Intramolecular Electron Transfer between Tyrosyl Radical and Cysteine Residue Inhibits Tyrosine Nitration and Induces Thyl Radical Formation in Model Peptides Treated with Myeloperoxidase, $H_2O_2$, and $NO_2^-$

EPR SPIN TRAPPING STUDIES

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We investigated the effects of a cysteine residue on tyrosine nitration in several model peptides treated with myeloperoxidase (MPO), $H_2O_2$, and nitrite anion ($NO_2^-$) and with horseradish peroxidase and $H_2O_2$. Sequences of model peptides were acetyl-Tyr-Cys-amide (YC), acetyl-Tyr- Ala- Ala- Cys-amide (YAC), and acetyl-Tyr- Ala- Ala- Ala- Cys-amide (YAAAAC). Results indicate that nitration and oxidation products of tyrosyl residue in YC and other model peptides were barely detectable. A major product detected was the corresponding disulfide (e.g. YCysCysY). Spin trapping experiments with 5,5'-dimethyl-1-pyrroline $N$-oxide (DMPO) revealed a thiyl adduct (e.g. DMPO-Cys-Tyr) from formation of peptides (e.g. YC) treated with MPO/$H_2O_2$ and MPO/$H_2O_2$/NO$_2^-$. The steady-state concentrations of DMPO-thiyl adducts decreased with increasing chain length of model peptides. Blocking the sulfydryl group in YC with methylmethanethiosulfonate (that formed YCSSCH$_3$) totally inhibited thiyl radical formation as did substitution of Tyr with Phe (i.e. FC) in the presence of MPO/$H_2O_2$/NO$_2^-$. However, increased tyrosine nitration, tyrosine dimerization, and tyrosyl radical formation were detected in the MPO/$H_2O_2$/NO$_2^-$/YCSSCH$_3$ system. Increased formation of S-nitrosated YC (YCysNO) was detected in the MPO/$H_2O_2$/NO$_2^-$ system. We conclude that a rapid intramolecular electron transfer reaction between the tyrosyl radical and the Cys residue impedes tyrosine nitration and induces corresponding thiyl radical and nitrosocysteine product. Implications of this novel intramolecular electron transfer mechanism in protein nitration and nitrosation are discussed.

There is increasing evidence for generation of inflammatory oxidants including the reactive oxygen/nitrogen species in the progression and pathogenesis of cardiovascular, pulmonary, and neurodegenerative diseases (1–9). Supporting evidence came from the identification of the post-translational modification of protein and lipid oxidation/nitration marker products (10–12). Prominent nitrosative, nitrosative, and oxidative reactions in tissues include tyrosine nitration, cysteine and tryptophan nitrosation, tyrosine, tryptophan, histidine, and methionine oxidation and lipid oxidation/nitration (12–15). The goal of the present study was to monitor the influence of a cysteine residue on tyrosine nitration. Several studies have shown that tyrosine nitration is a selective process that is controlled by various microenvironmental factors (hydrophobicity, $CO_2$ levels, membrane oxygen concentration, acidic environment, and amino acid sequence) (16–21). Previously, we have shown, using membrane-incorporated tyrosine analogs and tyrosyl peptides, that dityrosine formation is not a significant reaction process for tyrosyl radicals in membranes due to hindrance of free diffusion of tyrosyl radicals (22). In addition, the location of the tyrosyl probe in the membrane determines the transmembrane nitration profile (23). Literature data show that the $CO_2$ levels greatly influence the kinetics of tyrosyl nitration in proteins (24, 25).

Factors influencing nitration of tyrosyl residues in protein are not fully known. As discussed in previous reviews (12, 21, 26), the local environment of tyrosine residues within the secondary and tertiary structure of the protein will probably influence the site of tyrosine nitration. Although no specific amino acid sequence criteria exist for predicting tyrosine nitration or lack thereof, it has been shown, as originally suggested, that protein tyrosyl nitration is (i) enhanced when tyrosine is situated closer to a negatively charged amino acid (i.e. glutamate or aspartate) and (ii) decreased when tyrosine residue is present in the vicinity of a cysteinyl or methionine residue (12, 21, 26). However, detailed quantitative analysis of these effects on tyrosyl nitration is lacking, although a more recent report focused on the effect of lysine residues on tyrosyl nitration (27). Understanding the biophysical/biochemical mechanisms that determine the motif for nitration-sensitive tyrosine residues has physiological and pathophysiological relevance (28).

Two major pathways were proposed to be responsible for tyrosine nitration in vivo (29–35). These involve either the catalytic action of heme peroxidases (e.g. myeloperoxidase, eosinophil peroxidase) using nitrite and $H_2O_2$ as substrates and/or the nitric oxide chemistry of peroxynitrite. In this study, we investigated the effects of cysteine on tyrosyl nitration (or tyrosine on cysteine oxidation) in model peptides subjected to oxidation by myeloperoxidase (MPO)$^3/H_2O_2$ and $NO_2^-$. Results indicate that a rapid intramolecular electron transfer between the tyrosyl radical and the cysteine residue controls the extent of tyrosine nitration and cysteine oxidation by MPO/$H_2O_2$/NO$_2^-$. The influ-
ence of intramolecular electron transfer reactions in protein nitration and nitrosation reactions is discussed.

**EXPERIMENTAL PROCEDURES**

**Materials**

The following chemicals and enzymes were purchased from various sources as indicated: tyrosine, hydrogen peroxide, sodium nitrite, sodium bicarbonate, 3-nitrotirosine, N-ethylmaleimide, cysteine, and GSH were from Sigma; myeloperoxidase was from Calbiochem. Rink amide methylbenzhydrylamine resin and all Fmoc-protected amino acids were from Calbiochem. Diisopropylcarbodiimide (DIC), 1-hydroxybezotriazole (HOBt), triisopropylsilane, methylmethanethiosulphonate (MMTS), N-methylpyrrolidione (NMP), and piperidine were purchased from Fisher.

**Peptide Synthesis and Purification**

The sequences of the peptides used in this study are given in Fig. 2S. The peptides were chemically synthesized using the standard Fmoc solid phase peptide-based synthetic procedure on an Advanced Chemtech model 90 synthesizer (Louisville, KY) (23). Rink amide methylbenzhydrylamine resin (loading 0.72 mmol/g) was used as a solid support. Fmoc-protected amino acids were coupled as HOBt-esters. All amino acids were double coupled using HOBt/DIC. The following steps were performed in the reaction vessel for each double coupling: deprotection of the Fmoc group with 20% piperidine in NMP for 30 min (twice), three NMP washes, two dichloromethane washes, first coupling with trifluoroacetic acid, and the combined trifluoroacetic acid filtrates for 30 min (twice). The resin was washed twice with dichloromethane and three times with methanol and then dried under vacuum prior to cleavage. The peptide was deprotected and cleaved from the resin with 90% trifluoroacetic acid containing triisopropylsilane for 3 h at room temperature. The resin was removed by filtration and washed with trifluoroacetic acid, and the combined trifluoroacetic acid filtrates were evaporated to dryness under a stream of dry N2 gas. The oily residue was washed three times with cold ether to remove the scavengers, and the dry crude peptide was dissolved in acetonitrile/H2O (1:1) and lyophilized. The crude peptides were purified by a semipreparative reverse phase HPLC on a RP-C18 (10 × 250 mm) column using a CH3CN/water gradient (5–25% CH3CN over 60 min) containing 0.1% trifluoroacetic acid at a flow rate of 3 ml/min. Detection was at 280 nm. The structure of the disulfide product was confirmed by liquid chromatography/mass spectrometry.

**Syntheses of Disulfide, Dityrosine, and Nitration Products of YC**

The peptides were chemically synthesized using the standard Fmoc solid phase peptide-based synthetic procedure on an Advanced Chemtech model 90 synthesizer (Louisville, KY) (23). Rink amide methylbenzhydrylamine resin (loading 0.72 mmol/g) was used as a solid support. Fmoc-protected amino acids were coupled as HOBt-esters. All amino acids were double coupled using HOBt/DIC. The following steps were performed in the reaction vessel for each double coupling: deprotection of the Fmoc group with 20% piperidine in NMP for 30 min (twice), three NMP washes, two dichloromethane washes, first coupling with trifluoroacetic acid, and the combined trifluoroacetic acid filtrates for 30 min (twice). The resin was washed twice with dichloromethane and three times with methanol and then dried under vacuum prior to cleavage. The peptide was deprotected and cleaved from the resin with 90% trifluoroacetic acid containing triisopropylsilane for 3 h at room temperature. The resin was removed by filtration and washed with trifluoroacetic acid, and the combined trifluoroacetic acid filtrates were evaporated to dryness under a stream of dry N2 gas. The oily residue was washed three times with cold ether to remove the scavengers, and the dry crude peptide was dissolved in acetonitrile/H2O (1:1) and lyophilized. The crude peptides were purified by a semipreparative reverse phase HPLC on a RP-C18 (10 × 250 mm) column using a CH3CN/water gradient (5–25% CH3CN over 60 min) containing 0.1% trifluoroacetic acid at a flow rate of 3 ml/min with detection at 280 nm.

**Syntheses of Disulfide, Dityrosine, and Nitration Products of YC**

The disulfide of YC peptide (Y(CysCysY)) was synthesized as follows. YC peptide (30 mM) in a phosphate buffer (100 mM, pH 7.4) containing 1 mM DTPA was incubated with 30 mM hydrogen peroxide at room temperature for 1 h. The reaction mixture was then mixed with a CH3CN/water gradient (5–25% CH3CN over 60 min) containing 0.1% trifluoroacetic acid at a flow rate of 3 ml/min. Detection was at 280 nm. The structure of the disulfide product was confirmed by ESI-MS analysis (M + H+ 469.3).

The YC peptide dityrosine (bis-N-acetyl tyrosylcysteine amide) was prepared as follows. The YC disulfide (15 mM) was incubated with 10 mM H2O2 and 100 μM of horseradish peroxidase (HRP) in a phosphate buffer (100 mM, pH 7.4) containing 100 μM DTPA for 20 min. The reaction mixture was then mixed with β-mercaptoethanol (500 mM), and HRP was removed by ultracentrifugation (M, 3000 cut-off). The product (YC dityrosine) was purified by a preparative HPLC (C-18, 250 × 10 mm) using a fluorescence detector (excitation 294 nm; emission, 410 nm). The YC dityrosine was eluted by a linear CH2CN gradient as above. The product was further confirmed by ESI-MS.

Nitrated YC peptide (Y(NO2)C) was prepared as follows. The YCyCysY peptide (1 mM) was mixed with peroxynitrite (10 mM) in a phosphate buffer (100 mM, pH 7.4) containing DTPA (100 μM) for 20 min. The reaction mixture was then mixed with 500 mM β-mercaptoethanol and incubated for 20 min. The resulting Y(NO2)C peptide was purified using a preparative HPLC. Nitro peptides show a characteristic UV-visible spectrum. Upon adding NaOH, the 350 nm absorption peak in MeOH was shifted to 430 nm with an extinction coefficient of 4100 M−1 m−1. The structure of Y(NO2)C was verified by liquid chromatography/mass spectrometry on an Agilent 1100 series liquid chromatograph/mass spectrometer.

**Synthesis of S-Nitroso-YC (YCysNO)**

20 mM YC was mixed with 20 mM sodium nitrite in 0.03 M HCl for 10 min at room temperature. The solution was then neutralized by 100 mM phosphate buffer, pH 7.4 containing 100 μM DTPA. YCysNO (yield >95%) was verified by ESI-MS (M + H+; 355), UV absorption (λmax = 335 nm) and by Hg2+-induced cleavage.

**Oxidation Induced by the MPO/H2O2/NO2 System**

Typically, peptides (0.3 mM) were incubated with NaNO2 (0.5 mM), H2O2 (0.1 mM), and MPO (30 nM) in a phosphate buffer (100 mM, pH 7.4) containing DTPA (100 μM) at room temperature for 30 min. Reactions were stopped by adding catalase (200 units) and analyzed by HPLC. Repeat injections in 24 h showed no significant oxidation of YC under these experimental conditions.

**S-Nitrosation of YC Induced by MPO/H2O2/NO2 System**

All reagents were purged with argon gas for 60 min before experiments. YC (150 μM) was incubated with H2O2 (50 μM), MPO (30 nM), and DEA-NO (10–50 μM) in a phosphate buffer (50 mM, pH 7.4) containing DTPA (100 μM) at room temperature for 10 min. Reactions were stopped by adding catalase (200 units) and analyzed by HPLC. Repeat injections within 24 h showed no significant loss of YCysNO under these experimental conditions.

**HPLC Analyses of Nitration and Oxidation Products**

Typically, 20 μl of sample was injected into an HPLC system (HP1100) with a C-18 column (250 × 4.6 mm) equilibrated with 5% CH3CN in 0.1% trifluoroacetic acid. The peptide and its product were separated by a linear increase of CH3CN concentration to 25% in 60 min at a flow rate of 1 ml/min. The elution was monitored using the on-line
UV-visible and fluorescence detectors. YC and nitrated YC were eluted at 12 and 17.5 min, respectively.

Nitration of tyrosine with or without cysteine by MPO/H2O2/NaNO2 was performed under the same conditions, and the products were analyzed as reported previously (36).

ESR Spin Trapping Experiments

**Thiyl Radical Trapping**—A typical incubation mixture consisted of a peptide or tyrosine (1 mM) and cysteine (1 mM), MPO (50 nM), H2O2 (1 mM), and 150 mM DMPO in a phosphate buffer (100 mM, pH 7.4) containing DTPA (100 μM). The reaction was initiated by adding H2O2. Samples were subsequently transferred to a 100-μl capillary tube, and EPR spectra were recorded within 30 s after starting the reaction. EPR spectra were recorded at room temperature on a Bruker ER 200 D-SRC spectrometer operating at 9.8 GHz and a cavity equipped with a Bruker Aquax liquid sample cell. Typical spectrometer parameters were as follows: scan range, 100 G; field set, 3505 G; time constant, 0.64 ms; scan time, 10 s; modulation amplitude, 1.0 G; modulation frequency, 100 kHz; receiver gain, 5 × 10^4; and microwave power, 10 milliwatts. The spectra shown were the average of 10 scans.

**Tyrosyl Radical Trapping**—Incubations consisting of a peptide or tyrosine (1 mM) and cysteine (1 mM), MPO (100 nM), and DBNBS (20 mM) in a phosphate buffer (0.1 M, pH 7.4) containing DTPA (0.1 mM) were rapidly mixed with H2O2 (1 mM). Samples were subsequently transferred to a 100-μl capillary tube, and EPR spectra were taken within 30 s after starting the reaction. Typical spectrometer parameters were as follows: scan range, 100 G; field set, 3505 G; time constant, 0.64 ms; scan time, 20 s; modulation amplitude, 2.0 G; modulation frequency, 100 kHz; receiver gain, 5 × 10^4; and microwave power, 10 milliwatts. The spectra shown were the average of 10 scans.
kHz; receiver gain, $5 \times 10^5$; and microwave power, 20 milliwatts. The spectra shown were the average of 30 scans.

**Energy Minimization**

The minimum energy conformations of the peptides and their global minima in the presence of water molecules were computed by the Metropolis Monte Carlo approach using a random variation of the randomly selected torsional angles (37). The starting configurations of the peptides were linear, corresponding to an all-trans backbone configuration with an N-terminal acetyl and C-terminal amide group. The molecular mechanics calculations were performed using an Amber 99 force field methodology. The structures calculated by Metropolis Monte Carlo were reminimized by the conjugate gradient method. Energy minimization was terminated when the gradient root mean square was below 0.01 mol/kcal. The selected lower energy conformers of each peptide were solvated by TIP3P water molecules and remimized again.

**RESULTS**

**Characterization of Tyrosylcysteine Peptides**—TABLE ONE lists the amino acid sequences of model peptides and oxidation/nitration products along with the mass spectral data. The intramolecular distances between the tyrosyl oxygen atom and the cysteiny1 sulfur atom calculated from the lowest energy conformations in an aqueous environment using the HyperChem 7.1 Package Program (Hypercube Inc.) are shown (see supplemental Fig. 15).

**MPO/H$_2$O$_2$/NaNO$_2$-dependent Oxidation and Nitration of Peptides Containing Tyrosine and Cysteine Residues**—The aim of these experiments was to determine the effect of the cysteine residue on nitration and oxidation of the tyrosine group present in model peptides (see supplemental Fig. 25). MPO/H$_2$O$_2$/NaNO$_2$-dependent nitration/nitration of YC peptide was monitored by HPLC with UV-visible detection at 280 nm for tyrosyl residue and at 350 nm for nitrotyrosyl residue. The HPLC/fluorescence (excitation, 290 nm; emission, 410 nm) was used for detecting the dityrosyl product of YC (Fig. 1). The authentic YC peptide (300 $\mu$M), YC nitration product (Y(NO$_2$)C; 0.1 $\mu$M), dityrosyl product (bis-N-acetyl tyroslylcysteine amide; 0.1 $\mu$M), and the disulfide product (YCysCysY; 150 $\mu$M) were detected at 16, 18, 29, and 32 min, respectively (Fig. 1, A–C). Incubation of YC peptide with MPO, H$_2$O$_2$, and NaNO$_2$ in a phosphate buffer (100 mM, pH 7.4) containing DTPA (100 $\mu$M) for 30 min at room temperature failed to yield detectable levels of nitrated tyrosine or the dityrosyl product (Fig. 1, A and C). However, upon UV-visible analysis at 280 nm, a new product eluting at 32 min was detected (Fig. 1A, a–d). By comparison with the HPLC profile of the authentic YC disulfide, the peak detected at 32 min was attributed to YCysCysY, a disulfide formed from YC. Incubations of YC with MPO/H$_2$O$_2$ also yielded YCysCysY but no dityrosine peptide (bis-N-acetyl tyroslylcysteine amide) (Fig. 1C, a–c). In the presence of H$_2$O$_2$ alone, a slight increase in disulfide YCysCysY was detected (Fig. 1A, a) due to H$_2$O$_2$-dependent oxidation of cysteine to the corresponding disulfide. These data are in contrast to the results obtained with tyrosine alone in the MPO/H$_2$O$_2$/NaNO$_2$ system, where a substantial but significant increase in nitrotyrosine and dityrosine formation was noted (see TABLE TWO).

Additional confirmation of disulfide formation in this system (i.e. YC/MPO/H$_2$O$_2$/NaNO$_2$) was obtained by incubating the reaction mixture with $\beta$-mercaptoethanol. As shown in Fig. 1A, the disulfide YCy$s$CysY was reduced back to the parent YC peptide. To further demonstrate that YC disulfide is the only oxidation product formed in this system, an additional aliquot of fresh reagents (MPO/H$_2$O$_2$/NaNO$_2$) was added to the original reaction mixture after 30 min and incubated further for another 30 min. HPLC analysis did not reveal any other products other than the disulfide YCysCysY (Fig. 1A).

Further confirmation of the product analysis was obtained by mass spectrometry. Fig. 1A (trace d, inset) shows the HPLC/ESI-MS analysis of the incubation mixture obtained 30 min after oxidation of YC by MPO/H$_2$O$_2$/NaNO$_2$. The peak eluting at 32 min in Fig. 1A was analyzed by HPLC/ESI-MS. The mass spectral analysis of this peak is identical to the $m/z$ pattern of the authentic YC disulfide (M + H$^+$: 649; M + 2H$^+$: 325).

### TABLE TWO

| Experimental conditions | Product | YC | YCysCysY | Y(NO$_2$)C | CYYC |
|-------------------------|---------|----|----------|-------------|------|
| MPO | NaNO$_2$ | H$_2$O$_2$ | YC | YCysCysY | Y(NO$_2$)C | CYYC |
| $\mu$M | $\mu$M | $\mu$M | $\mu$M | $\mu$M | $\mu$M | $\mu$M |
| 300 | 30 | 50 | 50 | 55 ± 20 | 115 ± 12 | ND$^a$ | ND |
| 300 | 30 | 0 | 50 | 182 ± 7 | 61 ± 10 | ND | ND |
| 300 | 0 | 0 | 50 | 271 ± 11 | 17 ± 4 | ND | ND |
| 300 | 30 | 500 | 100 | 41 ± 12 | 120 ± 10 | ND | ND |
| 300 | 30 | 0 | 100 | 129 ± 18 | 71 ± 4 | ND | ND |
| 300 | 0 | 0 | 100 | 252 ± 11 | 30 ± 6 | ND | ND |
| YC-SSCH$_3$ (300 $\mu$M) | 30 | 500 | 100 | ND | 22 ± 7 | 1.4 ± 0.1 |
| Tyr (300 $\mu$M) | 30 | 500 | 100 | 23 ± 2 | 5.3 ± 0.1 |
| YAAC (300 $\mu$M) | 30 | 500 | 50 | 117 ± 1 | 93 ± 8 | ND | ND |
| YAC (300 $\mu$M) | 30 | 0 | 50 | 216 ± 3 | 39 ± 1 | ND | ND |
| YAC (300 $\mu$M) | 0 | 0 | 50 | 277 ± 7 | 18 ± 1 | ND | ND |
| YAAC (300 $\mu$M) | 30 | 500 | 50 | 215 ± 2 | 42 ± 2 | ND | ND |
| YAAC (300 $\mu$M) | 30 | 0 | 50 | 248 ± 2 | 25 ± 1 | ND | ND |
| YAAC (300 $\mu$M) | 0 | 0 | 50 | 278 ± 3 | 12 ± 2 | ND | ND |
| YAAAC (300 $\mu$M) | 30 | 500 | 50 | 254 ± 3 | 22 ± 2 | ND | ND |
| YAAAC (300 $\mu$M) | 30 | 0 | 50 | 273 ± 7 | 12 ± 3 | ND | ND |
| YAAAC (300 $\mu$M) | 0 | 0 | 50 | 283 ± 3 | 8 ± 2 | ND | ND |

$^a$ All reactions were incubated at room temperature for 0.5 h, and concentrations were determined by HPLC.

$^b$ ND, not detected.
Incubation of other model peptides (YAC, YAAC, and YAAAAC) with MPO/H$_2$O$_2$/NaNO$_2$ also yielded exclusively the corresponding disulfide products (i.e., YACysCysAY, YAACysCysAAY, and YAAAA-CysCysAAAAY) with little or no formation of nitrated and dityrosyl products; as shown in TABLE TWO, the yields of disulfide decreased with increasing peptide chain lengths.

These results indicate that MPO/H$_2$O$_2$/NaNO$_2$-dependent oxidation of YC and related homologs yields the corresponding disulfide as the only major product and that the extent of formation of nitrated tyrosine and dityrosine oxidation products was negligible (TABLE TWO).

**Intermediacy of Thiyl Radicals Formed during MPO/H$_2$O$_2$/NO$_2$-dependent Oxidation of Tyrosylcysteine Model Peptides**—To investigate whether MPO/H$_2$O$_2$/NaNO$_2$ induces formation of disulfide from YC, YAAC, YAC, or YAAAAC peptides via a radical mechanism, we used the DMPO spin trap to detect the corresponding thiyl radicals (38, 39). DMPO-thiyl radical adducts exhibit a distinct EPR spectral pattern (38–41). Incubation of YC with MPO, H$_2$O$_2$, and NaNO$_2$ in a phos-
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FIGURE 3. Tyrosine enhances formation of DMPO-thiyl radical adducts formed from MPO/H\textsubscript{2}O\textsubscript{2}/NO\textsubscript{2}\textsuperscript{-}-dependent oxidation of thiols. A, incubation mixtures consisted of cysteine (1 mM), MPO (50 nM), DMPO (150 \mu M), H\textsubscript{2}O\textsubscript{2} (1 mM), NaNO\textsubscript{2} (1 mM), and tyrosine (1 mM) in a phosphate buffer (0.1 M, pH 7.4) containing DTPA (100 \mu M). Oxidation of FC in MPO/H\textsubscript{2}O\textsubscript{2} and DMPO did not yield a detectable EPR signal. B–D, same as above except that the incubation mixtures contained GSH (1 mM) instead of cysteine. C, same as above except that Phe-Cys (FC) (1 mM) was used instead of GSH or cysteine.

phate buffer containing DTPA (100 \mu M) yielded a four-line EPR spectrum (Fig. 2A) with the hyperfine coupling constants, \( \alpha_{\text{H}} = 15.2 \) G and \( \alpha_{\text{H}} = 16.2 \) G, that are similar to the values reported for the DMPO-glutathionyl adduct (\( \alpha_{\text{H}} = 15.2 \) G and \( \alpha_{\text{H}} = 16.4 \) G). Pretreatment of the YC peptide with N-ethylmaleimide, a thiol-blocking reagent, inhibited the DMPO-thiyl adduct formation. To verify that tyrosyl residue is required for inducing thiyl radical formation in YC peptide, we performed the oxidation of a dipeptide, FC (tyrosine replaced by a phenylalanine) in the MPO/H\textsubscript{2}O\textsubscript{2}/NO\textsubscript{2}\textsuperscript{-} system. Oxidation of FC in MPO/H\textsubscript{2}O\textsubscript{2} and DMPO did not yield a detectable ESR signal (Figs. 2A and 3C). A small amount of DMPO-SCysF adduct (\( \alpha_{\text{H}} = 15.1 \) G and \( \alpha_{\text{H}} = 16.3 \) G) detected in the presence of added NO\textsubscript{2}\textsuperscript{-} was presumably from NO\textsubscript{2}\textsuperscript{-}-mediated oxidation of the sulfydryl group in FC. This suggests that the tyrosine residue is essential for inducing thiyl radical formation in this system (i.e. MPO/H\textsubscript{2}O\textsubscript{2}/YC).

Incubation of the YC peptide with MPO and H\textsubscript{2}O\textsubscript{2} alone also formed the DMPO-SCysY adduct that showed a signal intensity 20\% smaller than the signal intensity of the same adduct induced by MPO/H\textsubscript{2}O\textsubscript{2}/NaNO\textsubscript{2}. In the absence of MPO or H\textsubscript{2}O\textsubscript{2}, no spectrum was detected (Fig. 2A). Incubations containing N-ethylmaleimide-treated YC or FC in the presence of MPO, H\textsubscript{2}O\textsubscript{2}, and DMPO completely eliminated the DMPO adduct spectra (Fig. 2A).

Oxidation of other peptides (YAC, YAAC, and YAAAAAC) in the MPO/H\textsubscript{2}O\textsubscript{2}/NO\textsubscript{2}\textsuperscript{-} system containing DMPO yielded the corresponding DMPO-SCysAY (\( \alpha_{\text{H}} = 15.1 \) G and \( \alpha_{\text{H}} = 16.0 \) G), DMPO-SCysAAY (\( \alpha_{\text{H}} = 15.1 \) G and \( \alpha_{\text{H}} = 15.95 \) G), and DMPO-SCysAAAAY (\( \alpha_{\text{H}} = 15.1 \) G and \( \alpha_{\text{H}} = 15.75 \) G) adducts (Fig. 2, B–D). These results indicate that thiyl radicals are formed from these model peptides during MPO/H\textsubscript{2}O\textsubscript{2}-dependent oxidation reactions and that both tyrosine and cysteine residues are required for inducing thiyl radical formation.

The spectral intensity of the DMPO-thiyl adducts decreased with increasing peptide chain (Fig. 2). It has been previously reported that the rate of reaction with MPO-compound I and -compound II decreased with increasing chain length of peptide tyrosine. Consistent with this finding, we found that the amount of disulfide formed from oxidation of YAAAAC decreased as compared with that formed from oxidation of YC in MPO/H\textsubscript{2}O\textsubscript{2} system (TABLE TWO).

MPO/H\textsubscript{2}O\textsubscript{2} has been shown to effectively oxidize tyrosine to the tyrosyl radical (36). Incubation of Cys or GSH with MPO/H\textsubscript{2}O\textsubscript{2}/NaNO\textsubscript{2}/DMPO in the presence of tyrosine yielded DMPO-SCys (\( \alpha_{\text{H}} = 15.05 \) G and \( \alpha_{\text{H}} = 17.2 \) G) (Fig. 3A) and DMPO-SGlu adducts (Fig. 3B). Incubation of Cys or GSH with MPO/H\textsubscript{2}O\textsubscript{2}/NaNO\textsubscript{2}/DMPO in the absence of tyrosine yielded a much smaller EPR signal for DMPO-SCys and DMPO-SGlu adducts. Incubation of Cys or GSH with MPO/H\textsubscript{2}O\textsubscript{2}/DMPO in the presence of tyrosine greatly enhanced the formation of DMPO-SCys and DMPO-SGlu adducts (Fig. 3A). The DMPO-SCysF signal intensity was significantly elevated by tyrosine in the MPO/H\textsubscript{2}O\textsubscript{2}/FC system, indicating that the tyrosyl radical formed is responsible for stimulating thiyl radical (Phe-Cys) formation via a hydrogen atom abstraction reaction from HSCysF. These results clearly indicate that exogenously added tyrosine or the presence of a tyrosyl residue in the vicinity of a cysteinyl residue exacerbates thiyl radical formation in MPO/H\textsubscript{2}O\textsubscript{2}- and MPO/H\textsubscript{2}O\textsubscript{2}/NO\textsubscript{2}\textsuperscript{-}-dependent oxidation of cysteine-containing peptides.

Since superoxide formed during autoxidation of thiols will react with DMPO to yield both DMPO-OOH and DMPO-OH, we performed

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experiments in the presence of superoxide dismutase to exclude the role of oxy radicals. The addition of superoxide dismutase did not alter the ESR spectrum of the DMPO-SCys adduct (data not shown), indicating that there was little or no contribution from both DMPO-OH and DMPO-OOH adducts. Previously, it was shown that tyrosyl radical yield was considerably inhibited through scavenging of the tyrosyl radical by superoxide anion (42). In the presence of superoxide dismutase (100 μg/ml), we found that disulfide was the only major product formed during the incubation of YC with MPO/H2O2/NaNO2 (data not shown).

**FIGURE 4.** DBNBS spin trapping of tyrosyl radicals formed from MPO/H2O2/NO2-dependent oxidation of tyrosine and model peptides. Incubation mixtures contained tyrosine (1 mM) or peptides (1 mM) as indicated, MPO (100 nM), DBNBS (20 mM), and H2O2 (1 mM) in a phosphate buffer (0.1 M, pH 7.4) containing DTPA (100 μM), and ESR spectra were immediately analyzed. The *dashed lines* denote the computer simulation of the EPR spectrum corresponding to two different spin adducts.

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**Intermediacy of Tyrosyl Radicals during MPO/H2O2-dependent Oxidation of Tyrosylcysteine Model Peptides—**DBNBS spin trap was previously used to detect tyrosyl radical generated by metmyoglobin/H2O2 (43). Fig. 4 shows the EPR spectrum of the DBNBS-tyrosyl adduct formed from tyrosine or dipeptide YC oxidation by MPO/H2O2 or MPO/H2O2/NO2. Based on the computer simulation, the EPR spectrum was attributed to a mixture of two spin adducts with different EPR parameters (α1 = 13.6 G, α2 = 13 G, and α3 = 6 G). Previous reports indicate that two DBNBS-tyrosyl adducts exist centered at the C1 and C3 or C5 positions of tyrosine (42). The addition of GSH or Cys (1 mM) inhibited the signal intensity of the DBNBS-tyrosyl adduct that is attributed to scavenging of tyrosyl radicals by GSH or Cys.

In contrast to tyrosine, incubation of YC with MPO/H2O2/DBNBS failed to generate the DBNBS-Tyr-Cys adduct (Fig. 4). This indicates that the Cys residue in YC and in YAC, YAAC, and YAAAAC peptides considerably inhibited the formation of the corresponding DBNBS-tyrosyl adduct (Fig. 4). However, under these conditions, thyl radical formation was induced as shown by DMPO spin trapping (Fig. 3). These results indicate that the presence of a nearby Cys residue dramatically lowers the concentration of tyrosyl radicals formed during MPO/H2O2- or MPO/H2O2/NO2-mediated oxidation of YC, YAC, and YAAAAC.

From these results, we surmised that the presence of a sulfydryl-blocking agent will increase the stability of tyrosyl radicals formed from YC and related peptides. The peptide YC was treated with MMTS to block the sulfydryl group. Moreover, oxidation of MMTS-treated YC by MPO/H2O2 or MPO/H2O2/NO2 in the presence of DTPA totally abolished the formation of the DMPO-SCysY signal, as compared with YC oxidation by MPO/H2O2 (Fig. 5A). Blocking of the sulfydryl group inhibited the formation of the DMPO-SCysY adduct. However, oxidation of MMTS-modified YC by MPO/H2O2 or MPO/H2O2/NO2 in the presence of DBNBS spin trap resulted in the formation of the corresponding DBNBS-tyrosyl adducts (Fig. 5B). Thus, as expected, blocking of the sulfydryl group with MMTS increased the stability of the tyrosyl radical such that it could be trapped by DBNBS and detected as the corresponding tyrosyl adduct. These results led us to conclude that there is a rapid electron transfer from the tyrosyl radical to the cysteinyl sulfydryl group during MPO/H2O2/NO2-mediated oxidation of YC, YAC, YAAC, and YAAAAC peptides. This electron transfer reaction is respon-
sible for the lack of nitration of tyrosyl residue and enhanced oxidation of the cysteinyl -SH group. Thus, blocking the -SH group enhances tyrosyl nitration of YC and related peptide homologs (TABLE ONE).

Intramolecular Versus Intermolecular Electron Transfer Mechanism between Tyrosyl Radical and Cysteinyl Sulfydryl Residue in Model Peptides—To investigate whether thyl radicals in YC, YAC, YAAC, and YAAAAC are formed from an intermolecular hydrogen abstraction reaction (i.e. TyrO-·HSCys → TyrOH-·SCys → TyrOH-·HSCys) or from an intramolecular hydrogen abstraction reaction between tyrosyl radical and the cysteinyl sulfydryl group (TyrO-·HSCys → TyrOH-·SCysY), we incubated YC, YAC, YAAC, and YAAAAC peptides with MPO, H2O2, and NaNO2 in a phosphate buffer containing DTPA and DMPO in the presence of added cysteine. The rationale for this experiment is as follows. Tyrosyl radical reacts fairly rapidly with cysteine (k = 10^6 M^-1 s^-1), forming the cysteiny l radical, which is trapped by DMPO (k = 10^6 M^-1 s^-1) to form the DMPO-SCys adduct that exhibits a characteristic EPR spectral pattern (38). Thus, cysteine should inhibit intermolecular formation of YCys5' by scavenging the tyrosyl cysteiny l radical (TyrO-·HSCys). EPR spin trapping data showed that there was no change in the EPR spectra of the DMPO-SCysY adduct formed during oxidation of YC by MPO/H2O2/NO2 in the presence of DMPO and 1 mM cysteine. Only when the concentration of cysteine exceeded 5 mM did we detect a change in the EPR spectrum of the DMPO-SCysY adduct (Fig. 6A). Under the same experimental conditions, the addition of cysteine (1 mM) had no effect on the DMPO-SCysAY, DMPO-SCysAA, and DMPO-SCysAAAAY spectra (Fig. 6, C–E). Alternatively, YC peptide concentration was varied. As shown in Fig. 6G, even when the concentration of the YC peptide was reduced by 50%, the addition of cysteine had little or no effect on the formation of DMPO-SCysY adduct. These findings suggest that the intramolecular reaction between the tyrosyl radical and the cysteinyl sulfydryl group (TyrO-·HSCys → TyrOH-·SCys) is quite facile and that this reaction rate must be greater than 10^3–10^4 s^-1.

In contrast, tyrosyl radicals induced thyl radical formation from exogenously added cysteine via an intermolecular hydrogen abstraction reaction in MPO/H2O2/Tyr/Cys and MPO/H2O2/Tyr/GSH systems, as demonstrated by DMPO spin trapping results (Fig. 3 and Figs. 6B and 7F).

The EPR results were confirmed by HPLC. The reaction mixture was analyzed by HPLC after incubating YC in the presence of MPO/H2O2 or MPO/H2O2/NO2 with and without DMPO for 15 min. A new peak that eluted at 22 min was detected (Fig. 7, A and B, trace 2). This particular peak was not detected in the absence of YC dipeptide from mixtures of Cys with MPO/H2O2/DMPO or MPO/H2O2/NO2 with and without DMPO for 15 min. A new peak that eluted at 22 min was detected (Fig. 7, A and B, trace 2). This particular peak was not detected in the absence of YC dipeptide from mixtures of Cys with MPO/H2O2/DMPO or MPO/H2O2/NO2 with and without DMPO for 15 min. A new peak that eluted at 22 min was detected (Fig. 7, A and B, trace 2). This particular peak was not detected in the absence of YC dipeptide from mixtures of Cys with MPO/H2O2/DMPO or MPO/H2O2/NO2 with and without DMPO for 15 min. A new peak that eluted at 22 min was detected (Fig. 7, A and B, trace 2). This particular peak was not detected in the absence of YC dipeptide from mixtures of Cys with MPO/H2O2/DMPO or MPO/H2O2/NO2 with and without DMPO for 15 min. A new peak that eluted at 22 min was detected (Fig. 7, A and B, trace 2). This particular peak was not detected in the absence of YC dipeptide from mixtures of Cys with MPO/H2O2/DMPO or MPO/H2O2/NO2 with and without DMPO for 15 min.

We also compared the DMPO-SCysY yields at two YC concentrations (1 and 0.5 mM) in the presence of excess cysteine. Even under those conditions, only in the presence of large excess of cysteine (10 mM) did...
we detect any difference in signal intensity of the DMPO-SCysY adduct. The diminution in the signal intensity of DMPO-ScysY in the presence of a large excess of cysteine could be due to the reduction of nitroxide adducts to the corresponding hydroxylamine. Results from these experiments also support the conclusion that the majority of DMPO-thiyl adduct formation occurs via an intramolecular reaction. Additional
pulse-radiolysis experiments need to be performed for absolute quantitation of intramolecular versus intermolecular pathways.

Next, we determined whether S-nitrosylated product could be formed during anaerobic oxidation of YC in MPO/H₂O₂/NO system. Fig. 8, A and B, shows the HPLC optical traces (detected at 280 and 336 nm in Fig. 8, C and D) of the authentic S-nitrosylated product derived from MPO/H₂O₂-dependent oxidation of YC in the presence of DEA-NO (TABLE THREE). We used several concentrations of DEA-NO that released 'NO at different rates from 1 to 5 μM/min. In the absence of MPO, S-nitrosated product was not detected. Fig. 8D shows the HPLC trace using the FC peptide (TABLE THREE).

The effects of 'NO generated from DEA-NO on the steady-state concentrations of DMPO-thiyl adducts are shown in Fig. 9. As can be seen, DEA-NO strongly inhibited the formation of the corresponding DMPO-thiyl adducts formed from incubations containing MPO, H₂O₂, Y(A),C, and DMPO. This is attributed to a rapid reaction between 'NO and thiyl radicals formed from YC, YAAC, and YAAAAC.

Collectively, these results imply that the presence of a Cys residue adjacent to a tyrosyl group in peptides facilitates the intramolecular radical transfer mechanism between the tyrosyl radical and the Cys residue forming the corresponding thiyl radical and the S-nitrosated product.
It is well known that the peroxidatic activity of the enzyme HRP will oxidize a variety of structurally different substrates (44). Fig. 10A shows that HRP/H$_2$O$_2$ oxidized YC to the corresponding thiyl radical that was readily trapped by DMPO. HRP/H$_2$O$_2$ also induced nearly the same amount of DMPO-thiyl adduct formed from YC, YAAC, and YAAAAC (Fig. 10B). This is in contrast to MPO/H$_2$O$_2$-dependent oxidations of YC and its analogs, where we observed a steady decrease in the concentration of DMPO-thiyl adducts with increasing size of the model peptide. This suggests that the rate of intramolecular electron transfer reaction between the tyrosyl radical and cysteine residue in these peptides is
nearly similar, which is consistent with the intermolecular distance calculations as shown in TABLE ONE. Fig. 10C shows the DMPO/glutathiyl adduct formation via an intermolecular reaction between the tyrosyl radical and GSH in the HRP/H$_2$O$_2$ system. Comparison between Fig. 10, B and C, indicates that the intramolecular mechanism of radical transfer between the tyrosyl radical and the cysteine residue is very facile.

**Table Three**

| S-Nitrosation and oxidation products of YC dipeptide in the MPO/ H$_2$O$_2$/NO system |
|-----------------------------------------------|
| All reactions were performed under anaerobic conditions in phosphate buffer (purred with argon gas). ND, not detected. |

| Peptide | Concentration μM | NO μM/min | RSNO μM | Disulfide μM |
|---------|------------------|------------|----------|--------------|
| YC      | 150              | 5          | 21 ± 3   | 17 ± 2       |
| YC      | 150              | 2.5        | 14 ± 1   | 20 ± 1       |
| YC      | 150              | 1          | 9 ± 3    | 14 ± 4       |
| FC      | 150              | 5          | ND       | ND           |

**DISCUSSION**

Results from this study show that the presence of a cysteine residue in the vicinity of a tyrosyl group in model peptides inhibits tyrosine nitration and enhances thiol oxidation and thiol nitrosation in the MPO/H$_2$O$_2$/NO and MPO/H$_2$O$_2$/NO systems. ESR spin trapping studies show that under these conditions, thiol radicals are formed via an intramolecular electron transfer reaction between the tyrosyl radical and the cysteine residue in model peptides. Treatment with a thiol-blocking agent increased the stability of the tyrosyl radicals, facilitating the intermolecular radical reactions with NO, leading to enhanced tyrosyl nitration.

**Nitration/Oxidation/Nitrosation Reactions of Tyrosine and Tyrosyl-cysteinyl Peptide by MPO/H$_2$O$_2$**—In the presence of H$_2$O$_2$, both MPO and eosonophil peroxidase form higher oxidants (compounds I and II) that are capable of oxidizing a variety of inorganic ions (Cl$^-$, Br$^-$, NO$_2^-$, etc.), forming one- and two-electron oxidation products (29, 45, 46). Compound I and II derived from MPO oxidized NO to nitrogen dioxide free radical (NO$_2^+$) and tyrosine to tyrosyl radical (Tyr'). Nitration of tyrosine occurs following a rapid recombination of NO$_2^+$ and tyrosyl
radical (47). As shown in Scheme 1, tyrosyl radicals oxidize cysteine to the cysteinyl radical. From peptides containing a Cys residue closer to the Tyr residue, the tyrosyl radical forms the corresponding cysteinyl radical (Tyr-Cys/HSCys). These radicals will react with NO to form the corresponding S-nitrosated cysteine and the oxidation/dimerization products of tyrosylcysteinyl thiyl radical.

It is becoming increasingly clear that the local environment of the tyrosine residue determines the product profile in the MPO/H₂O₂ system (12, 21, 26).

Intramolecular Electron Transfer between Tyrosyl Radical and Cysteine Residue in Model Peptides—The EPR spin trapping data show that oxidation of YC and other model peptides with MPO-compounds I and II leads exclusively to the formation of respective disulfides via the intermediacy of thiyl radicals. The plausible mechanisms include the initial formation of a tyrosyl cysteiny1 radical (TyrO⁻·HSCys) from the oxidation of YC and its analogs by MPO-compounds I and II or 'NO₂, which then rapidly undergoes electron transfer, either intra- or intermolecularly, with the cysteinyl moiety (Reactions 1–4).

\[
\text{MPO-compound I + TyrOH-HSCys} \rightarrow \text{TyrO⁻·HSCys + H}^+ \\
\text{REACTION 1}
\]

\[
\text{TyrO⁻·HSCys} \rightarrow \text{TyrOH⁻·SCys} \\
\text{REACTION 2}
\]

\[
\text{TyrSCys}^- + \text{TyrSCys}^- \rightarrow \text{TyrCys-CysTyr} \\
\text{REACTION 3}
\]

\[
\text{TyrO⁻·HSCys} + \text{TyrOH⁻·HSCys} \rightarrow \text{TyrOH⁻·SCys} \\
\text{REACTION 4}
\]

In the presence of added cysteine (1 mM), there was little or no change in the EPR spectra of DMPO-SHCys adducts (Fig. 6). This suggests that there was little or no formation of cysteinyl thiol radical due to an intramolecular hydrogen abstraction mechanism in the presence of 1 mM cysteine (Reaction 5).
Effect of Intramolecular Electron Transfer on Tyr Nitration and Cys Oxidation/Nitrosation

| Table Four: Sequences surrounding S-nitrosylated cysteines |
|-------------------------------------------------------------|
| **Protein** | **S-Nitrosylation target** | **Sequence** | **References** |
| Human hypoxia-inducible factor 1α | Cys<sup>300</sup> | QLTSY<sup>798</sup>De<sup>800</sup>EVNAP | 57 |
| NF-κB p50 | Cys<sup>62</sup> | FRFRY<sup>56</sup>VY<sup>57</sup>QGPSPH | 58 |
| Creatine kinase (B) | Cys<sup>583</sup> | GY<sup>275</sup>ILTC<sup>283</sup>PSNLG GY<sup>275</sup>VLTC<sup>283</sup>PSNLG | 59 |
| Creatine kinase (M) | Cys<sup>283</sup> | DC<sup>263</sup>FLHLY<sup>264</sup>C<sup>265</sup>Y<sup>266</sup>VTRLN | 60 |
| Parkin | Cys<sup>63</sup>, Cys<sup>268</sup> | RFELSC<sup>131</sup>Y<sup>132</sup>YSLAP | 61 |
| Argininosuccinate synthetase | Cys<sup>312</sup> | Not identified | 62 |
| β-actin | Not identified | IEKLC<sup>211</sup>Y<sup>212</sup>V<sup>213</sup>VALD | 62 |
| Adenylate cyclase (type VI, human) | Not identified | RAGGF<sup>38</sup>TPRY<sup>42</sup>M GDC<sup>429</sup>YYC<sup>432</sup>VSGA<sup>472</sup>V<sup>473</sup>AC<sup>474</sup>ALLVF | 63 |

TyrO·HSCys + HSCys → ‘SCys + TyrOH-HSCys

**REACTION 5**

‘SCys + DMPO → DMPO-SCys

**REACTION 6**

However, the addition of higher cysteine concentrations (5 mM) generated the EPR spectra (Fig. 6A) consisting of a mixture of DMPO-SCysY and DMPO-SCys adducts formed from the intermolecular reaction. These results suggest that the tyrosyl cysteinyl radical is relatively unstable and rapidly undergoes an intramolecular electron transfer (k > 10<sup>4</sup>–10<sup>5</sup> s<sup>-1</sup>), forming the corresponding thyl radicals (Reaction 2).

There are a number of factors that determine the efficiency of intramolecular electron transfer, including the distance between the radical site and the hydrogen atom donor amino acid, the flexibility of the peptide side chain, the amino acid sequence, and its diastereomeric conformations. Fig. 1S shows the minimum energy conformations of the model peptides in a solvated environment used in this study. As shown, the distance between the tyrosyl oxygen atom and the cysteinyl sulfur atom is not significantly altered with increasing length of the peptide chain. Thus, it is possible to envision long range electron transfer reactions in protein due to a folded structure in aqueous solutions.

Radiation-induced intramolecular electron transfer reactions between tryptophan and tyrosine were reported many years ago (48–50). The rate constants of intramolecular electron transfer reactions for the tryptophan-tyrosine pair (Trp<sup>TyrOH</sup> → Trp<sup>HTyrO</sup>) are in the microsecond time scale (k = 10<sup>7</sup>–10<sup>8</sup> s<sup>-1</sup>) (48–50). The insertion of glycines between Trp<sup>H</sup> and Tyr<sup>OH</sup> slightly changed the intramolecular electron transfer rate but not its efficiency. A similar type of intramolecular electron transfer reaction between tryptophan and tyrosine was reported many years ago (48–50). The rate constants of intramolecular electron transfer reactions in protein due to a folded structure in aqueous solutions.

Biological Implications—Protein tyrosine nitration has long been used as a diagnostic marker of oxidative and nitrative stress in several pathological processes. As shown in Scheme 1, protein tyrosine nitration reactions are induced by NO<sub>2</sub> and the protein tyrosyl radical. Tyrosine nitration appears to be a selective process in that not all tyrosine residues present in a protein are avidly nitrated (26, 51). Sequence evaluation surrounding the nitrated tyrosine in proteins reveals one common feature, a relative scarcity of cysteine or methionine residues in the immediate vicinity (52). Another factor that should be taken into consideration in examining the selective nitration of tyrosines in proteins is electron transfer processes via oxidation-sensitive amino acids. The present data show that the presence of a cysteiny1 group in the peptide chain modulates or negates tyrosine nitration mediated by MPO/H<sub>2</sub>O<sub>2</sub>-derived nitrating species. Alternatively, the presence of a tyrosyl residue may stimulate intramolecular electron transfer-mediated thiol oxidation in the MPO/H<sub>2</sub>O<sub>2</sub>/NO<sub>2</sub> system.

In ribonucleotide reductase-catalyzed reactions, the electron transfer occurs over a 35-angstrom distance between a tyrosyl radical and a cysteine via a well defined electron transfer pathway (53). Recently, it has been suggested that the mechanism of S-nitrosation is probably mediated by “oxidative nitrosylation,” a pathway by which reactive nitrogen species (ONOO<sup>-</sup>/NO<sub>2</sub>) generate reactive intermediates that can oxidize thiols to thyl radicals, which can directly react with NO to form the nitratosothiol (54, 55). The intramolecular electron transfer mechanism proposed here will promote “oxidative nitrosylation” favoring nitrosation of thiols in peptides. MPO/H<sub>2</sub>O<sub>2</sub> was recently shown to potentiate NO-mediated N-nitrosation via a radical mechanism (56). We propose that intramolecular electron transfer reactions between tyrosyl radical and cysteine residues in proteins may have a profound effect on the selective nitration of tyrosine residues and oxidation/nitrosation of cysteinyl residues.

Peptide sequences involving Tyr-Cys residues are more prone to undergo cysteinyl oxidation or nitrosation. In order to determine whether this phenomenon is restrictive or more general, we investigated the sequence specificity of S-nitrosation of cysteinyl targets (57–63) (TABLE FOUR). S-Nitrosation of proteins is one of the major post-translational modifications induced by reactive nitrogen species (58). Nevertheless, the biophysical basis for selective nitrosation of cysteines still remains unclear. From looking at the peptide sequences of S-nitrosylated proteins (TABLE FOUR), it is evident that S-nitrosation occurs predominantly when cysteine residues are present adjacent to or closer to a tyrosine residue (boldface type in TABLE FOUR). This feature is conspicuous in many prominent proteins of significance in signal transduction (NF-κB, HIF-1α, etc.). Where the actual S-nitrosylation has not been identified, as in the case of β-actin and adenylate cyclase, the sequence surrounding the predicted S-nitrosylation target is shown in TABLE FOUR.

New insights into mitochondrial protein S-nitrosylation were recently provided using a proteomic approach (64, 65). Several proteins were S-nitrosylated by NO<sub>2</sub> under aerobic and anaerobic/oxidative conditions. Although the actual S-nitrosylation site(s) have not been identified, it is noteworthy that most S-nitrosylated mitochondrial proteins reported share a Tyr and Cys sequence as follows: sarcosine dehydrogenase (C<sub>Y</sub>), catalase (C<sub>XY</sub>), dihydrolipoamide dehydrogenase (C<sub>CX</sub>Y), hydroxymethylglutarly-CoA synthase (C<sub>XY</sub>), glutamate oxaloacetate transaminase (CXX<sub>XY</sub>Y), and malate dehydrogenase 2 (C<sub>CX</sub>Y). S-Nitrosylation in this system was proposed to occur following a one-electron oxidation (64). Clearly, tyrosyl radical-assisted S-nitrosylation is a possibility.

The proteomic approach was also utilized in the identification of S-nitrosylated proteins using lipopolysaccharide-stimulated RAW264.7.
murine macrophages (65). Multiple S-nitrosylated proteins were identified. From the protein data base, we found that several S-nitrosylated target proteins detected in this study indeed share a common Tyr and Cys sequence: β-actin (CY), FRAG-6 (CY), elongation factor-1 (CYX), α-tubulin (CXXY), GTP-binding protein (CYX), heat shock protein hsp86 (C1), and SMC-6 (CXXXTY). Lipopolysaccharide stimulation of macrophages had previously been shown to induce protein-derived tyrosyl radical formation that could facilitate oxidative S-nitrosylation (66).

Additional pulse-radiolysis studies are clearly required to fully substantiate the present proposal. However, results shown in this study demonstrate the significance of tyrosine oxidative chemistry and electron transfer mechanism in S-nitrosylation of several key proteins involved in signal transduction mechanism. The present data show that the intramolecular mechanism of electron transfer between the tyrosyl radical and cysteine residue is very facile and is dependent, to a large degree, on the efficiency of formation of tyrosyl radicals in the MPO/H2O2 system.

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