THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 273, No. 48, Issue of November 27, pp. 32030–32037, 1998
Printed in U.S.A.

Electron Paramagnetic Resonance Detection of Free Tyrosyl Radical Generated by Myeloperoxidase, Lactoperoxidase, and Horseradish Peroxidase*

(Received for publication, August 12, 1998)

Michael L. McCormick‡§¶, Joseph P. Gaut§§, Tien-Sung Lin‖, Bradley E. Britigan‡§, Garry R. Buettner‡‡, and Jay W. Heinecke**§§**

From the ‡Research Service, Veterans Affairs Medical Center, the §Department of Internal Medicine and ‡‡EPR Facility, University of Iowa, Iowa City, Iowa 52246, and the Departments of ‖Chemistry and §§Medicine, Washington University, St. Louis, Missouri 63110

Phagocytes secrete the heme protein myeloperoxidase, which is present and active in human atherosclerotic tissue. These cells also generate hydrogen peroxide (H_2O_2), thereby allowing myeloperoxidase to generate a range of oxidizing intermediates and stable end products. When this system acts on L-tyrosine *in vitro*, it forms *o*,*o*′-dityrosine, which is enriched in atherosclerotic lesions. Myeloperoxidase, therefore, may oxidize artery wall proteins *in vivo*, cross-linking their L-tyrosine residues. In these studies, we used electron paramagnetic resonance (EPR) spectroscopy to identify an oxidizing intermediate in this reaction pathway and in parallel reactions catalyzed by horseradish peroxidase and lactoperoxidase. Using an EPR flow system to rapidly mix and examine solutions containing horseradish peroxidase, H_2O_2, and L-tyrosine, we detected free tyrosyl radical ($a_{2,6}^{H}$ = 6.3 G, $a_{3,5}^{H}$ = 1.6 G, and a_{β}^{H} = 15.0 G). We then used spin trapping techniques with 2-methyl-2-nitrosopropane (MNP) to further identify this intermediate. The resulting three-line spectrum (a^{N} = 15.6 G) was consistent with an MNP/tyrosyl radical spin adduct. Additional MNP spin trapping studies with ring-labeled L-[$^{13}C_6$]tyrosine yielded a characteristic eight-line EPR spectrum (a^{N} = 15.6 G, $a^{13}C$ (2) = 8.0 G, $a^{13}C$ (1) = 7.1 G, $a^{13}C$ (1) = 1.3 G), indicating that the MNP adduct resulted from trapping a carbon-centered radical located on the aromatic ring of L-tyrosine. This same eight-line spectrum was observed when human myeloperoxidase or bovine lactoperoxidase was substituted for horseradish peroxidase. Furthermore, a partially immobilized MNP/tyrosyl radical spin adduct was detected when we exposed a synthetic polypeptide composed of glutamate and L-tyrosine residues to the myeloperoxidase-H_2O_2-L-tyrosine system. The broadened EPR signal resulting from this MNP/polypeptide adduct was greatly narrowed by proteolytic digestion with Pronase, confirming that the initial spin-trapped radical was protein-bound. Collectively, these results indicate that peroxidases use H_2O_2 to convert L-tyrosine to free tyrosyl radical. They also support the idea that free tyrosyl radical initiates cross-linking of L-tyrosine residues in proteins. We suggest that this pathway may play an important role in protein and lipid oxidation at sites of inflammation and in atherosclerotic lesions.

Phagocytic white blood cells generate partially reduced oxygen species, which play a critical role in host defenses against invading microorganisms (1–3). This well established pathway involves a membrane-associated NADPH oxidase and the secreted heme enzyme myeloperoxidase. The oxidase converts oxygen to superoxide, which dismutates to hydrogen peroxide (H_2O_2). Myeloperoxidase then converts H_2O_2 into more potent microbicidal oxidants. This process has a deleterious side, however, because these reactive species can also damage host tissue at sites of inflammation. Indeed, many lines of evidence implicate phagocytes in diseases ranging from ischemia-reperfusion injury to cancer to atherosclerosis (4).

A high level of LDL,[1] the major carrier of blood cholesterol, is one important risk factor for atherosclerosis (5). *In vitro* studies suggest that this lipoprotein must be oxidized to mimic the events that contribute to the pathogenesis of this disease (6–8). Moreover, oxidized LDL is present in animal and human atherosclerotic vascular lesions (9, 10), and lipid-soluble antioxidants retard atherosclerotic plaque development in hypercholesterolemic animals (11–13). The pathways that promote LDL oxidation *in vivo*, however, have not yet been identified (14). One mechanism may involve myeloperoxidase, which is active in human atherosclerotic tissue (15). Immunohistochemical studies reveal that the enzyme co-localizes in part with lipid-laden macrophages, the cellular hallmark of the early atherosclerotic lesion (15).

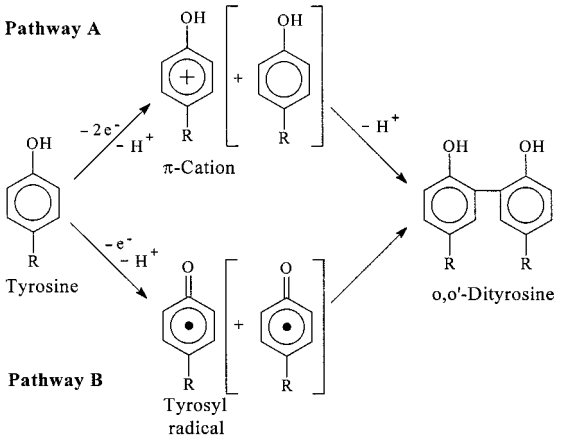
Myeloperoxidase cannot directly oxidize large molecules such as LDL because its active site is buried in a hydrophobic cleft (1–3). Instead, it relies on low molecular weight intermediates to convey oxidizing equivalents from its heme group to its target. One of these potential oxidizing intermediates is derived from L-tyrosine, a phenolic amino acid (16, 17). When LDL is exposed to myeloperoxidase, H_2O_2, and L-tyrosine, its lipids are peroxidized (18) and its L-tyrosine residues are cross-linked into *o*,*o*′-dityrosine residues (16, 19). We recently detected a remarkable 100-fold greater concentration of *o*,*o*′-dityrosine in LDL isolated from human atherosclerotic tissue than in circulating LDL (19). This raises the possibility that myeloperoxidase converts L-tyrosine to a freely diffusible, low

* This work was supported in part by National Institutes of Health Grants AG12293 (to J. W. H.), AG15013 (to J. W. H.), DK02456 (to J. W. H.), CA66081 (to G. R. B.), and AI28412 (to B. E. B.) and a Merit Review Award from the Department of Veterans Affairs (to B. E. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: Research-151, VA Medical Center, Iowa City, IA 52246. Tel.: 319-338-0581 (ext. 7548); Fax: 319-339-7162; E-mail: mccormic@jerry.icva.gov.

** Established Investigator of the American Heart Association.

[1] The abbreviations used are: LDL, low density lipoprotein; EPR, electron paramagnetic resonance; E-Y, synthetic polypeptide containing L-glutamic acid and L-tyrosine in a 4:1 (mol/mol) ratio; MNP, 2-methyl-2-nitrosopropane.



SCHEME 1

molecular weight oxidizing intermediate *in vivo* and that this reactive species plays a critical role in the pathogenesis of vascular disease.

The oxidizing intermediate derived from L-tyrosine has not yet been identified. *In vitro* studies suggest that it may be tyrosyl radical, because the reactions of myeloperoxidase, H_2O_2, and L-tyrosine show a striking resemblance to those of the phenoxyl radical (16, 17). For example, the myeloperoxidase system generates a spectrum of stable products (*o,o′*-dityrosine, trityrosine, pulcherosine, and isodityrosine) that could result from radical-radical coupling at the predominant sites of electron density on the oxygen and aromatic carbons (20). A second possibility is that the coupling reaction could proceed through an ionic mechanism, generating a reactive cation.

These two potential mechanisms are outlined in Scheme 1. Pathway A is an ionic mechanism involving an initial two-electron oxidation of L-tyrosine. Through an electrophilic reaction, the resulting π-cation attacks a second molecule of L-tyrosine to generate *o,o′*-dityrosine. Pathway B is a radical mechanism involving a one-electron oxidation. Two tyrosyl radicals then combine to form *o,o′*-dityrosine.

We now report direct evidence using electron paramagnetic resonance (EPR) spectroscopy that myeloperoxidase and other peroxidases generate free tyrosyl radical from L-tyrosine, strongly suggesting that *o,o′*-dityrosine forms via a radical mechanism, pathway B. We have also shown that this radical serves as a diffusible catalyst that cross-links tyrosyl residues in proteins. These observations have important implications for the mechanism of *o,o′*-dityrosine formation *in vivo*. Our results also indicate that free tyrosyl radical might be an agent of damage at sites of inflammation.

EXPERIMENTAL PROCEDURES

Materials

Horseradish peroxidase, H_2O_2, lactoperoxidase, 2-methyl-2-nitrosopropane (MNP), catalase, 3-aminotriazole, L-tyrosine, and the synthetic, random sequence polypeptide E-Y (glutamate/tyrosine; 4:1, mol/mol; catalog no. P0275) were obtained from Sigma. Human myeloperoxidase was provided by Dr. William M. Nauseef (University of Iowa, Iowa City, IA). Cambridge Isotope Laboratories (Andover, MA) supplied uniformly ring-labeled L-[$^{13}C_6$]tyrosine. Mass spectrometric analysis confirmed that the isotopic purity of the compound was >98%.

Methods

Tyrosine Oxidation—All reactions were performed at room temperature in Buffer A (50 mM Na_2HPO_4, 100 μM diethylenetriaminepentaacetic acid, pH 8). Phosphate buffer was treated with Chelex 100 analytical grade resin (Bio-Rad); diethylenetriaminepentaacetic acid was then added to chelate residual redox active metals (21).

EPR Spectroscopy—EPR spectra were obtained at room temperature using a Bruker model ESP 300 EPR spectrometer equipped with a TM_{110} cavity. Instrument settings were as follows: receiver gain, 2.5×10^5; microwave power, 20 milliwatts; modulation frequency, 100 kHz; modulation amplitude, 1.056 G; sweep rate, 1.9 G/s; and response time, 0.6 s.

Spin trapping experiments used MNP, a nitroso compound that effectively reacts with carbon-centered radicals (22). MNP was dissolved in Buffer A in the dark to a concentration of 20 mM. It is important to note that MNP solutions have an inherent three-line background spectra (a^N = 16.87 G) due to the light-induced formation of the MNP-derived spin adduct, di-*tert*-butylnitroxide (22). To minimize formation of these artifactual EPR signals, MNP solutions were kept in foil-covered tubes, and room lights were kept to a minimum. Solid L-tyrosine or L-[$^{13}C_6$]tyrosine was dissolved in the MNP solution to a final concentration of 2 mM.

For detection of free tyrosyl radical, an aqueous flow cell was used in the EPR spectrometer. Solutions were infused at a rate of 2 ml/min using a Harvard double syringe infusion pump (model 944). One syringe (50 ml) contained horseradish peroxidase (3 mg/ml in Buffer A), and the other (50 ml) contained H_2O_2 (3 mM in Buffer A). Both buffers contained 2 mM L-tyrosine, yielding a final concentration in the flow cell of 1.5 mg/ml horseradish peroxidase, 1.5 mM H_2O_2, and 2 mM L-tyrosine. The EPR spectrometer was set to acquire and signal average for 45 scans (41 s/60-G scan).

Peroxidase Modification of Synthetic Polypeptides—A synthetic polypeptide containing glutamic acid and L-tyrosine in a 4:1 (mol/mol) ratio, (E-Y, molecular mass 20,000–50,000 Da) was dissolved in Buffer A containing 20 mM MNP and then exposed to either the myeloperoxidase- or horseradish peroxidase-H_2O_2-L-tyrosine system described above. After a 15-min incubation at room temperature, samples were subjected to EPR analysis. Concurrent with this EPR analysis, a portion of the reaction mixture was subjected to size exclusion chromatography using a DG10 column (Bio-Rad) equilibrated with Buffer A. This step removes any low molecular mass (<10,000 Da) components, including free L-tyrosine, MNP, H_2O_2, and free MNP/tyrosyl radical spin adducts. The eluate, containing peroxidase, polypeptide E-Y, and MNP/tyrosyl radical adducts on polypeptide E-Y, was subjected to EPR analysis. The formation of a faint brown color in the complete systems, probably due to *o,o′*-dityrosine formation, facilitated identification of the fractions containing high molecular weight components. A portion of this eluate was treated with Pronase (1 mg/ml; Calbiochem) for 15 min at room temperature and subsequently analyzed by EPR.

RESULTS

EPR Spin Trapping of Tyrosyl Radical—In the presence of H_2O_2, myeloperoxidase can oxidize L-tyrosine, leading to the formation of *o,o′*-dityrosine (17), a reaction that might proceed through either a free tyrosyl radical or an ionic intermediate (Scheme 1). To determine whether tyrosyl radical might play a role in *o,o′*-dityrosine generation by this system, we used an EPR spin trapping system containing the nitroso spin trap MNP. MNP solutions typically demonstrate a small three-line background spectra (a^N = 16.9 G) due to the light-induced formation of di-*tert*-butylnitroxide (Fig. 1*A*) (22). Exposure of L-tyrosine to the myeloperoxidase-H_2O_2 system in the presence of MNP yielded the larger, narrower (a^N = 15.6) three-line spectrum, consistent with formation of the MNP/tyrosyl radical spin adduct (Fig. 1*B*). Removal of either L-tyrosine (Fig. 1*C*) or H_2O_2 (Fig. 1*D*) from the system led to spectra showing only the background di-*tert*-butylnitroxide signal, demonstrating the requirement for these factors in formation of the MNP spin adduct. Inclusion of the myeloperoxidase inhibitor azide (2 mM) also prevented tyrosyl radical formation (Fig. 1*E*), illustrating the requirement for active peroxidase in this process. Importantly, the addition of 150 mM NaCl did not appreciably decrease the MNP/tyrosyl radical signal observed, suggesting that myeloperoxidase can form tyrosyl radical in the presence of physiological levels of chloride ion (a known substrate for myeloperoxidase). These EPR spin trapping studies demonstrate that myeloperoxidase is capable of oxidizing tyrosine to tyrosyl radical by a process dependent on H_2O_2 and active peroxidase.

To provide further evidence that peroxidases generate free

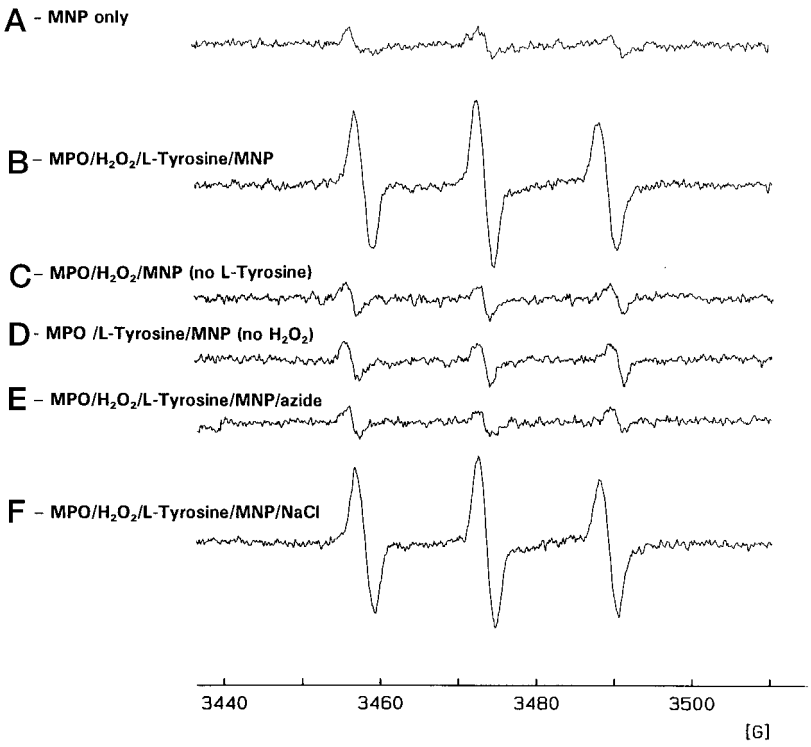


FIG. 1. **EPR spectra of MNP/tyrosyl radical spin adducts formed by the myeloperoxidase-H_2O_2-L-tyrosine system.** *A*, di-*tert*-butylnitroxide background signal from MNP stock solution (20 mM); *B*, myeloperoxidase (*MPO*; 200 μg/ml), H_2O_2 (1.5 mM), L-tyrosine (2 mM), and MNP (20 mM); *C*, as in *B*, but without L-tyrosine; *D*, as in *B*, but without H_2O_2; *E*, as in *B*, but in the presence of 2 mM azide; *F*, as in *B*, but in the presence of 140 mM NaCl. Reactions were initiated by the addition of H_2O_2 and performed in Buffer A. After a 15-min incubation at room temperature, the reaction mixture was subjected to EPR spectroscopy as described under "Methods." Spectra shown are representative of at least three independent experiments.

tyrosyl radical, we repeated the spin trapping experiments outlined above with horseradish peroxidase. The horseradish peroxidase-H_2O_2-L-tyrosine-MNP system yielded the same three-line MNP/tyrosyl radical spin adduct as the myeloperoxidase system (Fig. 2*A*). As before, the removal of peroxidase (Fig. 2*B*) or H_2O_2 (Fig. 2*C*) left only the MNP background signal. Interestingly, when L-tyrosine was removed from this system, the resulting spectrum lacked the di-*tert*-butylnitroxide background spectrum (Fig. 2*D*), suggesting that this species is unstable in the horseradish peroxidase-H_2O_2 system. Again, the di-*tert*-butylnitroxide background spectrum could be seen when azide was included in the reaction mixture (Fig. 2*E*). These experiments demonstrate that the horseradish peroxidase-H_2O_2 system can also lead to L-tyrosine oxidation and formation of MNP/tyrosyl radical spin adducts.

Substitution of ^{13}C ring-labeled L-tyrosine for L-tyrosine provides confirmation that the species responsible for the EPR spectrum generated by myeloperoxidase was tyrosyl radical, because ^{13}C has a spin of ½ compared with the ^{12}C spin of 0. Fig. 3*A* represents the same three-line MNP/tyrosyl radical spectra seen above with the myeloperoxidase-H_2O_2-L-tyrosine system. When L-[$^{13}C_6$]tyrosine was added to this system in place of nonisotopically labeled L-tyrosine, a distinct eight-line spectrum was observed (Fig. 3*B*). The resulting spectrum for the MNP/[$^{13}C_6$]tyrosyl radical adduct (a^N = 15.6 G, $a^{13}C$ (2) = 8.0 G, $a^{13}C$ (1) = 7.1 G, $a^{13}C$ (1) = 1.3 G) was clearly different from that seen with the same system in the presence of nonisotopic L-tyrosine. This eight-line spectrum was also seen when lactoperoxidase or horseradish peroxidase replaced myeloperoxidase in the presence of L-[$^{13}C_6$]tyrosine (Fig. 3, *C* and *D*). As with myeloperoxidase, these eight-line spectra were not seen if L-[$^{13}C_6$]tyrosine or H_2O_2 were omitted from the reaction mixture (data not shown). The experiments with L-[$^{13}C_6$]tyrosine provide strong evidence that the product of L-tyrosine oxidation by a peroxidase/H_2O_2 system is tyrosyl radical.

Formation of Free Tyrosyl Radical by Horseradish Peroxidase-H_2O_2—Chemical oxidation of L-tyrosine to tyrosyl radical has previously been demonstrated using a flow injection system and EPR (23). We used this same experimental approach to demonstrate that a peroxidase-H_2O_2 system converts free tyrosine into tyrosyl radical (Fig. 4*A*). In these experiments, no spin trap is added, and the resulting signal is representative of free tyrosyl radical. Horseradish peroxidase was employed for these studies because it is available in large quantities. Generation of tyrosyl radical required all of the components of the complete horseradish peroxidase-H_2O_2-L-tyrosine system; no EPR spectrum was observed when L-tyrosine (Fig. 4*B*), horseradish peroxidase (Fig. 4*C*), or H_2O_2 (data not shown) was omitted. To confirm the identity of the enzymatically generated radical species, we oxidized L-tyrosine chemically with $KMnO_4$. The resulting signal (Fig. 1*D*) was virtually identical to that generated by the horseradish peroxidase-H_2O_2-L-tyrosine system. Computer simulation of the enzymatically generated tyrosyl radical yielded EPR splitting constants of $a_{2,6}^H$ = 6.3 G, $a_{3,5}^H$ = 1.6 G, and a_β^H = 15.0 G (Fig. 4*E*), consistent with those previously reported for the chemically oxidized free tyrosyl radical (23). It is important to note that the assignment of 15.3 G to the β-hydrogens at C-7 assumes equivalency for these nonidentical protons. Line broadening at ambient temperatures yields the apparent coupling value (15.3 G), which is actually the sum of the couplings of these nonidentical protons (23). These results provide direct evidence for the peroxidase-dependent formation of freely diffusible tyrosyl radical.

Formation of Protein-bound Tyrosyl Radical—Previous studies have demonstrated that *o*,*o*′-dityrosine cross-linking of proteins by myeloperoxidase requires active enzyme, H_2O_2, and free L-tyrosine (16, 19). The requirement for free L-tyrosine in protein *o*,*o*′-dityrosine cross-linking suggests that a low molecular weight oxidizing species produced by myeloperoxidase from L-tyrosine might be acting as a diffusible radical catalyst. This diffusible species could then react with tyrosine residues on proteins, generating protein-bound tyrosyl radicals that would form *o*,*o*′-dityrosine cross-links. Our results demonstrating that various peroxidases convert free L-tyrosine into tyrosyl radical implicate this reactive intermediate as the diffusible oxidant produced by myeloperoxidase.

To investigate whether free tyrosyl radical generated by myeloperoxidase and H_2O_2 could react with protein to yield

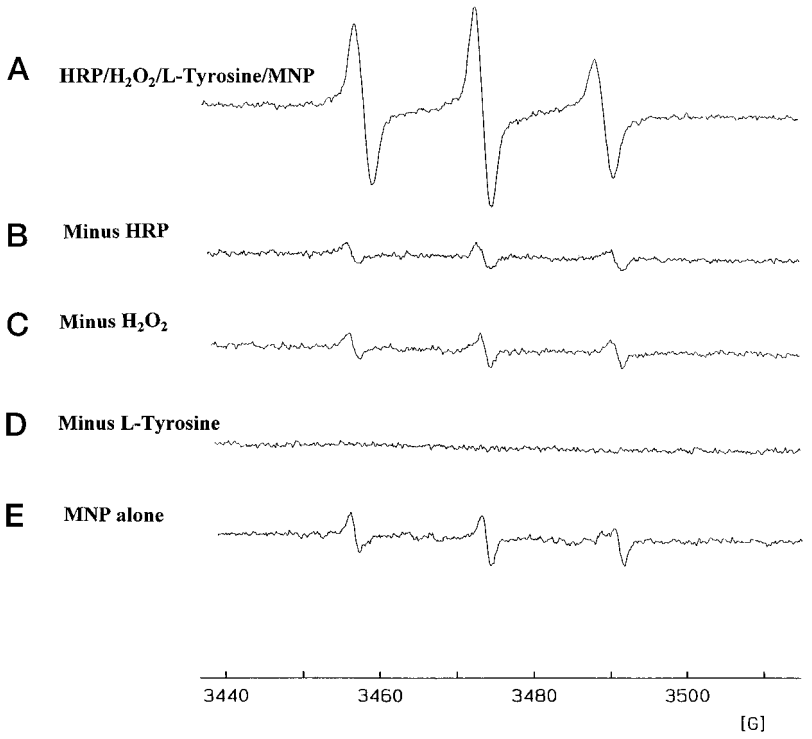


FIG. 2. **EPR spectra of MNP/tyrosyl radical spin adducts formed by the horseradish peroxidase-H_2O_2-L-tyrosine system.** *A*, horseradish peroxidase (*HRP*; 200 μg/ml), H_2O_2 (1.5 mM), L-tyrosine (2 mM), and MNP (20 mM); *B*, as in *A*, but without horseradish peroxidase; *C*, as in *A*, but without H_2O_2; *D*, as in *A*, but without L-tyrosine. *E*, di-*tert*-butylnitroxide background signal from MNP stock solution (20 mM). Reactions were initiated by the addition of H_2O_2 and performed in Buffer A. After a 15-min incubation at room temperature, the reaction mixture was subjected to EPR spectroscopy as described under "Methods." Spectra shown are representative of at least three independent experiments.

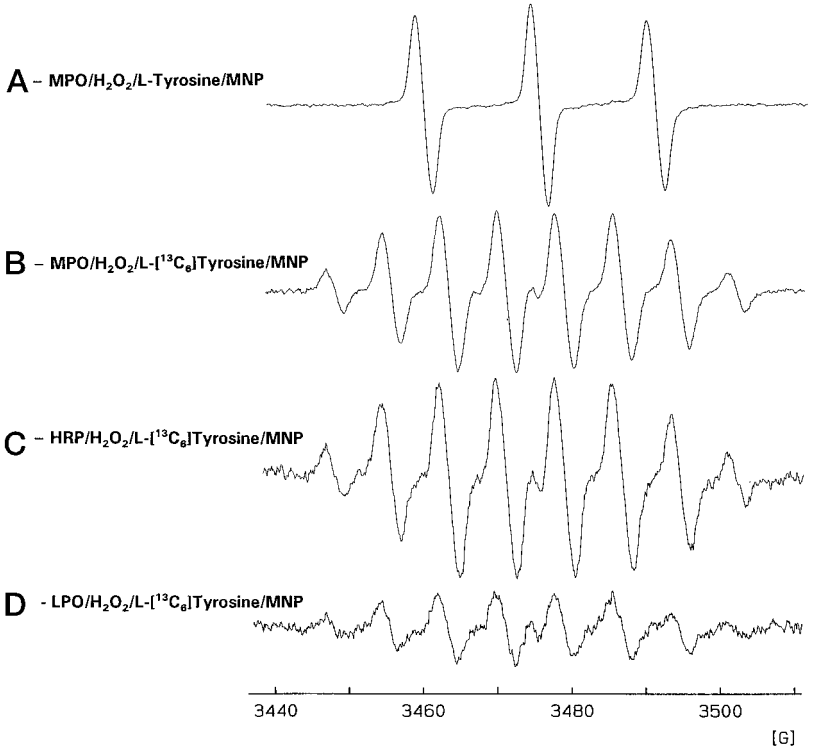


FIG. 3. **EPR spectra of MNP/tyrosyl radical and MNP/[$^{13}C_6$]tyrosyl radical formed by myeloperoxidase, horseradish peroxidase, or lactoperoxidase in the presence of H_2O_2 and L-tyrosine or L-[$^{13}C_6$]tyrosine.** *A*, myeloperoxidase (*MPO*; 200 μg/ml), H_2O_2 (1.5 mM), L-tyrosine (2 mM), and MNP (20 mM); *B*, as in *A*, but with L-[$^{13}C_6$]tyrosine (2 mM) in place of L-tyrosine; *C*, as in *B*, but with horseradish peroxidase (*HRP*; 200 μg/ml) in place of myeloperoxidase; *D*, as in *B*, but with lactoperoxidase (*LPO*, 80 μg/ml) in place of myeloperoxidase. Reactions were initiated by the addition of H_2O_2 and performed in Buffer A. After a 15-min incubation at room temperature, the reaction mixture was subjected to EPR spectroscopy as described under "Methods." Spectra shown are representative of at least three independent experiments.

protein-bound tyrosyl radical, we exposed a synthetic polypeptide containing glutamate and L-tyrosine (E-Y polypeptide, molecular mass 20,000–50,000 Da) to the myeloperoxidase-H_2O_2-L-tyrosine system in the presence of MNP. When this sample was subjected to EPR analysis, we observed an isotropic three-line spectrum, consistent with formation of free MNP/tyrosyl radical (Fig. 5*A*), with slightly broadened peaks and a shoulder on the high field peak between 3480 and 3485 Gauss. This is consistent with a fraction of the MNP/tyrosyl radical spin adducts being unable to rotate freely, suggesting that these adducts were bound to the E-Y polypeptide.

To determine whether MNP spin adducts were present on the E-Y polypeptide, the reaction mixture was fractionated by size exclusion chromatography. EPR analysis of the fraction containing the reisolated polypeptide yielded a spectrum characteristic of an immobilized nitroxide (Fig. 5*B*). Note that the spectrum in Fig. 5*A* and the dominant three-line spectrum seen in Fig. 5*B* exhibit the same g values and hyperfine splitting constants; however, the high field peak in the spectrum of the fraction following size exclusion chromatography (Fig. 5*B*) was markedly broadened compared with that of the complete reaction mixture (Fig. 5*A*). Formation of the immobilized MNP

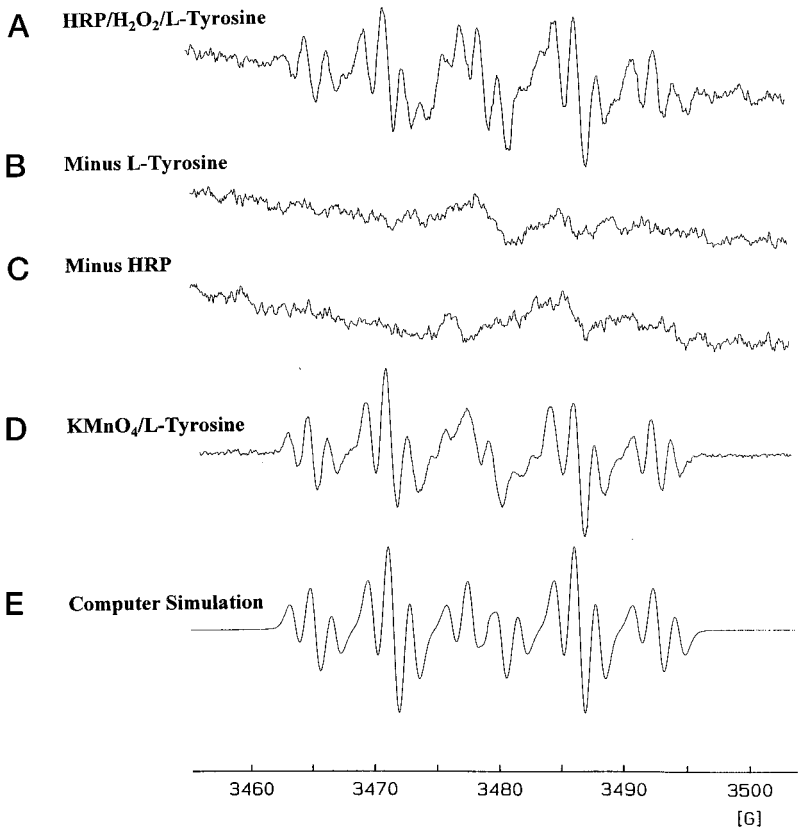


FIG. 4. **EPR spectra of free tyrosyl radical formed by the horseradish peroxidase-H_2O_2-L-tyrosine system.** *A*, horseradish peroxidase (*HRP*; 1.5 mg/ml), H_2O_2 (1.5 mM), and L-tyrosine (2 mM); *B*, as in *A*, but without L-tyrosine; *C*, as in *A*, but without horseradish peroxidase; *D*, L-tyrosine (2 mM) and $KMnO_4$ (1 mM); *E*, computer simulation of the spectrum shown in *A*, assuming ($a_{2,6}^{H}$ = 6.30 G, $a_{3,5}^{H}$ = 1.61 G, and a_{β}^{H} = 15.0 G). Reactions were performed at room temperature in Buffer A. Flow EPR was carried out at a flow rate of 2 ml/min as described under "Methods." Spectra are representative of two independent experiments.

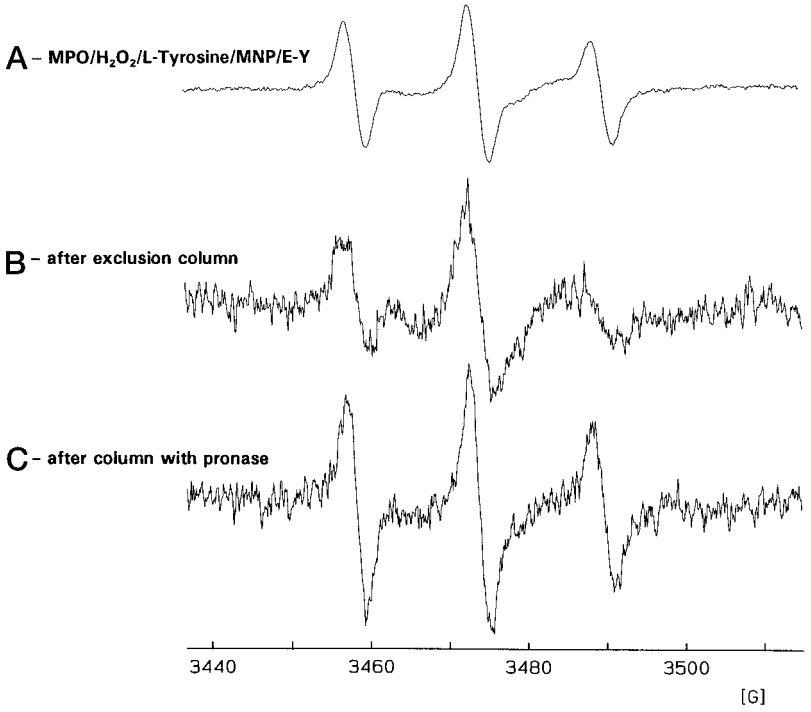


FIG. 5. **EPR spectra of protein-bound MNP/tyrosyl radical spin adducts formed by the myeloperoxidase-H_2O_2-L-tyrosine system.** *A*, synthetic polypeptide E-Y (1 mg/ml; glutamate/tyrosine, 1:1, mol/mol), myeloperoxidase (*MPO*, 200 μg/ml), H_2O_2 (1.5 mM), L-tyrosine (2 mM), and MNP (20 mM); *B*, sample as described in *A*, reisolated by size exclusion chromatography as described under "Methods"; *C*, sample as described in *B*, subjected to digestion with Pronase (1 mg/ml). Reactions were initiated by the addition of H_2O_2. Spectra shown are representative of three independent experiments.

polypeptide adducts required the presence of free tyrosine as well as MNP, active enzyme, H_2O_2, and polypeptide (data not shown).

To provide further evidence that the immobilized nitroxide adduct was formed on tyrosine residues of the E-Y polypeptide, the high molecular weight fraction containing the reisolated peptide was subjected to nonspecific proteolysis with Pronase, and the digest was subjected to EPR analysis. The resulting EPR signal of the Pronase-treated sample (Fig. 5*C*) was nearly isotropic and similar to that of the myeloperoxidase-MNP/L-tyrosine-H_2O_2-E-Y polypeptide system (Fig. 5*A*). These findings strongly suggest that MNP/tyrosyl spin adducts are formed with tyrosine residues in the E-Y polypeptide. The requirement for free tyrosine implies that tyrosyl radical generated by myeloperoxidase is catalyzing the formation of tyrosyl radical on the polypeptide.

We also determined whether horseradish peroxidase would form tyrosyl radical on the E-Y polypeptide. As with myeloper-

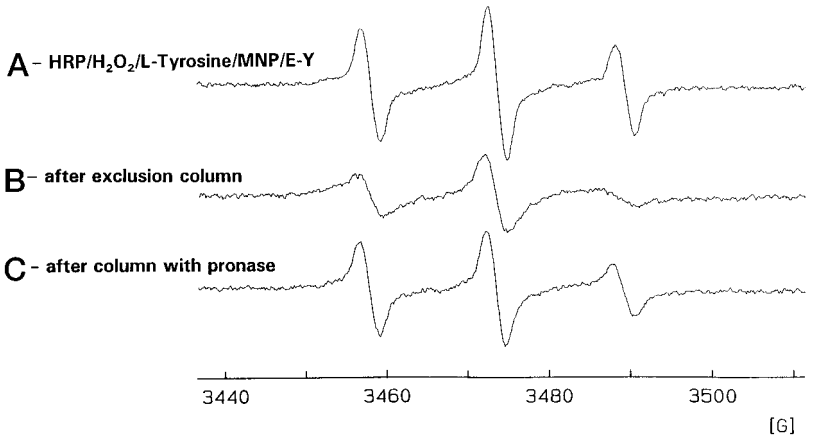


FIG. 6. **EPR spectra of protein-bound MNP/tyrosyl radical spin adducts formed by the horseradish peroxidase-H_2O_2-L-tyrosine system.** *A*, synthetic polypeptide E-Y (1 mg/ml; glutamate/tyrosine, 1:1, mol/mol), horseradish peroxidase (*HRP*; 200 μg/ml), H_2O_2 (1.5 mM), L-tyrosine (2 mM), and MNP (20 mM); *B*, sample as described in *A*, reisolated by size exclusion chromatography as described under "Methods"; *C*, sample as described in *B*, subjected to digestion with Pronase (1 mg/ml). Reactions were initiated by the addition of H_2O_2. Spectra shown are representative of three independent experiments.

oxidase, EPR analysis of the complete horseradish peroxidase-MNP/L-tyrosine-H_2O_2-E-Y polypeptide system revealed the formation of a slightly broadened three-line spectrum (Fig. 6*A*). EPR analysis of polypeptide E-Y isolated by size exclusion chromatography again showed the immobilized nitroxide spectrum (Fig. 6*B*). Treatment of the polypeptide with Pronase yielded a nearly isotropic three-line spectrum (Fig. 6*C*).

Previous studies have shown that horseradish peroxidase will directly oxidize protein-bound tyrosyl residues to *o*,*o*′-dityrosine. Consistent with these observations, horseradish peroxidase also generated MNP/tyrosyl radical spin adducts on polypeptide E-Y in the absence of free tyrosine (data not shown). Immobilized MNP adducts were also observed when horseradish peroxidase was incubated together with H_2O_2 and MNP alone, strongly suggesting that MNP/tyrosyl radical adducts were formed on the peroxidase itself. In contrast, MNP/tyrosyl radical adducts were undetectable in the myeloperoxidase system when free L-tyrosine was omitted from the reaction mixture, implying that the nitroxide spin adduct was generated on the synthetic polypeptide by a reaction that required free tyrosine and active peroxidase.

DISCUSSION

In the present report, we provide direct evidence that myeloperoxidase, lactoperoxidase, and horseradish peroxidase use H_2O_2 to convert free L-tyrosine into tyrosyl radical, a diffusible species that can react with tyrosine residues on proteins and lead to protein *o*,*o*′-dityrosine cross-linking. The spin trapping reagent MNP reacted with radical intermediates generated by each of the peroxidase-H_2O_2-L-tyrosine systems to yield a spin adduct, demonstrating a characteristic isotropic three-line EPR spectrum. Generation of the EPR spectrum by peroxidase required free L-tyrosine, suggesting that free tyrosyl radical was being generated. The myeloperoxidase-dependent reaction was not inhibited by plasma levels of NaCl, an alternative substrate for oxidation by the enzyme, suggesting that myeloperoxidase may promote the formation of tyrosyl radical *in vivo*.

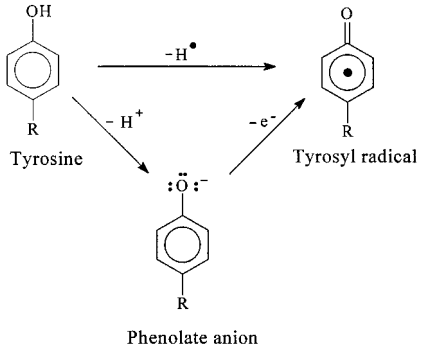
To further demonstrate that myeloperoxidase and the other peroxidases generate tyrosyl radical, we examined the EPR spectrum of ring-labeled L-[$^{13}C_6$]tyrosine oxidized by the different enzymes. When ring-labeled L-[$^{13}C_6$]tyrosine was substituted for L-tyrosine in each of the peroxidase-H_2O_2-MNP systems, an eight-line spectrum replaced the three-line spectrum. The appearance of additional hyperfine splitting in the EPR spectrum of the MNP spin adduct indicates that the radical site is located on the aromatic ring of L-tyrosine, strongly suggesting that free tyrosyl radical is generated by myeloperoxidase and the other enzymes.

EPR spectroscopy using a flow cell provided direct evidence of free tyrosyl radical formation by the horseradish peroxidase-H_2O_2-L-tyrosine system. Formation of tyrosyl radical required each of the components of the reaction system and was not observed when horseradish peroxidase, H_2O_2, or L-tyrosine was omitted from the reaction mixture. The EPR spectrum generated by enzymatic oxidation of L-tyrosine by peroxidase was essentially identical to that generated by direct one-electron oxidation of L-tyrosine by $KMnO_4$ (23). Moreover, a computer simulation using splitting constants of $a_{2,6}^{H} = 6.3$ G, $a_{3,5}^{H} = 1.6$ G, $a_{\beta}^{H} = 15.0$ G yielded a spectrum that was essentially identical to that seen with the enzymatic system. These results provide unambiguous evidence that horseradish peroxidase converts free L-tyrosine to tyrosyl radical by a reaction requiring H_2O_2.

Previous studies indicate that human phagocytes use myeloperoxidase and H_2O_2 to convert L-tyrosine into a family of L-tyrosine oxidation products, including *o*,*o*′-dityrosine, pulcherosine, isodityrosine, and trityrosine (17, 20). Oxidizing intermediates generated in these reactions also generate *o*,*o*′-dityrosine cross-links in proteins and stimulate the peroxidation of LDL lipids (16, 18, 24). Crystallographic studies of myeloperoxidase indicate that the heme group of the enzyme is located deep within the enzyme's core (3). These observations, together with the results of the current studies, suggest that myeloperoxidase acts on tyrosine residues of proteins and LDL lipids by converting free L-tyrosine into tyrosyl radical, which serves as a freely diffusible, low molecular weight radical catalyst. This species then attacks protein tyrosine residues, converting them to tyrosyl radical. Additional reactions lead to *o*,*o*′-dityrosine formation or protein *o*,*o*′-dityrosine cross-linking.

To test this hypothesis, we exposed an L-tyrosine-rich, synthetic polypeptide to either myeloperoxidase or horseradish peroxidase in the presence of H_2O_2, L-tyrosine, and the spin trap MNP. Following isolation of the polypeptide by size exclusion chromatography, the three-line MNP/tyrosyl radical spectrum was greatly broadened, suggesting formation of an immobilized adduct. This observation suggested that tyrosine residues on the polypeptide had been oxidized to tyrosyl radicals that subsequently reacted with MNP. This hypothesis was strongly supported by the demonstration that the broadened, nonisotropic three-line spectrum became nearly isotropic after the polypeptide was digested to low molecular weight peptides and free amino acids with a nonspecific protease.

Protein *o*,*o*′-dityrosine cross-linking is commonly found in cell wall proteins that are modified after translation, as in bacterial and plant cell walls, yeast spore coats (25), and the fertilization envelope of sea urchin embryos (26). In all cases where the biochemical mechanism is known, the reaction involves a heme protein and takes place in the absence of free



SCHEME 2

tyrosine. High concentrations of horseradish peroxidase and H_2O_2 form *o,o′*-dityrosine cross-links in proteins *in vitro* (27). Also, protein-bound tyrosyl radical has been detected by EPR spin trapping in cytochrome *c* exposed to H_2O_2 (28). Collectively, these observations strongly suggest that the mechanism involves the direct oxidation of protein-bound L-tyrosine residues by a heme protein. In contrast, myeloperoxidase requires free L-tyrosine to generate *o,o′*-dityrosine in proteins, implying that a species derived from L-tyrosine is the actual oxidizing intermediate. Our results strongly implicate tyrosyl radical as the reactive species.

Although this study suggests that coupling of tyrosyl radicals generates *o,o′*-dityrosine, the reaction pathway that generates tyrosyl radical is not yet established. The mechanism could involve hydrogen atom abstraction from L-tyrosine, or it could be a one-electron oxidation of the phenolate anion (Scheme 2). The latter may be kinetically favored, because alkaline pH is optimal for the formation of *o,o′*-dityrosine by horseradish peroxidase and myeloperoxidase (17). These conditions favor loss of a proton from the hydroxyl group, as would happen in a one-electron oxidation of the phenolate anion.

Based on our observations, peroxidase chemistry, and the crystal structure of myeloperoxidase (1–3, 16–20), we propose that compound I, a strongly oxidizing complex of peroxidase and H_2O_2, converts L-tyrosine into tyrosyl radical as follows.

Peroxidase + H_2O_2 → *Compound I* + H_2O

REACTION 1

Compound I + L-tyrosine → Compound II + tyrosyl radical

REACTION 2

Tyrosyl radical then diffuses out of the active site of the enzyme, where it subsequently undergoes a number of possible reactions. One involves a radical-radical recombination reaction between two tyrosyl radicals, generating *o,o′*-dityrosine, isodityrosine, and other products.

2 tyrosyl radicals → o,o′-Dityrosine + other products

REACTION 3

Tyrosyl radical might also attack other readily oxidized substrates like vitamin E and polyunsaturated fatty acids, initiating lipid peroxidation (18, 29, 30).

Tyrosyl radical + vitamin E ⇔ L-Tyrosine + tocopheryl radical

REACTION 4

Alternatively, it might exchange a hydrogen atom or electron with protein-bound L-tyrosine, forming protein-bound tyrosyl radical and regenerating L-tyrosine (16, 24).

Tyrosyl radical + protein-tyrosine ⇔

L-Tyrosine + protein-tyrosyl radical

REACTION 5

Our detection of MNP bound to the synthetic polypeptide strongly supports this hypothesis and further suggests that tyrosyl radical is the true intermediate in the reaction. The protein-bound tyrosyl radical might then react with free tyrosyl radical or a second protein-bound tyrosyl radical to generate a protein-bound *o,o′*-dityrosine cross-link. Experiments with radiolabeled free L-tyrosine suggest that the predominant reaction involves free tyrosyl radical in solution (24).

Protein-tyrosyl radical + tyrosyl radical

→ Protein o,o′-dityrosine cross-links

REACTION 6

The direct demonstration that peroxidases generate free tyrosyl radical may have important implications for the mechanism of tissue damage by activated phagocytes at sites of inflammation. Myeloperoxidase is present in atherosclerotic lesions, a chronic inflammatory condition (15). Protein-bound levels of *o,o′*-dityrosine are greatly elevated in atherosclerotic tissue and in LDL isolated from such lesions (19). Therefore, L-tyrosine may be an important substrate for myeloperoxidase. The resulting tyrosyl radical may render LDL atherogenic by peroxidizing its lipids and cross-linking its proteins. This connection between myeloperoxidase and L-tyrosine oxidation raises the possibility that the enzyme plays a key role in vascular tissue damage at sites of inflammation. Moreover, it is likely that heme proteins also oxidize L-tyrosine to tyrosyl radical in other biological systems.

Acknowledgments—We thank Dr. L. Sage for critical reading of the manuscript and Dr. W. M. Nauseef for providing human myeloperoxidase.

REFERENCES

1. Hurst, J. K., and Barrette, W. C., Jr. (1989) *CRC Crit. Rev. Biochem. Mol. Biol.* **24,** 271–328
2. Klebanoff, S. J., and Clark, R. A. (1978) *The Neutrophil: Function and Clinical Disorders*, Elsevier/North Holland Biomedical Press, New York
3. Zeng, J., and Fenna, R. E. (1992) *J. Mol. Biol.* **226,** 185–207
4. Ames, B. N., Shigenaga, M. K., and Hagen, T. M. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 7915–7922
5. Brown, M. S., and Goldstein, J. L. (1986) *Science* **232,** 34–47
6. Berliner, J. A., and Heinecke, J. W. (1996) *Free Radical Biol. Med.* **20,** 707–727
7. Steinberg, D., Parthasarathy, S., Carew, T. E., Khoo, J. C., and Witztum, J. L. (1989) *N. Engl. J. Med.* **320,** 915–924
8. Esterbauer, H., Gebicki, J., Puhl, H., and Jurgens, G. (1992) *Free Radical Biol. Med.* **13,** 341–390
9. Yla-Herttuala, S., Palinski, W., Rosenfeld, M. E., Parthasarathy, S., Carew, T. E., Butler, S., Witztum, J. L., and Steinberg, D. (1989) *J. Clin. Invest.* **84,** 1086–1095
10. Daugherty, A., Zweifel, B. S., Sobel, B. E., and Schonfeld, G. (1988) *Arteriosclerosis* **8,** 768–777
11. Kita, T., Nagano, Y., Yokode, M., Ishii, K., Kume, N., Ooshima, A., Yoshida, H., and Kawai, C. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 5928–5931
12. Carew, T. E., Schwenke, D. C., and Steinberg, D. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 7725–7729
13. Sparrow, C. P., Doebber, T. W., Olszewski, J., Wu, M. S., Ventre, J., Stevens, K. A., and Chao, Y. S. (1998) *J. Clin. Invest.* **89,** 1885–1891
14. Heinecke, J. W. (1997) *Curr. Opin. Lipidol.* **8,** 268–274
15. Daugherty, A., Dunn, J. L., Rateri, D. L., and Heinecke, J. W. (1994) *J. Clin. Invest.* **94,** 437–444
16. Heinecke, J. W., Li, W., Francis, G. A., and Goldstein, J. A. (1993) *J. Clin. Invest.* **91,** 2866–2872
17. Heinecke, J. W., Li, W., Daehnke, H. L., III, and Goldstein, J. A. (1993) *J. Biol. Chem.* **268,** 4069–4077
18. Savenkova, M. I., Mueller, D. M., and Heinecke, J. W. (1994) *J. Biol. Chem.* **269,** 20394–20400
19. Leeuwenburgh, C., Rasmussen, J. E., Hsu, F. F., Mueller, D. M., Pennathur, S., and Heinecke, J. W. (1997) *J. Biol. Chem.* **272,** 3520–3526

20. Jacob, J. S., Cistola, D. P., Hsu, F. F., Muzaffar, S., Mueller, D. M., Hazen, S. L., and Heinecke, J. W. (1996) *J. Biol. Chem.* **271,** 19950–19956
21. Britigan, B. E., Rosen, G. M., Chai, Y., and Cohen, M. S. (1986) *J. Biol. Chem.* **261,** 4426–4431
22. Buettner, G. R. (1987) *Free Radical Biol. Med.* **3,** 259–303
23. Sealy, R. C., Harman, L., West, P. R., and Mason, R. P. (1985) *J. Am. Chem. Soc.* **107,** 3401–3406
24. Francis, G. A., Mendez, A. J., Bierman, E. L., and Heinecke, J. W. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 6631–6635
25. Briza, P., Winkler, H., Kalchhauser, H., and Breitenbach, M. (1986) *J. Biol. Chem.* **261,** 4288–4294
26. Foerder, C. A., and Schapiro, B. M. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74,** 4214–4218
27. Amado, R., Aeschbach, R., and Neukom, H. (1984) *Methods Enzymol.* **107,** 377–388
28. Barr, D. P., Gunther, M. R., Deterding, L. J., Tomer, K. B., and Mason, R. P. (1996) *J. Biol. Chem.* **271,** 15498–15503
29. Karthein, R., Dietz, R., Nastainczyk, W., and Ruf, H. H. (1988) *Eur. J. Biochem.* **171,** 313–320
30. Bowry, V. W., and Stocker, R. J. (1993) *Am. Chem. Soc.* **115,** 6029–6044