Nitric Oxide Trapping of the Tyrosyl Radical of Prostaglandin H Synthase-2 Leads to Tyrosine Iminoxyl Radical and Nitrotyrosine Formation*

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The determination of protein nitrotyrosine content has become a frequently used technique for the detection of oxidative tissue damage. Protein nitration has been suggested to be a final product of the production of highly reactive nitrogen oxide intermediates (e.g. peroxynitrite) formed in reactions between nitric oxide (NO) and oxygen-derived species such as superoxide. The enzyme prostaglandin H synthase-2 (PHS-2) forms one or more tyrosyl radicals during its enzymatic catalysis of prostaglandin formation. In the presence of the NO-generator diethylamine nonoate, the electron spin resonance spectrum of the PHS-2-derived tyrosyl radical is replaced by the spectrum of another free radical containing a nitrogen atom. The magnitude of the nitrogen hyperfine coupling constant in the latter species unambiguously identifies it as an iminoxyl radical, which is likely formed by the oxidation of nitrosotyrosine, a stable product of the addition of NO to tyrosyl radical. Addition of superoxide dismutase did not alter the spectra, indicating that peroxynitrite was not involved. Western blot analysis of PHS-2 after exposure to the NO-generator revealed nitrotyrosine formation. The results provide a mechanism for nitric oxide-dependent tyrosine nitration that does not require formation of more highly reactive nitrogen oxide intermediates such as peroxynitrite or nitrogen dioxide.

The detection of nitrotyrosine in proteins or tissues that have been exposed to conditions of oxidative stress is rapidly becoming an assay of choice in the implication of oxidative tissue damage as a mechanism of disease. Nitrotyrosine has been detected in samples from a wide variety of disease states, including acute lung injury (1, 2), atherosclerosis (3, 4), neurodegenerative diseases (5–8), bacterial and viral infections (9–11), aging (12), chronic inflammation (13), and exposure to cigarette smoke (14) or carbon monoxide (15). In most of the above cases, the nitrotyrosine detection was presumed to be the result of the reaction of tyrosine residues with peroxynitrite/peroxynitrous acid. Peroxynitrite is formed in the reaction between nitric oxide (NO) and superoxide. Recently, however, other mechanisms for the production of nitrotyrosine that are independent of peroxynitrite have been identified (16, 17), raising doubts about the specificity of nitrotyrosine detection as an assay for peroxynitrite (18).

Nitric oxide has been shown to react with the stable tyrosyl radical residue that is involved in the catalytic mechanism of ribonucleotide reductase, quenching the tyrosyl radical signal in the electron spin resonance (ESR) spectrum of the enzyme (19). Nitric oxide, a free radical, is expected to form a radical-radical adduct with organic radicals such as the phenoxyl radical of a tyrosine residue. The inhibition of ribonucleotide reductase and the quenching of its tyrosyl radical ESR spectrum are reversible with time (19–22), suggesting that NO forms a complex with the radical that can decay back to the radical pair. Oxidation of such a complex, however, would likely lead to the formation of modified tyrosine residues, including nitrotyrosine. Prostaglandin H synthase, like ribonucleotide reductase, has a tyrosine residue oxidized to the corresponding phenoxyl radical during its catalysis. To test the hypothesis that nitrotyrosine can be formed from the NO-adduct of a tyrosyl radical, the tyrosyl radical formed during the catalytic production of prostaglandins by prostaglandin H synthase-2 was exposed to the nitric oxide-generating compound diethylamine nonoate.

MATERIALS AND METHODS

Prostaglandin H Synthase Preparation—Human prostaglandin H synthase-2 (PHS-2) was expressed in a baculovirus expression system and purified as the apoprotein (23). Protein concentrations were determined by the method of Bradford (24). After adjustment of the enzyme concentration to that desired for a particular experiment, the apoprotein was reconstituted at room temperature for 10 min with either heme or Mn-heme to a concentration of 1 heme/ubiquitin. Heme and Mn-heme were dissolved in Me3SO at sufficient concentration to allow reconstitution of the enzyme with 10 μl of heme stock solution. Protein samples were then frozen and stored at −70 °C until just before use.

ESR Spectroscopy—PHS-2 samples were thawed and transferred (sample volume 200 μl) into a 3-mm quartz ESR sample tube using a Hamilton syringe equipped with a long needle. Reactions were performed at room temperature and were initiated by the addition of arachidonic acid (acquired from NuChek Prep, Elysian, MN) at the desired concentration contained in 10 μl of ethanol (arachidonic acid stock solution concentrations varied). After brief mixing with a nichrome wire, the samples were frozen 4 s after addition of arachidonic acid in liquid nitrogen and were transferred to a quartz fingertip Dewar, which was then placed in a TM110 cavity of a Bruker ESP900 ESR spectrometer. Subsequent time points were acquired by thawing the sample to room temperature by immersion in a water bath for the desired interval, followed by refreezing in liquid nitrogen. In that manner, a single sample could be used to follow the time course of the...
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reaction. The NO\textsuperscript{+} donor diethylamine nonoate (DEA/NO; Cayman Chemical, Ann Arbor, MI) was dissolved in water to a concentration of 25 mM immediately before use and was added to the enzyme at a final concentration of 500 \mu M. Arachidonic acid was added after a 3-min incubation with DEA/NO at room temperature. ESR spectra were acquired using the following instrument settings: modulation amplitude, 1 G; modulation frequency, 100 kHz; time constant, 1.3 s; scan time, 1342 s; receiver gain, 1 \times 10\textsuperscript{5} unless otherwise noted. Spectral simulations were calculated using the Powfit program of the NIEHS public ESR software tools package.\textsuperscript{a} Determination of g values was accomplished by comparison to a Cr\textsuperscript{3+} in MgO g standard (g\textsubscript{iso} = 1.9800 \pm 0.0005).\textsuperscript{25}

**Western Blot Analysis—**PHS-2 was recovered from the ESR sample tubes and was analyzed by Western blot after dilution to 10 \mu M. Each sample was diluted 1:1 with Laemmli sample buffer, boiled for 5 min, and immediately loaded onto a gel. Samples were run on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were electrophotographically transferred to pure nitrocellulose membrane in 25 mM Tris, 192 mM glycine, and 20% methanol. Membranes were blocked overnight at 4°C in Tris-buffered saline containing 0.2% Tween 20 (TBST) with 5% BSA (Life Technologies, Inc.). The blots were then incubated for 1 h with either anti-nitrotyrosine antibody (1 \mu g/ml) (Upstate Biotechnology Inc., Lake Placid, NY) or anti-human PHS-2 antibody (1:2000) (Oxford Biomedical Research Inc., Oxford, MI) in 1% BSA at room temperature. Blots were then washed five times in TBST with 0.1% BSA, and incubated for 1 h with peroxidase-conjugated anti-rabbit IgG (1:5000 or 1:10000) (Amersham) in 1% BSA at room temperature. The blots were again washed five times in TBST with 0.1% BSA and then visualized using the Enhanced Chemiluminescence detection (ECL) system (Amersham).

**RESULTS**

The ESR spectrum of PHS-2 acquired 4 s after addition of arachidonic acid exhibited a single line with a peak-to-trough line width of 27.4 G and with indications of resolved hyperfine structure (Fig. 1, spectrum A), which has been assigned to a tyrosyl radical residue (26). The ESR spectra obtained after additional incubation at room temperature maintained the linewidth observed at the first time point, but the lineshape increased in doublet character (Fig. 1, spectra B–D). The signal intensity also decreased at the later time points. No ESR spectrum was detected in the absence of added arachidonic acid (data not shown).

Inclusion of the NO\textsuperscript{-} generator DEA/NO in the reaction mixture resulted in the replacement of the tyrosyl radical spectrum with that of another species, which exhibited hyperfine coupling to an atom with a nuclear spin of 1, e.g., a nitrogen atom (Fig. 1, spectrum E). The concentration of this species persisted for another 10 s at room temperature but decreased to undetectable levels after further incubation at room temperature (Fig. 1, spectra G and H). The spectrum detected at 77 K in the presence of NO\textsuperscript{-} is typical of an immobilized nitroxide, but the nitrogen hyperfine coupling constants were much larger than those of simple nitroxides, approximately A\textsubscript{1} = 39 G and A\textsubscript{2} = 20 G (see Fig. 2). The magnitude of the isotropic nitrogen hyperfine coupling constant (\alpha\textsubscript{iso} = (A\textsubscript{1} + 2A\textsubscript{2})/3 = 26.3 G) identifies the free radical detected in the presence of NO\textsuperscript{-} as an iminoxyl radical (27). The spectrum was simulated starting with the nitrogen hyperfine coupling constants measured from the spectrum by inspection. Slight variations of the hyperfine coupling constants by the computer program to minimize the difference between the experimental and the calculated spectra gave a best-fit simulation with the following parameters: A\textsubscript{1} = 19.9 G, A\textsubscript{3} = 23.7 G, and A\textsubscript{2} = 40.1 G (A\textsubscript{iso} = 27.9 G); g\textsubscript{x} = 2.0078, g\textsubscript{y} = 2.0053, g\textsubscript{z} = 2.0042 (the simulation is presented in Fig. 2). In some experiments the ESR spectra obtained from samples that had been exposed to DEA/NO exhibited characteristics of both the tyrosyl radical and the iminoxyl radical discussed above, with the iminoxyl radical becoming the dominant species after longer room temperature incubations. The use of lower concentrations of DEA/NO resulted in correspondingly increased tyrosyl radical character of the ESR spectra (data not shown). Depletion of the DEA/NO stock solution by overnight incubation at room temperature (16 h) before use prevented iminoxyl radical formation (data not shown).

Peroxynitrite forms in the reaction between superoxide and NO\textsuperscript{-} under physiological conditions (28). To eliminate the possibility that the iminoxyl radical formation arose from a reaction involving peroxynitrite, superoxide dismutase was added to the reaction mixture to prevent peroxynitrite formation. Inclusion of 1000 units/ml superoxide dismutase in the reaction mixture did not modify either the ESR spectrum or the kinetics of its changes as a function of time in the NO\textsuperscript{-} experiments (data not shown).

Reconstitution of PHS-2 with manganese-heme rather than iron-heme results in a protein that retains cyclooxygenase activity but has greatly decreased peroxidase activity (29). Some published evidence has suggested the formation of an enzymatic tyrosyl radical during the catalysis of Mn-PHS-1 (29), but that suggestion has not been supported with unequivocal ESR data (30, 31). To test whether tyrosyl radicals are formed in the Mn-enzyme and to determine whether iminoxyl radical formation is a more general phenomenon, some exposure of tyrosyl radicals to NO\textsuperscript{-} was reconstituted with Mn-PHS-2 (Fig. 3). Addition of arachidonic acid to PHS-2 that had been reconstituted with manganese-heme resulted in the generation of an ESR spectrum with a peak-to-trough linewidth of 30 G (Fig. 3) and a g value of 2.005, which is slightly lower than that of the tyrosyl radical of Fe-PHS (2.006). No hyperfine structure was resolved in the ESR spectrum of Mn-PHS-2 (Fig. 3, spectra

\textsuperscript{a} This software is available via the World Wide Web (http://alfred.niehs.nih.gov/LMB/pest/).
FIG. 2. Experimental and computer-simulated spectra of the tyrosyl iminoxyl radical resulting from the reaction of PHS-2 tyrosyl radical and nitric oxide. Upper spectrum, arachidonic acid (250 μM) was added to PHS-2 (25 μM) and DEA/NO (500 μM), and the sample was frozen within 4 s of the addition of arachidonic acid. Lower spectrum, the computer simulation of the tyrosine iminoxyl radical calculated using the following hyperfine coupling tensor: $A_1 = 19.9$ G; $A_2 = 23.7$ G; $A_3 = 40.1$ G and the following $g$ tensor: $g_z = 2.0078$, $g_y = 2.0053$, $g_x = 2.0042$. The estimated values for $A_1 (A_2$ and $A_3$) and for $A_2$ ($A_3$) measured from the experimental spectrum were 20 and 39 G, respectively, as indicated. The value of $g_z$ was also measured from the spectrum. The simulation program was allowed to adjust the initial nitrogen hyperfine coupling tensor to minimize the difference between the experimental and simulated spectra resulting in the final hyperfine coupling tensor.

A–E). Previous experiments have indicated that the signal arises from multiple radicals (30).

Upon reaction of Mn-PHS with arachidonic acid in the presence of DEA/NO, the ESR spectrum of the protein is much less intense than the spectrum obtained from Mn-PHS-2 in the absence of DEA/NO (12 ± 6% of the integrated area of the Mn-PHS-2 spectrum). Increasing intensity in the low field region of the spectrum is observed upon longer incubation at room temperature, with the spectrum detected after 3 min strongly resembling the iminoxyl radical spectrum observed with Fe-PHS (Fig. 3, spectra F–K). Apparently, the tyrosyl and the tyrosine iminoxyl radicals coexist at intermediate time points. As was the case with Fe-PHS-2 in the presence of DEA/NO, hyperfine coupling to a nitrogen atom with $A_1 = 38$ G and $A_2 = 22$ G (compare with 39 and 20 G, respectively, for Fe-PHS) was observed, consistent with iminoxyl radical formation. The greater linewidth of the ESR signal detected from Mn-PHS-1 (29, 30) has been suggested to arise from magnetic coupling between the paramagnetic manganese heme system and the radical (31). This proposal is consistent with the greater linewidth observed in the iminoxyl radical detected from Mn-PHS-2.

The conversion of the tyrosyl radical to an iminoxyl radical to ESR-silent products in Fe-PHS-2 suggests a continuing series of oxidations. The 1-electron oxidation of an iminoxyl radical would lead to nitrotyrosine residues in PHS-2. To test this hypothesis, both Fe-PHS-2 and Mn-PHS-2 samples, subsequent to the ESR studies, were subjected to Western analysis using an antibody specific for nitrotyrosine. A triplet staining pattern was observed for both the Fe-PHS-2 and Mn-PHS-2 samples that were exposed to NO at the appropriate molecular weight for human PHS-2 (Fig. 4). Furthermore, the triplet band was also detected in the Fe-PHS-2 with NO’ sample in the presence of superoxide dismutase (Fig. 4). Increasing the concentration of superoxide dismutase from 6 μM to 1 mM did not prevent nitrotyrosine formation (data not shown). However, no nitrotyrosine was detected in the samples that had not been exposed to NO (Fig. 4). This is consistent with the nitration of one or more tyrosine residues on the protein. The triplet band observed is due to the differential glycosylation states of the protein (32). The blots were then stripped and reprobed with an anti-human PHS-2 antibody (data not shown). PHS protein was observed in all lanes at the same molecular weight as the nitrotyrosine proteins detected for both the Fe- and Mn-PHS samples, indicating that the nitrated proteins observed are indeed PHS-2. The detection of nitrotyrosine in the Mn-PHS samples in the Western blot indicates that at least part of the
ESR signal observed in the absence of NO arises from a tyrosyl radical (Fig. 3, A–E).

**DISCUSSION**

The free radical signal detected in the presence of the NO donor can be identified as an iminoxyl radical by the magnitude of its isotropic hyperfine coupling constant (27.9 G), which is substantially greater than the 15–16 G normally seen for amionoxyl nitroxides (>NO) but is similar to those normally seen for iminoxyl radicals (>C=NO) (33, 34). The relatively large hyperfine coupling constant has been suggested to result from a relatively localized electron density on the nitrogen atom (33). The detected A_νv is markedly similar to the value of 26.12 G detected from the product of the oxidation of 2-nitroso-4-methylphenol, a reasonable analog of tyrosine (27).

The combination of the ESR experiments in which an iminoxyl radical is detected and the Western analysis for nitrotyrosine residues in PHS-2 strongly suggests the mechanism outlined in Scheme 1. The reaction occurring between the PHS tyrosyl radical and NO results in the formation of an ESR-silent diamagnetic adduct, and has been shown to occur with free tyrosyl radical at a rate that is nearly diffusion controlled (35). Formation of the ESR-silent complex between NO and tyrosyl radical shown in Scheme 1 has also been demonstrated in photosystem II (36) and ribonucleotide reductase (19). The initial complex formed between tyrosyl radical and NO can be decomposed back to tyrosyl radical and NO by evacuation of the sample, which effectively removes NO (35). Rearrangement of the initial, reversible adduct between the tyrosyl radical and NO to form 3-nitrosotyrosine, as shown in the scheme, results in the rearomatization of the system, and as such is highly energetically favored even if it is somewhat slow kinetically due to the required breakage of a C-H bond (37). The proposal that 3-nitrosotyrosine can decompose to form nitric oxide and tyrosyl radical (35) is mistaken in that the cleavage of the C-N bond would form a σ radical rather than the π-phenol x tyrosyl radical.

The 1-electron oxidation of the minor oxime tautomer of nitrosotyrosine shown in Scheme 1 (38, 39) results in the formation of the iminoxyl radical that is detected. Iminoxyl radicals have been chemically synthesized by oxidation of the corresponding oximes (33, 34). Free nitrosophenols and nitrosonaphthols have been shown to be oxidized to the corresponding iminoxyl radicals both chemically (27) and with horseradish peroxidase (40). Iminoxyl radical formation has also been detected in the photosystem II system, although the time scale for iminoxyl radical formation is hours instead of seconds in that system (37).

Further oxidation of the tyrosine iminoxyl radical results in the formation of nitrotyrosine, an ESR-silent product that was detected in Western blots. The source of the oxidizing equivalents for the oxidations of nitrosotyrosine and its corresponding iminoxyl radical has not been identified. The strongly oxidizing peroxidase activity of PHS-2 represents one reasonable source. This conclusion is supported by the data from the Mn-substituted enzyme, in which the conversion of the tyrosyl radical to the iminoxyl radical is greatly slowed (compare Fig. 1 with Fig. 3) since the Mn-enzyme has only 4% of the peroxidase activity of the Fe-enzyme (28), but other oxidants such as nitrogen dioxide may also be important and have not been excluded.

The recently reported detection of a tyrosine iminoxyl radical resulting from the reaction between the tyrosyl radical of photosystem II and NO (37) and our evidence for tyrosine nitration via an iminoxyl radical in PHS-2 provide at least two examples of this chemistry in biological systems. The absence of inhibition of tyrosine nitration by the addition of a large concentration of superoxide dismutase indicates that the superoxide-dependent peroxynitrite/peroxynitrous acid is not involved in tyrosine nitration by NO in PHS-2.

There are numerous reports of tyrosyl radical formation in different systems throughout biology. In addition to the unstable tyrosyl radical of prostaglandin H synthase (41), stable tyrosyl radicals in ribonucleotide reductase and photosystem II (42, 43) have been detected. Furthermore, tyrosyl radicals have been detected in oxidatively damaged hemoproteins such as myoglobin (44), hemoglobin (45), and cytochrome c (46). Since NO is made by a variety of cell types, the opportunity for tyrosine iminoxyl free radical formation exists in any protein where tyrosyl radical formation occurs, making this a potentially general mechanism for tyrosine nitration.

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