lower nitrating efficiencies than authentic peroxynitrite (Pfeiffer, S. and Mayer, B. (1998) J. Biol. Chem. 273, 27280–27285). The present study extends those earlier findings to several alternative NO/O2-generating systems and provides evidence that the apparent lack of tyrosine nitration by NO/O2 is due to a pronounced decrease of nitration efficiency at low steady-state concentrations of authentic peroxynitrite. The decrease in the yields of 3-nitrotyrosine was accompanied by an increase in the recovery of dityrosine, showing that dimerization of tyrosine radicals outcompetes the nitration reaction at low peroxynitrite concentrations. The observed inverse dependence on peroxynitrite concentration of dityrosine formation and tyrosine nitration is predicted by a kinetic model assuming that radical formation by peroxynitrous acid homolysis results in the generation of tyrosyl radicals that either dimerize to yield dityrosine or combine with NO2 radical to form 3-nitrotyrosine. The present results demonstrate that very high fluxes (>2 μmol) of NO/O2 are required to render peroxynitrite an efficient trigger of tyrosine nitration and that dityrosine is a major product of tyrosine modification caused by low steady-state concentrations of peroxynitrite.

Tyrosine nitration is a well established protein modification occurring in vivo in a number of inflammatory diseases associated with oxidative stress and increased activity of NO synthases (1, 2). Nitration of specific tyrosine residues has been reported to affect protein structure and function (3), suggesting that 3-nitrotyrosine formation may not only be a disease marker but could be causally involved in the pathogenesis of certain disease states.

Peroxynitrite, formed in a nearly diffusion-controlled reaction from NO and O2, is considered a potent pathophysiological relevant cytotoxin. Besides oxidation reactions resulting in dysfunction of various biomolecules, nitration of free and protein-bound tyrosine to yield 3-nitrotyrosine is a well established reaction of peroxynitrite that may contribute to NO cytotoxicity (1). The nitration reaction has been extensively studied in vitro by bolus addition of synthetic peroxynitrite to tyrosine-containing samples including purified proteins, cells, and tissues (3–6). In situ, 3-nitrotyrosine was most frequently visualized with monoclonal or polyclonal antibodies (2), but the identity of the product has been confirmed by several laboratories using sophisticated gas chromatography/mass spectrometry and HPLC1 methods (7, 8).

Thus, there is general agreement that (i) authentic peroxynitrite is a potent nitrating agent that converts free and protein-bound tyrosine to the corresponding 3-nitro derivative, and that (ii) 3-nitrotyrosine does occur in vivo. The conclusion that peroxynitrite is the main cause for in vivo nitration may thus seem obvious, but is not supported by experimental data. In fact, several recent studies have identified alternative pathways of tyrosine nitration (9), and we found that nitration by simultaneously generated NO and O2 is much less efficient than the reaction triggered by authentic peroxynitrite (10). The interpretation of the latter results has been disputed, and a number of points have been raised questioning their validity. One point was related to the possibility that urate formed in the XO reaction might have scavenged peroxynitrite and thus prevented tyrosine nitration in long term (12 h) experiments. Concerning data interpretation, we had suggested that NO and O2 may combine to trans-peroxynitrite, the rapid protonation of which prevents formation of the nitrating CO2 adduct. However, thermodynamic calculations have unambiguously identified the cis-rotamer as the more stable conformation of peroxynitrite in both gas phase (11) and aqueous solution,2 rendering our initial hypothesis untenable. We hope to settle both issues with the present study in which we extend our earlier findings to other, urate-free NO/O2-generating systems and demonstrate that the low nitrating efficiency of NO/O2 is explained by an unexpected dependence of nitration yields on peroxynitrite steady-state concentrations. It is shown that the reaction between tyrosine and peroxynitrite yields almost exclusively dityrosine at peroxynitrite concentrations ≤5 μM, whereas product yields were progressively shifted toward 3-nitrotyrosine at higher concentrations of peroxynitrite.

1 The abbreviations used are: HPLC; high performance liquid chromatography; DEANO, 2-(N,N-diethylamino)diazeneolate-2-oxide; DHR, dihydrodihydrodiamine 123; FMN, flavine mononucleotide; NO, nitric oxide; O2, superoxide anion; SOD, superoxide dismutase; SPER/NO; (Z)-1-[N-[3-aminopropyl]-N-[4-(3-aminopropylammonio)butyl]-amino]diazene-1-ium-1,2-dioolate; Tyr, tyrosyl radical; XO, xanthine oxidase.
2 R. Janoschek, S. Pfeiffer, and B. Mayer, unpublished data.
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TABLE I

| NO/O₂⁻-generating systems | NO | O₂⁻ | ONOO⁻ | 3-Nitrotyrosine | Nitrating efficiency |
|---------------------------|----|-----|-------|-----------------|---------------------|
| SPER/NO (1 mM)            | None|      |       |                 |                     |
| SPER/NO (1 mM)            | XO (28 milliliters/ml) |      |       |                 |                     |
| SPER/NO (1 mM)            | Hypoxanthine (1 mM) |      |       |                 |                     |
| SPER/NO (0.3 mM)          | None|      |       |                 |                     |
| SPER/NO (0.3 mM)          | XO (50 milliliters/ml) CH₃CHO (1 mM) |      |       |                 |                     |
| DEA/NO (0.1 mM)           | None|      |       |                 |                     |
| DEA/NO (0.1 mM)           | FMN (0.1 mM) |      |       |                 |                     |

**a** 100% = accumulated ONOO⁻ measured as DHR oxidation.

**b** 100% = 1 mM.

**EXPERIMENTAL PROCEDURES**

**Materials**—SPER/NO and DEA/NO were from Alexis (Vienia, Austria). Hypoxanthine, DHR, and 3-nitrotyrosine were from Fluka (Vienia, Austria). XO (from buttermilk, 1.4 units/mg), uricase (from Arthrobacter globiformis, 18 units/mg), and all other chemicals were from Sigma. A stock solution of authentic dityrosine (30 μM), synthesized as described previously (12, 13), was kindly provided by Dr. Jay W. Heinke from the Department of Internal Medicine, Washington University, School of Medicine, St. Louis, MO.

**Solutions**—All solutions were prepared freshly each day. Water was from a Milli-Q reagent water system from Millipore (Vienia, Austria; resistance ≥18 megohms × cm⁻¹). SPER/NO and DEA/NO were prepared as 10-fold stock solutions in 10 mM NaOH. DHR was dissolved to 10 mM in acetonitrile and kept in the dark until use. Alkaline solutions of peroxynitrite were prepared from acidified NaO₂ and H₂O₂ as described (14). The solutions were diluted with H₂O to 10 mM (pH 12.8) and further diluted in 10 mM NaOH to 10-fold stock solutions used for experiments.

**NO/O₂⁻-generating Systems**—XO, SPER/NO, and hypoxanthine or acetaldehyde were incubated at the indicated concentrations in the presence of tyrosine (1 mM) at ambient temperature for 3 h in 0.1 M KH₂PO₄/KH₂PO₄ buffer (pH 7.4). Reactions were terminated by the addition of 5% (v/v) NaOH (10 μl, pH 1-10). Uricase activity was measured by monitoring urate consumption at 292 nm. The amount of oxidized urate was calculated using an extinction coefficient of 11 mM⁻¹ cm⁻¹ (15). DEA/NO (0.1 mM) and FMN (0.1 mM) were incubated with 1 mM tyrosine at ambient temperature for 1 h in 0.1 M KH₂PO₄/KH₂PO₄ buffer (pH 7.4) in the presence of visible light from a tungsten lamp (60 watts).

**Determination of NO and O₂⁻ Formation**—NO release from DEA/NO (0.1 mM) was measured with a Clark-type NO-sensitive electrode (ISO-NO, World Precision Instruments, Berlin, Germany) in the absence or presence of FMN as described (16). 5-μl aliquots of a 1 mM FMN solution were added to 495-μl of 0.1 mM KH₂PO₄/KH₂PO₄ buffer (pH 7.4). Reactions were terminated by the addition of 5% (v/v) NaOH (10 μl, pH 1–10). Uricase activity was measured by monitoring urate consumption at 292 nm. The amount of oxidized urate was calculated using an extinction coefficient of 11 mM⁻¹ cm⁻¹ (15). DEA/NO (0.1 mM) and FMN (0.1 mM) were incubated with 1 mM tyrosine at ambient temperature for 1 h in 0.1 M KH₂PO₄/KH₂PO₄ buffer (pH 7.4) in the presence of visible light from a tungsten lamp (60 watts).

**Oxidation of DHR**—Oxidation of DHR was monitored at 501 nm as described (10, 18). The amount of oxidized DHR was calculated using an extinction coefficient of 21 mM⁻¹ cm⁻¹ for reduced cytochrome c (17). Measurements were performed at ambient temperature in a total volume of 0.2 ml of a 50 mM KH₂PO₄/KH₂PO₄ buffer (pH 7.4) containing 1 mM hypoxanthine or acetaldehyde, 20 μM ferrocyanochrome c, and variable amounts of XO.

**Peroxynitrite Infusion**—The infusion experiments were performed with a Merck-Hitachi HPLC pump (655A-11) provided with Peek capillaries (internal diameter, 0.25 mm) under constant stirring of the tyrosine-containing solutions at ambient temperature. Peroxynitrite (2 ml of a 0.1 mM stock solution) was infused at increasing rates (0.1, 0.2, 0.4, 0.5, and 0.8 mM/min) into 18 ml of 0.1 M KH₂PO₄/KH₂PO₄ buffer (pH 7.4) containing 1 mM tyrosine, followed by the determination of 3-nitrotyrosine as described below.

**Determination of 3-Nitrotyrosine and Dityrosine**—HPLC analysis of 3-nitrotyrosine was performed on a C₁₈ reversed phase column with 0.1 M KH₂PO₄/H₂PO₄ buffer (pH 3) containing 6% (v/v) methanol at 0.5 ml/min and detection at 274 nm, as described (19). In some experiments peroxynitrite was detected with a dual-channel electrochemical detector (ESA, Coulochem II, Chelmsford, MA) set to 600 mV and 850 mV (20). Oxidation of 3-nitrotyrosine was followed at 850 mV. A guard cell placed between the solvent delivery system and injector was set to 1000 mV. Calibration curves were recorded daily with authentic 3-nitrotyrosine (2 mM–0.5 μM and 60 nM–5 μM for electrochemical and UV-visible detection, respectively). HPLC analysis of peroxynitrite was performed on a C₁₈ reversed phase column with 50 mM KH₂PO₄/H₂PO₄ buffer (pH 3) containing 1% (v/v) methanol at 0.7 ml/min and fluorescence detection (Hitachi fluorescence spectrophotometer F 1050; excitation 285 nm, emission 410 nm) as described (4). Calibration curves were recorded with authentic dityrosine (50 nM–5 μM).

**Kinetic Experiments**—The rate of peroxynitrite decay was determined by stopped-flow absorbance spectroscopy at 302 nm (Bio-Sequenator SX-17MV stopped-flow spectrophotometer, Applied Photophysics, Leatherhead, UK) at 22 °C. Reservoir 1 contained peroxynitrite (0.2 mM) in 0.1 M NaOH, and reservoir 2, the buffer solution (0.2 M KH₂PO₄/KH₂PO₄ buffer (pH 7.4), containing 3 mM tyrosine). A k₁ value of 0.27 ± 0.05 s⁻¹ (mean ± S.D.; n = 9) was calculated from the initial rates of first order peroxynitrite decay. The peroxynitrite steady-state concentrations obtained in the infusion experiments were calculated by dividing infusion rates (μM s⁻¹) by 0.27 s⁻¹.

**Kinetic Simulations**—Kinetic simulations of tyrosine nitration were performed using the software package Mathematica (Version 2.2.2., Wolfram Research Inc., Champaign, IL). The model was based on peroxynitrite decomposition (k₂ = 0.27 s⁻¹) via homolysis producing O₂⁻ and NO radicals with 30% yields (21–23). The following second order rate constants (M⁻¹ s⁻¹) were used to account for the reactions of NO₂⁻ with tyrosine, tyrosyl radical, and NO₂⁻, respectively: 3.2 × 10⁹ (24), 3.3 × 10⁸ (24), 9 × 10⁷ (24). A second order rate constant of 2.25 × 10⁹ M⁻¹ s⁻¹ was used to account for the dimerization of tyrosyl radicals to dityrosine (25).

**RESULTS**

The formation of 3-nitrotyrosine was measured in the presence of four different NO/O₂⁻-generating systems. As shown in Table I, incubation of SPER/NO (1 mM; initial rate of NO release ~100 nM s⁻¹) with tyrosine (1 mM) led to the formation of 1.8 μM ± 0.18 μM 3-nitrotyrosine within 3 h. As observed earlier (10), nitration yield was markedly reduced (54.7 ± 13.15 μM) upon simultaneous generation of O₂⁻ from XO/hypoxanthe. Based on the total amount of peroxynitrite that had been formed (32.5 ± 5.3 μM), the nitrating efficiency of this system was 0.17%. The XO reaction results in an accumulation of urate, a potent scavenger of peroxynitrite (26, 27). Therefore,
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Experiments were performed in the presence of 0.1 units/ml uricase, an enzyme that oxidizes urate to allantoin and \( \text{H}_2\text{O}_2 \) (28). Allantoin (0.1 mM) had no effect on tyrosine nitration mediated by authentic peroxynitrite (data not shown). In the presence of uricase, the amount of detectable peroxynitrite was approximately doubled, accompanied by a 6-fold increase in 3-nitrotyrosine formation. The corresponding nitrating efficiency was 0.54%. These results suggested that accumulation of urate does indeed contribute to the low nitrating efficiency of the applied NO/\( \text{O}_2^- \)-generating system, but the nitration yield was still about 10-fold lower than that obtained with 70 \( \mu \text{M} \) authentic peroxynitrite (3.41 \( \pm \) 0.63 \( \mu \text{M} \), corresponding to 4.9%) and about 5-fold lower than the nitration triggered with SPER/NO alone. It was conceivable that this difference was caused by residual urate, because urate did not completely consume the accumulated urate under our experimental conditions (data not shown). Therefore, two urate-free NO/\( \text{O}_2^- \)-generating systems were additionally tested.

Acetaldehyde is known to function as an alternative substrate of XO albeit at much lower turnover numbers (29). Incubation of 1 mM acetaldehyde with 50 milliunits/ml XO resulted in formation of 25.5 \( \pm \) 3.3 \( \mu \text{M} \) \( \text{O}_2^- \) s\(^{-1} \), a rate that was approximately matched by the rate of NO released from 0.3 mM SPER/NO (30 \( \pm \) 30 \( \mu \text{M} \) s\(^{-1} \)). At this concentration, the NO donor alone led to formation of 165.5 \( \pm \) 43.6 \( \mu \text{M} \) 3-nitrotyrosine (Tab.1). The 11-fold decrease in nitration upon an only 3.3-fold decrease in the SPER/NO concentration supports our earlier proposal (10) that NO-induced nitration is mediated by a product of NO autoxidation, a reaction that follows second order kinetics with respect to NO. Coincubation of SPER/NO with the XO/acetaldehyde system resulted in formation of 45.9 \( \pm \) 6.9 \( \mu \text{M} \) peroxynitrite (measured as DHR oxidation) and an about 11-fold reduction of 3-nitrotyrosine formation, corresponding to a nitrating efficiency of 0.04%. As expected from recent reports on the slow reaction of peroxynitrite with aldehydes (30, 31), tyrosine nitration by authentic peroxynitrite was only slightly inhibited to about 75% of controls by 1 mM acetaldehyde (data not shown).

Finally, we used illuminated FMN as a nonenzymatic source of \( \text{O}_2^- \) (32). Formation of \( \text{O}_2^- \) was assessed indirectly by measuring NO release from DEA/NO with a Clark-type NO electrode in the absence and presence of FMN in light and dark conditions with and without SOD (Fig. 1A). FMN had no significant effect on NO autoxidation in dark conditions (trace \( a \) versus \( b \)) but led to a rapid apparently zero order decay of NO upon illumination (trace \( c \)), an effect that was largely (\(-73\%\)) inhibited by SOD (trace \( d \)), confirming that FMN might be a useful source for \( \text{O}_2^- \) in our experiments.

Incubation of DEA/NO (0.1 mM) with 1 mM tyrosine in the absence and presence of 0.1 mM FMN led to formation of 86.1 \( \pm \) 29.5 \( \mu \text{M} \) and 678 \( \pm \) 90.8 \( \mu \text{M} \) 3-nitrotyrosine, respectively. The effect of FMN was concentration-dependent; maximal effects were obtained with \( \geq 0.1 \text{mM} \), the apparent EC\(_{50} \) was 57.5 \( \pm \) 7.0 \( \mu \text{M} \) (Fig. 1B). FMN did not significantly increase DEA/NO-mediated nitration in dark conditions (data not shown). Due to a strong interference of FMN with the DHR assay,\(^3\) it was not possible to measure apparent peroxynitrite formation by this system, but the DEA/NO-FMN system allowed us for the first time to demonstrate a stimulation of NO-mediated nitration by co-generation of \( \text{O}_2^- \), indicating that the \textit{in situ} generation of peroxynitrite does lead to tyrosine nitration under certain experimental conditions.

Intriguing data were obtained when the nitrating efficiencies of NO/\( \text{O}_2^- \)-generating systems were studied in the presence of bicarbonate (\( \text{CO}_3^- \)). \( \text{CO}_3^- \) is known to react rapidly with peroxynitrite to yield the potent nitrating adduct nitrosoperoxycarbonate (\( \text{ONO}_2\text{CO}_3^- \)) (33). Therefore, depending on the buffer concentrations of \( \text{CO}_3^- \) (34), tyrosine nitration by authentic peroxynitrite is increased 2- to 4-fold upon the addition of 0.25–50 mM bicarbonate (5, 35–37). The data obtained with four NO/\( \text{O}_2^- \)-generating systems tested for tyrosine nitration with and without 25 mM bicarbonate (5, 35–37) clearly demonstrated that \( \text{CO}_3^- \) had no effect whatsoever on nitration by NO/\( \text{O}_2^- \), although it produced a consistent stimulation of nitration by authentic peroxynitrite added as a bolus at concentrations approximately matching those formed by the respective NO/\( \text{O}_2^- \)-generating systems. The nitrating efficiency of NO/\( \text{O}_2^- \) varied from 0.04% (SPER/NO plus XO/acetaldehyde) to 0.68% (DEA/NO plus FMN) but never approached the efficiency of authentic peroxynitrite (7–10%). Since \( \text{CO}_3^- \) radicals formed by homolysis of \( \text{ONO}_2\text{CO}_3^- \) may be essential to nitration (38), the data suggest that \( \text{CO}_3^- \) was efficiently scavenged by an unknown competing reaction under our experimental conditions.

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\(^3\) S. Pfeiffer and B. Mayer, unpublished data.
We considered several possibilities to explain the poor nitrating efficiency of NO/O₂⁻. Unfortunately, however, most hypotheses, including the proposal of a distinct chemical species that is formed from NO/O₂⁻ in situ (10), are in conflict with the known theoretical background of NO/O₂⁻ and/or peroxynitrite chemistry. One remaining possibility was that tyrosine nitration required a certain threshold steady-state level of peroxynitrite to become significant. This would explain the observed differences between bolus addition and continuous generation of peroxynitrite. We have addressed this issue using two experimental approaches.

First we studied the nitrating efficiency of increasing peroxynitrite concentrations (5–1,000 µM) added as a bolus to buffer solutions containing 1 mM tyrosine. As expected, the total amount of 3-nitrotyrosine gradually increased with increasing concentrations of added peroxynitrite (46.23 ± 1.59 µM at 1 mM; data not shown). It was surprising, however, to find that the nitrating efficiency of peroxynitrite increased from 1.4 ± 0.3 to 5.4 ± 0.4% when the peroxynitrite concentration was increased from 5 µM to 100 µM and leveled off at higher concentrations (Fig. 3A). In another set of experiments 2 ml of a 0.1 mM stock solution of peroxynitrite was infused at increasing rates (3.33 nm s⁻¹, 16.67 nm s⁻¹, 33.33 nm s⁻¹, 41.67 nm s⁻¹, and 66.67 nm s⁻¹) into tyrosine-containing buffer solutions (10 µM peroxynitrite final in each case). The respective steady-state concentrations of peroxynitrite were calculated from the rate of first order decomposition measured by stopped-flow absorbance spectroscopy under identical conditions (k₆ = 0.27 ± 0.05 s⁻¹; data not shown). Fig. 3B shows that the nitrating efficiency of infused peroxynitrite increased about 3-fold (from 0.22 ± 0.05 to 0.64 ± 0.08%) when the steady-state concentrations were increased from 30.7 to 247 nM.

Since dityrosine is another product of the reaction between tyrosine and peroxynitrite (4, 35, 36), we speculated that the tyrosine dimerization reaction may be predominant at low peroxynitrite concentrations. To test this hypothesis, we measured dityrosine formation from 1 mM tyrosine treated with increasing concentrations of peroxynitrite. As shown in Fig. 4, a maximal yield of 17.0 ± 3.8% dityrosine was obtained with the lowest peroxynitrite concentration that has been tested (1 µM) and decreased down to less than 1% at ≥1 mM peroxynitrite. The replots of the 3-nitrotyrosine data (open symbols in Fig. 4) demonstrates that dityrosine is indeed the major product of tyrosine reacting with low concentrations of peroxynitrite.

**DISCUSSION**

This study extends our previous observations showing that the nitrating efficiency of simultaneously generated NO/O₂⁻ is much lower than that of authentic peroxynitrite (10). The potential scavenging effect of urate was minimized or excluded by generation of O₂⁻ in the presence of uricase or with urate-free systems such as XO/acetaldehyde or FMN. With all O₂⁻ generators investigated except FMN, we observed an inhibition of NO-mediated nitration by co-generation of O₂⁻. Co-incubation of DEA/NO with FMN, however, resulted in a significant stimulation of nitration by the O₂⁻ donor. Even though the nitrating...
efficiency of the combined system was still significantly lower than that of authentic peroxynitrite, these results suggested that peroxynitrite formed from NO/O$_2^*$ may indeed be capable of triggering nitration under certain conditions. It is conceivable that tyrosine nitration has been quenched by XO that was used for O$_2^*$ generation in the other experimental set-ups. Accordingly, the protein-free DEA/NO-FMN system apparently allowed the detection of the minor nitration reaction triggered by peroxynitrite at low steady-state concentrations.

The most interesting finding of this study was the observation that dityrosine formation almost completely outcompeted nitration at low concentrations of peroxynitrite. As a mechanistic explanation of these surprising results, we propose the scheme depicted in Fig. 5. Accordingly, the key event of both reactions, nitration and dityrosine formation, would be the formation of tyrosyl radicals by NO$_2$ in the course of homolytic cleavage of ONOOH (Equations 1 and 2, path a in Fig. 5). The tyrosyl radicals could either react with NO$_2$ to yield 3-nitrotyrosine (Equation 3, path b) or dimerize to give dityrosine (Equation 4, path c). A major competing reaction would be the dimerization of NO$_2$ yielding N$_2$O$_4$ (Equation 5, path d).

\[
\text{ONOOH} \rightarrow \text{OH} + \text{NO}_2 \quad \text{(Eq. 1)}
\]
\[
\text{Tyr} + \text{NO}_2 = \text{Tyr} + \text{NO}_2 + \text{H}^+ \quad \text{(Eq. 2)}
\]
\[
\text{Tyr} + \text{NO}_2 = \text{NO}_2^- \text{Tyr} \quad \text{(Eq. 3)}
\]
\[
\text{Tyr} + \text{Tyr} = \text{Tyr-Tyr} \quad \text{(Eq. 4)}
\]
\[
\text{NO}_2^- + \text{NO}_2 = \text{N}_2\text{O}_4 \quad \text{(Eq. 5)}
\]

Homolysis of ONOOH (Equation 1) has been questioned based on thermodynamical calculations (39), but recent evidence suggests that about 30% of ONOOH does indeed yield free NO$_2$ and OH, whereas the residual 70% undergoes rearrangement to nitric acid without escape of free radicals (22, 23, 40). Tyrosyl radical formation by NO$_2$ and subsequent combination of Tyr and NO$_2$, has been reported to occur with second order rate constants of $3.2 \times 10^9$ and $3 \times 10^9$ M$^{-1}$s$^{-1}$, respectively (24). Rate constants of $9 \times 10^6$ and $2.25 \times 10^8$ M$^{-1}$s$^{-1}$, respectively, were reported for the two major competing reactions, i.e. the dimerization of NO$_2$ (24) and the combination of two Tyr radicals to yield dityrosine (25).

Together with the rate of peroxynitrite decomposition determined by stopped-flow spectroscopy under our experimental conditions (0.27 s$^{-1}$), the published rate constants were used for the kinetic simulation of peroxynitrite reacting with excess free tyrosine, assuming 30% homolysis of ONOOH. Fig. 6 shows that the yields of 3-nitrotyrosine and dityrosine predicted by the model for tyrosine reacting with peroxynitrite at concentrations ranging from 1 µM to 2 mM are similar in shape to the measured data illustrated in Fig. 4. In agreement with our observations, the model predicts an inverse dependence on peroxynitrite concentration of tyrosine nitration and dimerization. At low peroxynitrite concentrations, dimerization of Tyr radicals (filled symbols) is the predominant pathway, whereas nitration (open symbols) and NO$_2$ dimerization (not shown), which both follow second order kinetics with respect to NO$_2$, become the predominant reactions at high peroxynitrite (and thus NO$_2$) concentrations. The measured yields of dityrosine agreed well with the predictions of the model, but the measured 3-nitrotyrosine levels were 2-fold below the theoretical expectation over the complete range of peroxynitrite concentrations. This quantitative mismatch suggests that reactions not considered in the kinetic simulation compete with tyrosine nitration. These reactions may involve OH radicals, as it was shown previously that OH radical scavengers significantly enhance peroxynitrite-triggered tyrosine nitration (4). Therefore, it is likely that the reactions of OH radicals with NO$_2$ to yield HNO$_3$ and with Tyr radical, resulting in the formation of 3-hydroxytyrosine (dopa) (36), compete with the nitration reaction. Since the rate constants of the reactions triggered by OH are not known, it was not possible to account for them in the kinetic model. Nonetheless, we think that, despite some quantitative uncertainties, the proposed model provides a simple and reliable mechanistic explanation for the insignificant nitration efficiency of peroxynitrite generated in situ.

What are the implications of the present study for the effects of peroxynitrite generated from NO/O$_2^*$ in vivo? Obviously, the oxidative chemistry of peroxynitrite, including dityrosine formation, would be expected to be predominant at the relatively low NO/O$_2^*$ fluxes that are likely to occur in most in vivo conditions. As a specific marker of oxidation, dityrosine has

**Fig. 4.** Dityrosine formation by authentic peroxynitrite. Increasing concentrations of peroxynitrite (1–2,000 µM) were added to 0.1 M K$_2$HPO$_4$/KH$_2$PO$_4$ buffer (pH 7.4) containing 1 mM tyrosine at ambient temperature and were analyzed for dityrosine as described under "Experimental Procedures." Data are mean values ± S.E. of seven separate experiments performed in duplicate. A replot of the 3-nitrotyrosine data from Fig. 3A (open symbols) is shown for comparison.

**Fig. 5.** Hypothetical mechanism of 3-nitrotyrosine and dityrosine formation by peroxynitrite.
been detected in human atherosclerotic plaques (41, 42) in the brain of elderly humans (43) or patients affected with Alzheimer's disease (44), in age-related nuclear cataract (45) and other pathologies thought to be associated with oxidative stress. Formation of dityrosine has been attributed mainly to the activation of the myeloperoxidase/H2O2 system of neutrophils and macrophages (46), but other peroxidases (47) and peroxynitrite (36, 48) have been recognized as additional sources of dityrosine. The present results agree with previous studies suggesting that dityrosine formation together with increased NO synthase expression may be a useful marker for peroxynitrite formation in tissues (49, 50).

With respect to tyrosine nitration, it seems unlikely that the high NO/O2 fluxes that are required to cause this reaction can occur in vivo. However, the rates of O2 production by activated neutrophils are typically in the range of 0.02–0.05 nmol s−1/106 cells (51–53). Based on a neutrophil cell volume of about 300 femtoliters (54) the upper limit of O2 release during the respiratory burst of neutrophils would be about 55–150 μM s−1, indicating that the rates of O2 release could be sufficiently high to cause nitration via the peroxynitrite pathway, even at 50-fold dilution of the cells in biological fluids or tissues. But in vivo measurements of NO, O3, and peroxynitrite in aorta of endotoxemic rats suggested that the maximal steady-state levels of O2, which apparently limited peroxynitrite formation, were about 50 nM (55), indicating that scavenging of O2 by SOD and other mechanisms may limit peroxynitrite formation in vivo. Another potential source of NO/O3 is neuronal NO synthase activated in the course of brain ischemia/reperfusion injury, resulting in cortical NO steady-state concentrations of up to 1.5 μM (56, 57). A pivotal role of NO/O3 interaction in neurotoxicity is suggested by studies on motor neuron degeneration (58), and reports with transgenic mice and SOD knockout mutants showing that both Cu,Zn and Mn-SOD are protective against stroke (59). Our data render it likely that the molecular mechanisms underlying these pathologies are related to protein oxidation and/or cross-linking rather than nitration. It is conceivable that latter reaction is triggered by peroxynitrite-independent pathways involving myeloperoxidase (60–62) or other peroxidases (63). As a further alternative, trapping of tyrosyl radicals by NO and subsequent peroxynitrite-mediated oxidation of nitroso tyrosine could result in the formation of 3-nitrotyrosine (64). The latter mechanism would imply that several pathways have to be activated at the same time to cause significant nitration. In inflammatory tissues, for example, induction of macrophage NO synthase together with the activation of neutrophil NADPH oxidase and secretion of myeloperoxidase would constitute a highly efficient nitrating system operating through several pathways. Further studies should clarify which of these pathways or which combinations of them are responsible for tyrosine nitration in human disease.

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