Leukocytes Utilize Myeloperoxidase-Generated Nitrating Intermediates as Physiological Catalysts for the Generation of Biologically Active Oxidized Lipids and Sterols in Serum†

David Schmitt,‡ Zhongzhou Shen,‡ Renliang Zhang,‡ Scott M. Colles,‡ Weijia Wu,‡,§ Robert G. Salomon,|| Yonghong Chen,§ Guy M. Chisolm,‡ and Stanley L. Hazen*,‡,§,⊥

Department of Cell Biology and Department of Cardiology, Cleveland Clinic Foundation, Cleveland, Ohio 44195, Chemistry Department, Cleveland State University, Cleveland, Ohio 44115, and Department of Chemistry, Case Western Reserve University, Cleveland, Ohio 44106

Received July 14, 1999; Revised Manuscript Received September 23, 1999

ABSTRACT: The initiation of lipid peroxidation and the concomitant formation of biologically active oxidized lipids and sterols is believed to play a central role in the pathogenesis of inflammatory and vascular disorders. Here we explore the role of neutrophil- and myeloperoxidase (MPO)-generated nitrating intermediates as a physiological catalyst for the initiation of lipid peroxidation and the formation of biologically active oxidized lipids and sterols. Activation of human neutrophils in media containing physiologically relevant levels of nitrite (NO_2^-), a major end product of nitric oxide (nitrogen monoxide, NO) metabolism, generated an oxidant capable of initiating peroxidation of lipids. Formation of hydroxy- and hydroperoxyoctadecadienoic acids [H(P)ODEs], hydroxy- and hydroperoxyeicosatetraenoic acids [H(P)-ETEs], F_2-isoprostanes, and a variety of oxysterols was confirmed using on-line reverse phase HPLC tandem mass spectrometry (LC/MS/MS). Lipid oxidation by neutrophils required cell activation and NO_2^-, occurred in the presence of metal chelators and superoxide dismutase, and was inhibited by catalase, heme poisons, and free radical scavengers. LC/MS/MS studies demonstrated formation of additional biologically active lipid and sterol oxidation products known to be enriched in vascular lesions, such as 1-hexadecanoyl-2-oxovalaryl-*sn*-glycero-3-phosphocholine, which induces upregulation of endothelial cell adhesion and chemoattractant proteins, and 5-cholesten-3β-ol 7β-hydroperoxide, a potent cytotoxic oxysterol. In contrast to the oxidant formed during free metal ion-catalyzed reactions, the oxidant formed during MPO-catalyzed oxidation of NO_2^- readily promoted lipid peroxidation in the presence of serum constituents. Collectively, these results suggest that phagocytes may employ MPO-generated reactive nitrogen intermediates as a physiological pathway for initiating lipid peroxidation and forming biologically active lipid and sterol oxidation products in vivo.

The process of lipid peroxidation is implicated in the pathogenesis of inflammatory and vascular diseases (*1*−*5*). For example, a wealth of evidence demonstrates that glycerophospholipid and sterol oxidation products are enriched within atherosclerotic lesions (*1*−*3*, *6*−*8*). Moreover, many lipid oxidation products are vasoactive and/or possess potent proinflammatory biological activities (*1*−*4*, *6*−*9*). Despite evidence that products of lipid peroxidation are generated during the evolution of inflammatory and vascular disorders, and their potential role in contributing to the pathophysiological sequelae of these conditions, the mechanisms that initiate lipid oxidation in vivo remain poorly understood.

Cell culture models using endothelial cells (*10*, *11*), smooth muscle cells (*10*, *12*), neutrophils (*13*, *14*), monocytes (*13*), macrophages (*15*), and mixed mononuclear cells (*16*) have been shown to oxidize low-density lipoprotein (LDL)[1] lipids ex vivo. In many of these models, lipid peroxidation occurs through reactions that require superoxide (O_2^-) and trace levels of redox active transition metal ions, and is inhibited by superoxide dismutase (SOD) and both low-molecular weight and proteinaceous chelators (*12*, *16*−*18*). Consequently, addition of plasma or interstitial fluid components to these reaction mixtures potently blocks lipid oxidation by chelating free or loosely bound metal ions and rendering them redox inactive (*18*). Redox active metal ions presented in carrier proteins such as ceruloplasmin, the major carrier of copper in plasma, may be one mechanism for promoting lipid peroxidation in vivo since serum constituents did not block lipid oxidation via this pathway (*19*). The physiological significance of free metal ions in promoting lipid peroxidation in vivo has been questioned (*18*, *20*), and lipid peroxidation mediated by cells may occur via multiple pathways.

† This work was supported in part by the American Heart Association and by National Institutes of Health Grants HL62526 and HL61878 (to S.L.H.) and HL29582 (to G.M.C.).

* To whom correspondence should be addressed: Department of Cell Biology, Lerner Research Institute, Cleveland Clinic Foundation, 9500 Euclid Ave., NC-10, Cleveland, OH 44195. Telephone: (216) 445-9763. Fax: (216) 444-9404. E-mail: hazens@ccf.org.

‡ Department of Cell Biology, Cleveland Clinic Foundation.

§ Cleveland State University.

|| Case Western Reserve University.

⊥ Department of Cardiology, Cleveland Clinic Foundation.

10.1021/bi991623w CCC: $18.00
Published on Web 12/03/1999

Lipoxygenases represent another potential mechanism for catalyzing lipid peroxidation in vivo. These cytosolic nonheme iron-containing enzymes catalyze the direct insertion of molecular oxygen into polyenoic fatty acids, forming hydroperoxides (*21*, *22*). Evidence of a role for lipoxygenases in lipid oxidation in vivo is based on chiral phase high-performance liquid chromatography (HPLC) analysis of oxygenated species of linoleic and arachidonic acids recovered from tissues and fluids. Stereospecific accumulation of isomers generated by lipoxygenases during polyenoic fatty acid oxidation in vitro has been observed (*21*, *22*).

Another potential pathway for initiating lipid peroxidation in vivo may involve reactive nitrogen species derived from nitric oxide (nitrogen monoxide, NO), a long-lived free radical which is formed by multiple inflammatory and vascular wall cells (*23*). NO is relatively unreactive toward most biomolecules (*24*); however, a variety of reactive nitrogen species derived from NO are capable of damaging biological targets (*24*−*28*). Phagocytic cells exploit the reactivity of nitrogen oxides by generating microbicidal and tumoricidal NO-derived oxidants as part of their normal function during host defenses (*29*, *30*). However, these oxidizing agents may also damage normal tissue and contribute to inflammatory injury (*31*, *32*). The most well-studied pathway for generating nitrating intermediates from NO involves formation of peroxynitrite ($ONOO^-$), the product of NO and O_2^- (*27*, *28*). This potent oxidizing agent nitrates protein tyrosine residues (*28*), depletes lipid soluble antioxidants (*33*, *34*), and initiates lipid peroxidation (*26*). Many inflammatory and vascular wall cells produce both O_2^- and NO (*23*), suggesting that $ONOO^-$ may contribute to oxidant stress and lipid peroxidation in vivo. The role of this pathway in mediating protein nitration and lipid peroxidation in vivo, particularly under conditions of enhanced NO flux, has recently been questioned (*15*, *35*).

Myeloperoxidase (MPO), an enzyme implicated in inflammatory injury and vascular disease, has recently been suggested as another potential pathway for generating reactive nitrogen species in vivo (*36*−*38*). MPO is an abundant heme protein that is secreted from activated phagocytes (*39*). It amplifies the oxidizing potential of H_2O_2 by using it as a cosubstrate to generate a variety of reactive oxidants and diffusible radical species (*37*, *38*, *40*, *41*). Formation of phenoxyl radicals by MPO, such as during oxidation of the free amino acid L-tyrosine, can promote protein cross-linking (*42*) and oxidation of LDL lipids (*14*). MPO may also use H_2O_2 and nitrite (NO_2^-), a major end product of NO metabolism, to generate a microbicidal oxidant (*30*) capable of nitrating phenolic compounds and proteins in vitro (*36*−*38*, *43*−*45*). NO_2^- is ubiquitous in biological tissues and fluids (*37*, and references therein). During inflammatory and infectious processes where NO production is enhanced, plasma and extracellular levels of NO_2^- are markedly increased (*46*−*48*). Both isolated MPO and human neutrophils mediate aromatic nitration reactions in media containing concentrations of NO_2^- approximating those found in biological fluids (*38*, *43*, *45*). Thus, MPO can utilize NO_2^- as a physiological substrate in the presence of plasma levels of chloride.

Studies thus far on the reactive nitrogen species generated by MPO have focused on the nitrated products formed using aromatic trapping agents and protein tyrosine residues as targets. However, the ability of leukocyte-generated nitrogen oxides to modify lipids and initiate lipid peroxidation remains essentially unexplored. We recently demonstrated that incubation of LDL in media containing NO_2^- and either activated monocytes or the MPO/H_2O_2 system converts the lipoprotein into a form which is readily taken up and degraded by macrophages, resulting in cholesterol accumulation and foam cell formation (*45*). Nitrogen dioxide (NO_2), the one-electron oxidation product of NO_2^-, has been proposed to be the oxidant formed by the MPO/H_2O_2/NO_2^- pathway (*37*). This free radical is produced as an environmental contaminant when organic compounds undergo combustion (*49*, *50*). Gas phase NO_2 reacts with both alkanes and alkenes at 25 °C via free radical mechanisms (*51*). It initiates the autoxidation of unsaturated fatty acids in vitro, and increased levels of lipid peroxidation products are observed when NO_2 is inhaled in vivo (*49*, *50*, *52*). Whether reactive nitrogen species generated by neutrophils via MPO-dependent oxidation of NO_2^- behave similarly has not yet been tested.

We now report that nitrating intermediates generated during neutrophil- and MPO-dependent oxidation of NO_2^- act as physiological catalysts for the initiation of lipid peroxidation and the formation of biologically active oxidized lipids and sterols. Reverse phase HPLC with on-line electrospray ionization tandem mass spectrometry (LC/MS/MS) was used to confirm that specific glycerophospholipid and sterol oxidation products present in inflammatory and vascular disorders were formed. Initiation of lipid peroxidation by the MPO/H_2O_2/NO_2^- system was observed in reactions performed in the presence of serum. Thus, phagocyte activation and subsequent reactive nitrogen species formation by MPO represent a physiologically plausible and novel pathway for the initiation of lipid peroxidation and the formation of biologically active lipid oxidation products in vivo.

EXPERIMENTAL PROCEDURES

Materials

(*E*,*Z*)-9-Hydroxy-10,12-octadecadienoic acid (9-HODE), (*E*,*Z*)-9-hydroxy-5,7,11,14-eicosatetraenoic acid (9-HETE),

[1] Abbreviations: APCI, atmospheric-pressure chemical ionization; BAEC, bovine aortic endothelial cells; BHT, butylated hydroxytoluene; cholesterol α-epoxide, 5-cholesten-5α,6α-epoxy-3*β*-ol; cholesterol *β*-epoxide, 5-cholesten-5*β*,6*β*-epoxy-3*β*-ol; cholesteryl 9-H(P)ODE, cholesteryl 9-hydroxy-10,12-octadecadienoate and cholesteryl 9-hydroperoxy-10,12-octadecadienoate; DTPA, diethylenetriaminepentaacetic acid; ESI, electrospray ionization; GC, gas chromatography; GGOx, glucose/glucose oxidase; H_2O_2, hydrogen peroxide; 9-H(P)ETE, 9-hydroxy-5,7,11,14-eicosatetraaenoic acid and 9-hydroperoxy-5,7,11,14-eicosatetraaenoic acid; 9-H(P)ODE, 9-hydroxy-10,12-octadecadienoic acid and 9-hydroperoxy-10,12-octadecadienoic acid; HPLC, high-performance liquid chromatography; 7-keto cholesterol, 5-cholesten-3*β*-ol-7-one; LC/MS/MS, reverse phase HPLC with on-line tandem mass spectrometry; LDL, low-density lipoprotein; MDA, malondialdehyde; MS, mass spectrometry; MPO, myeloperoxidase; MRM, multiple reaction monitoring; L-NMMA, N^G-monomethyl-L-arginine; NO, nitrogen monoxide (nitric oxide); NO_2^-, nitrite; NO_2, nitrogen dioxide; $ONOO^-$, peroxynitrite; 7-OH cholsterol, 5-cholesten-3*β*,7*β*-diol; 25-OH cholesterol, 5-cholesten-3*β*,25-diol; 7-OOH cholesterol, 5-cholesten-3*β*-ol 7*β*-hydroperoxide; PAPC, 1-hexadecanoyl-2-eicosatetra-5′,8′,11′,14′-enoyl-*sn*-glycero-3-phosphocholine; PMA, phorbol myristate acetate; POxvPC, 1-hexadecanoyl-2-oxovalaryl-*sn*-glycero-3-phosphocholine; O_2^-, superoxide; SOD, superoxide dismutase; TBA, thiobarbituric acid; triol, cholestan-3*β*,5α,6*β*-triol.

(*E*,*Z*)-cholest-5-en-3-ol-9-hydroxy-10,12-octadecadienoate (CE-9-HODE), 8-epiprostaglandin $F_{2\alpha}$ (8-epi-$PGF_{2\alpha}$), and 8-epi-[$^2H_4$]$PGF_{2\alpha}$ standards were purchased from Cayman Chemical (Ann Arbor, MI). 5-Cholesten-3β-ol 7β-hydroperoxide (7-OOH cholesterol) was synthesized and isolated as described previously (*53*). Cholestan-3β,5α,6β-triol (triol), 5-cholesten-3β,25-diol (25-OH cholesterol), 5-cholesten-3β-ol-7-one (7-keto cholesterol), 5-cholesten-3β,7β-diol (7-OH-cholsterol), 5-cholesten-5α,6α-epoxy-3β-ol (cholesterol α-epoxide), and 5-cholesten-5β,6β-epoxy-3β-ol (cholesterol β-epoxide) and were obtained from Steraloids Inc. (Wilton, NH). [^{3}H]Arachidonate (40 mCi/mmol) was obtained from ICN Pharmaceutical, Inc. (Costa Mesa, CA). Organic solvents (HPLC grade) and sodium phosphate were obtained from Fisher Chemical Co. (St. Louis, MO). Catalase (bovine liver, thymol-free) and glucose oxidase (grade II) were purchased from Boehringer Mannheim (Indianapolis, IN). Synthetic 1-hexadecanoyl-2-oxovalaryl-*sn*-glycero-3-phosphocholine (POxvPC) was a generous gift from R. Soloman (Case Western Reserve University). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise specified.

Methods

General Procedures. All buffers and media were treated with Chelex-100 resin (Bio-Rad, Hercules, CA) and supplemented with diethylenetriaminepentaacetic acid (DTPA) to remove trace levels of potential redox active transition metal ions. Protein content was determined by the Markwell-modified Lowry protein assay (*54*) with bovine serum albumin as the standard. Production of H_2O_2 by glucose/glucose oxidase was quantified by oxidation of Fe(II) and formation of an Fe(III)−thiocyanate complex (*37*). The concentration of reagent H_2O_2 was determined spectrophotometrically ($\epsilon_{240} = 39.4\ M^{-1}\ cm^{-1}$; *55*). LDL concentrations are expressed as milligrams of protein per milliliter. Myeloperoxidase (donor hydrogen peroxide oxidoreductase, EC 1.11.1.7) was isolated from human leukocytes as described previously (*56*). MPO utilized in this study demonstrated a RZ of 0.74 (A_{430}/A_{280}), and possessed no evidence of eosinophil peroxidase contamination upon analysis by SDS−PAGE with Coomassie Blue staining, as well as following native PAGE with in-gel peroxidase staining (*57*). LDL was isolated from fresh human plasma by sequential ultracentrifugation as a fraction with a *d* between 1.019 and 1.063 g/mL (*58*). Neutrophils were isolated by buoyant density centrifugation as described previously (*59*). Low levels of contaminating eosinophils were then removed by fluorescence-activated cell sorting (*60*). NO_2^-/NO_3^- production by cultured endothelial cells was assessed using a modified Griess assay (*61*).

Oxidation Reactions. Isolated human neutrophils were incubated at 37 °C under air in medium A [Ca^{2+}/Mg^{2+}/phenol/bicarbonate-free Hank's Balanced Salt Solution (Gibco Life Technologies, Gaithersburg, MD) supplemented with 200 μM DTPA (pH 7.2)] containing 0.2 mg/mL LDL protein. Neutrophils (1×10^6/mL) were activated with 200 nM phorbol myristate acetate (PMA) and maintained in suspension by gentle mixing every 5 min. After 2 h, reactions were stopped by pelleting cells by centrifugation at 4 °C and addition of 50 μM butylated hydroxytoluene (BHT) and 300 nM catalase to the supernatants. Oxidation products were then assayed in supernatants as described below. In several studies, neutrophils were activated with either fMLP or opsonized zymosan. Zymosan for those studies was first pretreated with Chelex resin to remove trace metal ion contaminants and then opsonized by conventional methods (*41*).

Reactions with isolated MPO were typically performed at 37 °C in sodium phosphate buffer (50 mM, pH 7.0) supplemented with 200 μM DTPA using 30 nM MPO, 100 μg/mL glucose, 20 ng/mL glucose oxidase, and 50 μM $NaNO_2$ for the indicated periods of time. Under these conditions, a constant flux of H_2O_2 (0.18 μM/min) was generated by the glucose/glucose oxidase system, as assessed by the oxidation of Fe(II) and the formation of an Fe(III)−thiocyanate complex (*37*). Unless otherwise stated, reactions were terminated by addition of 50 μM BHT (from a 100 mM ethanolic stock) and 300 nM catalase to the reaction mixture, and extraction of lipids into organic solvents as described below.

LDL oxidation by endothelial cell/neutrophil cocultures was performed as follows. Bovine aortic endothelial cells (BAEC, passages 2−4) were grown to confluence in 16-well plates in phenol red-free RPMI 1640 medium supplemented with 5% fetal calf serum and 100 μM L-arginine at 37 °C in humidified 5% CO_2. Twenty-four hours before the addition of neutrophils, fresh medium supplemented with 5% human serum and either L-arginine (100 μM) or the nitric oxide synthase inhibitor N^G-monomethyl-L-arginine (L-NMMA, 500 μM) was added. Lipoprotein oxidation was initiated by adding native LDL (0.2 mg of protein/mL final concentration) to medium with either PMA-stimulated (200 ng/mL phorbol ester final concentration) or unstimulated isolated human neutrophils (2×10^6/mL). Cocultures were incubated for an additional 2 h at 37 °C in humidified 5% CO_2, and then the reaction was stopped by addition of BHT (100 μM final concentration). Medium was removed to borosilicate glass test tubes, and lipids were extracted as described below. Lipids remaining in tissue culture wells were also recovered by washing wells with similar organic solvents and the organic phases combined for further sample workup and LC/MS/MS analysis as described below.

Lipid Vesicle Preparation and Oxidation. Vesicles were prepared in sodium phosphate buffer (20 mM, pH 7.0) which was treated with Chelex-100 resin, supplemented with 200 μM DTPA, and sparged with argon prior to use. Stock solutions (2 mg/mL) of small unilamellar vesicles comprised of 1-hexadecanoyl-2-eicosatetra-5′,8′,11′,14′-enoyl-*sn*-glycero-3-phosphocholine (16:0,20:4-PC) were prepared by extrusion (10 times) through a 0.1 μm polycarbonate filter using an Avanti Mini-Extruder Set (Avanti Polar Lipids, Inc., Alabaster, AL) as described by the manufacturer. Vesicles were then diluted to a final concentration of 0.2 mg of lipid/mL and incubated in the presence of MPO (30 nM), glucose (100 μg/mL), and glucose oxidase (100 ng/mL) at 37 °C under air as described in the figure legends. Under these conditions, a constant flux of H_2O_2 (0.80 μM/min) was generated by the glucose/glucose oxidase system. Reactions were stopped by addition of 50 μM BHT and 300 nM catalase.

Analysis of Lipid Peroxidation Products. Diene conjugation of oxidized lipids was monitored as the increase in absorbance at 234 nm using a molar extinction coefficient ϵ

of 2.95×10^4 M^{-1} cm^{-1} (*62*). The content of thiobarbituric acid (TBA) reactive products was determined by fluorescence analysis (*63*) in the presence of 0.05% (w/v) BHT. Fluorescence measurements (2 nm slit width, excitation at 515 nm, and emission at 553 nm) were made on a Perkin-Elmer LS-3 fluorescence spectrometer using an external calibration curve constructed with known amounts of malondialdehyde (MDA) obtained from hydrolysis of 1,1,3,3-tetramethoxypropane in 20% acetic acid (pH 3.5).

For the assessment of 9-H(P)ODE, 9-H(P)ETE, and F_2-isoprostanes, hydroperoxides in reaction mixtures were reduced to their corresponding hydroxides during extraction utilizing a modified Dole procedure in which the reducing agent, triphenylphosphine, is present (*14*). These conditions also inhibit artifactual formation of isoprostanes and oxidized lipids (*4*). Lipids were dried under N_2 and resuspended in 2-propanol (2 mL), and then fatty acids were released by base hydrolysis with 1 N sodium hydroxide (2 mL) at room temperature under N_2 for 90 min. The samples were acidified (pH 3.0) with 2 N HCl; known amounts of internal standards were added, and free fatty acids were extracted twice with hexane (5 mL). The amounts of 9-H(P)ODEs, 9-H(P)ETEs, and F_2-isoprostanes were then determined by LC/MS/MS analysis as outlined below. Independent control experiments using [^{3}H]arachidonate confirmed that no detectable formation of ^{3}H-labeled 9-H(P)ODE, 9-H(P)ETE, or F_2-isoprostanes occurred under the conditions employed for sample preparation.

POxvPC and PAPC were quantified in vesicle and LDL lipids following extraction by the same modified Dole procedure used for 9-H(P)ODE, 9-H(P)ETE, and F_2-isoprostane analyses described above, but omitting addition of the reductant, triphenylphosphine. Lipids were dried under N_2, resuspended in methanol, and stored under argon at −70 °C until subsequent LC/MS analysis as outlined below.

Sterol oxidation products in 250 μL of LDL preparations were extracted by adding 4 M NaCl (150 μL) and acetonitrile (500 μL). Samples were vortexed and centrifuged, and the upper organic phase was removed. Extracts were dried under N_2, resuspended in 300 μL of methanol, and stored under argon at −70 °C until analysis by HPLC with on-line mass spectrometric analysis as described below.

Mass Spectrometry. Mass spectrometric analyses were performed on a Quatro II triple-quadruple mass spectrometer (Micromass, Inc.) interfaced with an HP 1100 HPLC (Hewlett-Packard). F_2-Isoprostanes were quantified by stable isotope dilution mass spectrometry using on-line reverse phase HPLC tandem mass spectrometry (LC/MS/MS) with 8-epi-[$^2H_4$]$PGF_{2\alpha}$ as a standard as described by Mallat (*7*). For 9-HODE and 9-HETE analyses, lipid extracts generated following base hydrolysis of reduced lipids (above) were dried under N_2 and reconstituted in 100 μL of methanol. An aliquot of the mixture was then injected on an Ultrasphere ODS C18 column (4.6 mm × 250 mm, 5 μm, Beckman Instruments, Fullerton, CA) equilibrated and run under isocratic conditions at 1 mL/min employing methanol/H_2O (85:15, v/v) as a solvent. The column eluent was split (rates of 930 μL/min to the UV detector and 70 μL/min to the mass detector) and analyzed with the mass spectrometer. LC/MS/MS analyses of 9-HODE, 9-HETE, and F_2-isoprostanes in column effluents were performed using electrospray ionization mass spectrometry (ESI-MS) in the negative ion mode with multiple reaction monitoring (MRM) and monitoring the transitions *m/z* 295 → 171 for 9-HODE, *m/z* 319 → 151 for 9-HETE, *m/z* 353 → 309 for F_2-isoprostanes, and *m/z* 357 → 313 for [$^2H_4$]$PGF_{2\alpha}$. The endogenous content of 9-H(P)ODE and 9-H(P)ETE in samples was initially determined by the method of standard additions utilizing known amounts of authentic 9-HODE and 9-HETE added to samples. Quantification in subsequent experiments was carried out by employing external calibration curves constructed with authentic 9-HODE and 9-HETE following preliminary LC/MS/MS experiments which produced results identical to those obtained by the method of standard additions.

Quantification of PAPC and POxvPC was performed on lipid extracts utilizing HPLC with on-line ESI-MS analysis in the positive ion mode and selected ion monitoring at *m/z* 782 and 594, respectively. An aliquot of lipid extract reconstituted in methanol (above) was mixed with 50 μL of 0.1% formic acid in methanol (mobile phase B) and loaded onto a Columbus C18 column (1 mm × 250 mm, 5 μm, P. J. Cobert, St. Louis, MO) pre-equilibrated in 70% mobile phase B and 30% mobile phase A (0.1% formic acid in water) at a flow rate of 30 μL/min. Following a 3 min wash period with 70% mobile phase B, the column was developed with a linear gradient to 100% mobile phase B over the course of 10 min, followed by isocratic elution with 100% mobile phase B. External calibration curves constructed with authentic POxvPC and PAPC were used for quantification following preliminary experiments which produced results identical to those obtained by the method of standard additions.

7-OH cholesterol, 7-keto cholesterol, and 7-OOH cholesterol where resolved on an Ultrasphere ODS C18 column (4.6 mm × 250 mm, 5 μm, Beckman Instruments) at a rate of 1.5 mL/min. The elution gradient consisted of 91:9 acetonitrile/water and 0.1% formate (v/v), and the column was washed between runs with acetonitrile and 0.1% formate. The column effluent was split (rates of 900 μL/min to the UV detector and 100 μL/min to the mass detector) and ionized by atmospheric-pressure chemical ionization (APCI) in the positive ion mode with selected ion monitoring. Identification of 7-OH cholesterol was performed by demonstrating comigration of ions at *m/z* 385.3 [$(M - H_2O)^+$] and *m/z* 367.3 [$(M - 2H_2O)^+$] with the same retention time as the authentic standard. The integrated area of the ion current for the peak monitored at *m/z* 367.3 was used for quantification. Identification of 7-OOH cholesterol was performed by demonstrating comigration of ions at *m/z* 401.3 [$(M - H_2O)^+$], *m/z* 383.3 [$(M - 2H_2O)^+$], and *m/z* 367.3 [$(M - H_2O - H_2O_2)^+$] with the same retention time as the authentic standard. The integrated area of the ion current for the peak monitored at *m/z* 401.3 was used for quantification. Identification of 7-keto cholesterol was performed by demonstrating comigration of ions at *m/z* 401.3 [$(M + H)^+$] and *m/z* 383.3 [$(M - H_2O)^+$] with the same retention time as the authentic standard. The integrated area of the ion current for the peak monitored at *m/z* 401.3 was used for quantification. External calibration curves constructed with authentic 7-OH cholesterol, 7-OOH cholesterol, and 7-keto cholesterol were used for quantification following preliminary APCI LC/MS experiments which produced results identical to those obtained by the method of standard

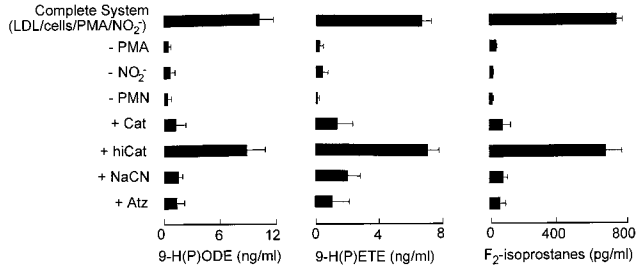


FIGURE 1: Neutrophils activated in medium containing NO_2^- initiate lipid peroxidation via the MPO/H_2O_2 system. Isolated human neutrophils (10^6/mL) were incubated at 37 °C in HBSS supplemented with DTPA (100 μM), LDL (0.2 mg of protein/mL), and NO_2^- (50 μM). Neutrophils were activated with 200 nM phorbol myristate acetate (PMA) and maintained in suspension by intermittent inversion (complete system) for 2 h. Additions or deletions to the complete system were as indicated. Reactions were stopped by the removal of cells via centrifugation, followed by addition of BHT and extraction of lipids in the supernatant. Lipids were reduced and hydrolyzed, and the levels of total (free and esterified) 9-H(P)-ODE, 9-H(P)ETE, and F_2-isoprostanes were then determined by LC/MS/MS as described in Experimental Procedures. The final concentrations of additions to the complete system were 300 nM catalase (Cat), 300 nM heat-inactivated catalase (hiCat), 1 mM NaCN, and 10 mM 3-aminotriazole (Atz). Data represent the means ± the standard deviations of triplicate determinations.

additions. The retention times for 25-OH cholesterol, 5,6-α- and -β-epoxides, and triol were determined by LC/MS analysis of authentic standards.

RESULTS

Reactive Nitrogen Intermediates Formed by Neutrophils Initiate Lipid Peroxidation. LDL incubated alone or with phorbol ester-activated human neutrophils in medium A (Chelex 100-treated Hank's Balanced Salt Solution supplemented with the metal chelator DTPA) exhibited little evidence of lipid oxidation (Figure 1). Activation of leukocytes in medium containing levels of NO_2^- observed in inflammatory fluids caused a marked increase in the extent of lipid peroxidation, as monitored by LC/MS/MS analysis of oxidation products of both linoleic acid [9-H(P)ODE] and arachidonic acid [9-H(P)ETE and F_2-isoprostanes] (Figure 1). Initiation of lipid peroxidation required cells, NO_2^-, and an activating stimulus (Figure 1). Catalase, but not heat-inactivated catalase, inhibited LDL lipid peroxidation by activated human neutrophils, indicating that cell-generated H_2O_2 was required for the reaction. Addition of peroxidase inhibitors, cyanide and 3-aminotriazole, inhibited lipid peroxidation, consistent with a role for MPO in promoting oxidation of glycerophospholipids by the cells. Qualitatively similar results were observed with neutrophils activated with alternative agonists such as fMLP and opsonized Chelex-treated zymosan (data not shown). Collectively, these results indicate that the reactive nitrogen intermediate formed by neutrophils during MPO-catalyzed oxidation of NO_2^- initiates lipid peroxidation.

The Mechanism of Neutrophil-Mediated Initiation of Lipid Peroxidation via MPO- and H_2O_2-Dependent Oxidation of NO_2^- Does Not Require Free Transition Metals, Thiols, or Superoxide. Endothelial cells, smooth muscle cells, and mononuclear phagocytes oxidize LDL lipids ex vivo by reactions that require transition metal ions (*10*−*12*, *16*), reactive intermediates produced from thiols (*64*), and reactive

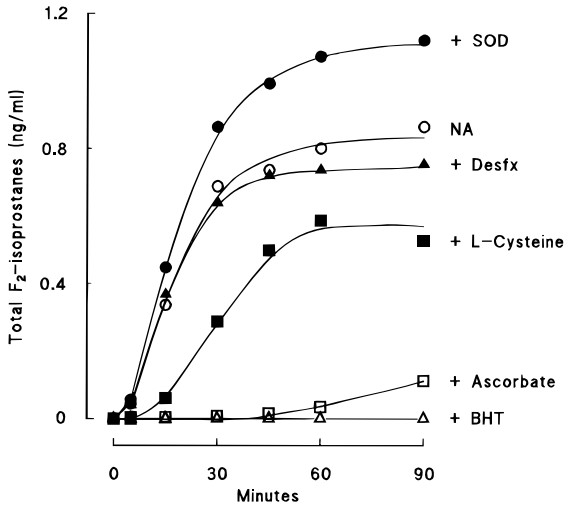


FIGURE 2: Reaction characteristics of neutrophil-dependent initiation of lipid peroxidation by the MPO/H_2O_2/NO_2^- system of neutrophils. Human neutrophils (10^6/mL) were incubated at 37 °C in HBSS supplemented with DTPA (100 μM), LDL (0.2 mg of protein/mL), and NO_2^- (50 μM) for the indicated periods of time following activation with 200 nM phorbol myristate acetate. The level of total F_2-isoprostanes formed in LDL was then determined by LC/MS/MS as described in the legend of Figure 1. Where indicated, superoxide dismutase (SOD, 10 μg/mL), desferroxamine (Desfx, 10 μM), L-cysteine (100 μM), ascorbate (100 μM), butylated hydroxytoluene (BHT, 100 μM), or no addition (NA) was included in the cell reaction mixtures. Data represent the means of duplicate determinations from a representative experiment performed three times.

oxidant species derived from NO and O_2^- (*15*, *26*). Lipid oxidation by all these pathways is sensitive to inhibition by SOD (*10*, *12*, *16*, *17*). To examine the nature of lipid peroxidation reactions mediated by neutrophils via MPO- and H_2O_2-dependent oxidation of NO_2^-, LDL was incubated with PMA-stimulated neutrophils in medium A supplemented with NO_2^- alone or in the presence of a variety of additions, and the time course of F_2-isoprostane production was determined by LC/MS/MS. The kinetics of lipid peroxidation was similar to that of O_2^- production (data not shown) and demonstrated an initial brief lag phase over the first 5 min, followed by a rapid increase in rate which then plateaued by approximately 1 h. Addition of SOD enhanced the extent of lipid peroxidation induced by neutrophils in the presence of NO_2^- by approximately 1.5-fold (Figure 2); thus, O_2^- is not required for neutrophil-mediated lipid peroxidation by this pathway. Control experiments suggested that SOD itself was not directly promoting lipid peroxidation since addition of both SOD and either peroxidase inhibitors (i.e., NaN_3, NaCN, or 3-aminotriazole) or catalase to cells activated in the presence of NO_2^- resulted in no significant formation of lipid oxidation products (data not shown). The mechanism-(s) responsible for the increased extent of lipid peroxidation observed in the presence of SOD therefore likely reflects either prevention of O_2^--dependent conversion of MPO into an inactive form (i.e., Compound III; *65*), enhanced conversion of O_2^- into the MPO substrate, H_2O_2 (*66*), or prevention of O_2^--dependent chain terminating reactions (*67*). Addition of desferroxamine (10 μM), a metal chelator which potently blocks lipid peroxidation catalyzed by free redox active transition metal ions, had no significant inhibitory effect on F_2-isoprostane production. Although L-cysteine promotes lipid peroxidation mediated by cultured smooth muscle cells

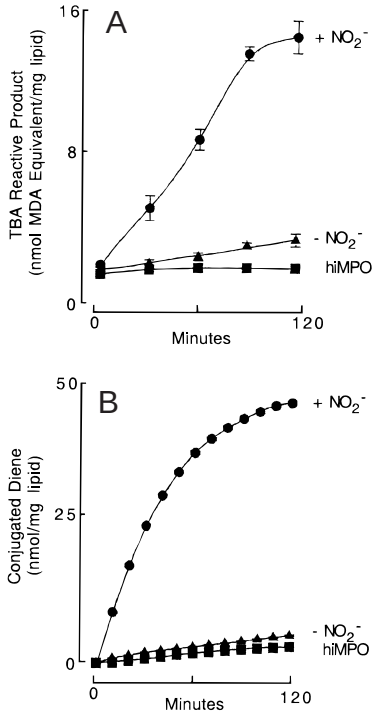


FIGURE 3: Oxidation of PAPC vesicles by the MPO/H_2O_2/NO_2^- system. Small unilamellar vesicles comprised of PAPC (0.2 mg/mL) were incubated with isolated human MPO (30 nM), glucose (100 μM), and glucose oxidase (100 ng/mL) in sodium phosphate buffer (50 mM, pH 7.0) supplemented with DTPA (200 μM) for the indicated periods of time in either the presence (+ NO_2^-) or absence (− NO_2^-) of 100 μM NO_2^-. The levels of formation of (A) thiobarbituric acid (TBA) reactive products and (B) conjugated dienes were determined as described in Experimental Procedures. Reactions performed in the presence of NO_2^- were also performed substituting heat-inactivated MPO (hiMPO) for catalytically active enzyme. Data represent the means ± the standard deviations of triplicate determinations (A) and the means of duplicate determinations from a representative experiment performed three times (B).

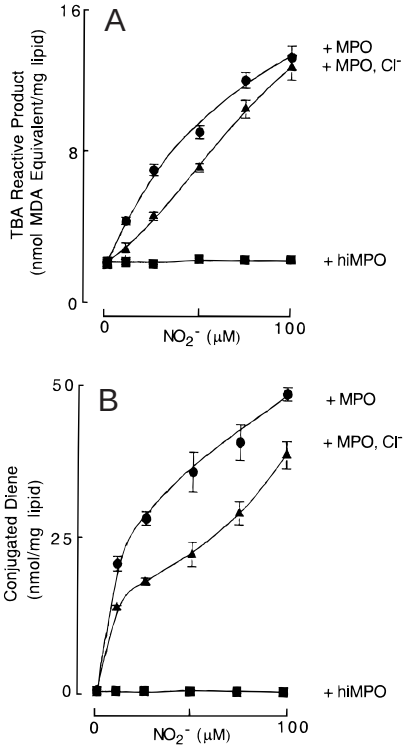


FIGURE 4: NO_2^- concentration dependence of the MPO-catalyzed oxidation of PAPC vesicles. Small unilamellar vesicles comprised of PAPC (0.2 mg/mL) were incubated for 120 min at 37 °C with glucose (100 μM), glucose oxidase (100 ng/mL), and the indicated concentrations of NO_2^- in sodium phosphate buffer (50 mM, pH 7.0) supplemented with DTPA (200 μM) and either 30 nM MPO (+ MPO), 30 nM MPO and 100 mM Cl^- (+ MPO, Cl^-), or 30 nM heat-inactivated MPO (+ hiMPO). The levels of (A) thiobarbituric acid (TBA) reactive products and (B) conjugated dienes formed were then determined as described in Experimental Procedures. Data represent the means ± the standard deviations of triplicate determinations.

(*64*, *68*), addition of the thiol to reaction mixtures modestly attenuated the extent of F_2-isoprostane formation by the reactive species formed during neutrophil-dependent oxidation of NO_2^-. Finally, addition of both water-soluble (ascorbate) and lipid-soluble (BHT) free radical scavengers dramatically attenuated F_2-isoprostane production (Figure 2). Thus, oxidation of NO_2^- by the MPO/H_2O_2 system of phagocytes promotes initiation of lipid peroxidation through mechanisms that do not require O_2^-, free redox active transition metal ions, or reactive intermediates derived from thiols.

Characterization of MPO- and NO_2^--Dependent Initiation of Lipid Peroxidation in a Model Vesicle System. To assess the reaction requirements for lipid peroxidation by the MPO/H_2O_2/NO_2^- system, we examined the time course (Figure 3) and NO_2^- concentration dependence (Figure 4) of lipid peroxidation using lipid vesicles and isolated MPO as a model system. Small unilamellar vesicles comprised of 1-hexadecanoyl-2-eicosatetra-5′,8′,11′,14′-enoyl-*sn*-glycero-3-phosphocholine (PAPC) were incubated with MPO, a H_2O_2-generating system (glucose/glucose oxidase), and NO_2^-, and the extent of lipid peroxidation was monitored by assessing conjugated diene formation and thiobarbituric acid reactive products. Both indices of lipid peroxidation increased in a time-dependent manner following exposure of PAPC vesicles to the complete MPO/H_2O_2/NO_2^- system. Initiation of lipid peroxidation required the presence of catalytically active MPO, NO_2^-, and a H_2O_2-generating source (Figure 3). Exposure of PAPC vesicles to MPO, a H_2O_2-generating system, and concentrations of NO_2^- that approximate those found in inflammatory tissues and fluids ($\leq$50 μM) promoted lipid peroxidation (Figure 4). Parallel reactions that included heat-inactivated MPO failed to result in lipid peroxidation. Finally, addition of plasma levels of chloride (100 mM), a major substrate for MPO, only modestly attenuated the extent of lipid peroxidation mediated by the reactive intermediate generated during MPO-dependent oxidation of NO_2^- (Figure 4). Thus, MPO can oxidize NO_2^- to form a reactive intermediate capable of initiating lipid peroxidation, even in the presence of plasma levels of chloride.

The MPO/H_2O_2/NO_2^- System Generates Biologically Active Oxidized Lipids and Sterols Known To Be Formed in Vivo. Multiple lipid and sterol oxidation products are formed during inflammatory and vascular disorders and often possess potent biological activities. For example, 1-hexadecanoyl-2-oxovalaryl-*sn*-glycero-3-phosphocholine (POxvPC) is a free radical-dependent oxidation product of PAPC. This platelet-activating factor (PAF)-like molecule induces up-regulation of adhesion and chemoattractant proteins on vascular endothelium and is present in atherosclerotic lesions (*9*). We therefore sought to determine whether MPO-

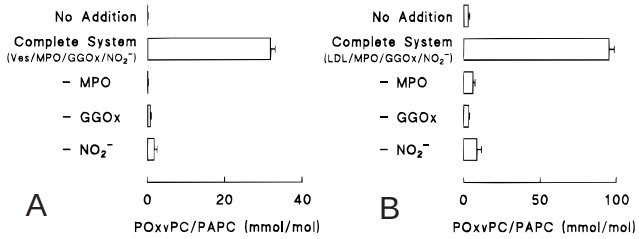


FIGURE 5: Quantification of POxvPC formation during PAPC vesicle and lipoprotein oxidation by the MPO/H_2O_2/NO_2^- system. Small unilamellar vesicles comprised of (A) PAPC (0.2 mg/mL) or (B) LDL (0.2 mg of protein/mL) were incubated for 4 h at 37 °C with glucose (100 μM), glucose oxidase (20 ng/mL), MPO (30 nM), and NO_2^- (50 μM) in sodium phosphate buffer (50 mM, pH 7.0) supplemented with DTPA (200 μM) (complete system) or the indicated deletions. Lipids were then extracted, and the level of POxvPC was determined by HPLC with on-line ESI-MS as described in Experimental Procedures. Data represent the mean ± the standard deviation of triplicate determinations.

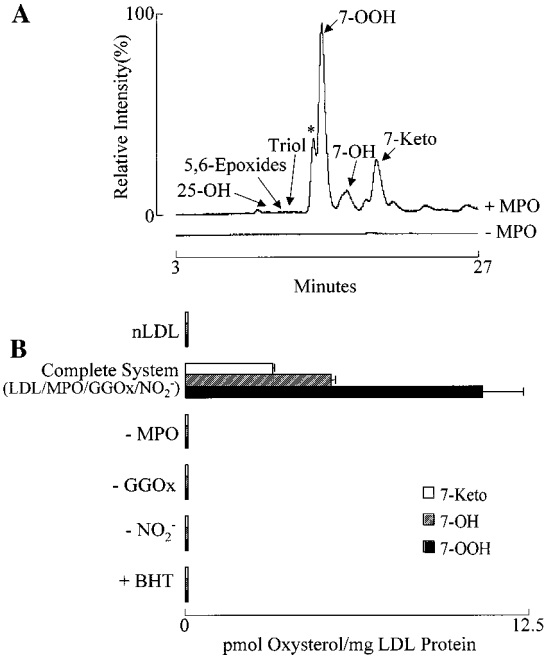


FIGURE 6: Analysis of oxysterol formation during LDL oxidation by the MPO/H_2O_2/NO_2^- system using HPLC with on-line APCI-MS. (A) LDL (0.2 mg of protein/mL) was incubated at 37 °C for 8 h with glucose (100 μM), glucose oxidase (20 ng/mL), and NO_2^- (50 μM) in the presence (+ MPO) or absence (− MPO) of isolated human MPO (30 nM) in sodium phosphate buffer (50 mM, pH 7.0) supplemented with DTPA (200 μM). Lipids were extracted and analyzed by reverse phase HPLC with on-line APCI-MS as described in Experimental Procedures. The chromatograms represent the sum of ion currents obtained by monitoring at *m*/*z* 367.3 and 401.3. The asterisk denotes an oxysterol of unknown identity, but its mass and retention time are consistent with 5-OOH cholesterol. (B) LDL (0.2 mg of protein/mL) was incubated at 37 °C for 8 h with MPO (30 nM), glucose (100 μM), glucose oxidase (20 ng/mL), and NO_2^- (50 μM) in sodium phosphate buffer (50 mM, pH 7.0) supplemented with DTPA (200 μM). Additions or deletions to the complete system were as indicated. Lipids were extracted and analyzed by reverse phase HPLC with on-line APCI-MS as described in Experimental Procedures. Butylated hydroxytoluene (BHT, 100 μM) was added to reaction mixtures where indicated. Data represent the means ± the standard deviations of triplicate determinations.

generated nitrating intermediates derived from oxidation of NO_2^- can form POxvPC using vesicles or LDL as targets. Exposure of either small unilamellar vesicles comprised of PAPC or lipoproteins to the complete MPO/H_2O_2/NO_2^- system resulted in the formation of POxvPC, as assessed by reverse phase HPLC with on-line ESI-MS analysis (Figure 5). Production of POxvPC required MPO, a H_2O_2-generating system, and NO_2^-. Similarly, LC/MS analyses with LDL exposed to phorbol ester-activated human neutrophils demonstrated that POxvPC was produced in the presence, but not absence, of NO_2^- via a peroxidase inhibitor (3-aminotriazole) and catalase sensitive pathway (data not shown). Taken together, these results suggest that leukocyte activation in vivo may result in the formation of specific oxidized lipids, such as POxvPC, which can serve to promote further recruitment of inflammatory cells to that site.

In a parallel set of experiments, we examined whether MPO-generated nitrating intermediates might serve as a potential mechanism for the generation of oxysterols in vivo. Oxysterols are present in plasma and human vascular lesions (*53*, *69*) and typically possess potent biological activities (*53*, *70*); however, the reaction pathways responsible for cholesterol oxidation in vivo have not yet been identified. We therefore assessed whether production of 7-OOH cholesterol, a particularly potent cytotoxic oxysterol present in human vascular lesions (*53*), as well as formation of other oxysterols identified in atherosclerotic lesions (e.g., 7-OH cholesterol and 7-keto cholesterol; *71*) occurred following exposure of LDL to the MPO/H_2O_2/NO_2^- system. HPLC with on-line ESI/MS analysis of lipids demonstrated that peroxidation of lipoprotein cholesterol by the MPO system formed multiple oxysterols, including 7-OOH cholesterol, 7-keto cholesterol, and 7-OH cholesterol. Oxysterol formation required each component of the MPO/H_2O_2/NO_2^- system and was inhibited by the free radical scavenger BHT (Figure 6). Taken together, these results strongly suggest that the MPO/H_2O_2/NO_2^- system may contribute to the formation of numerous lipid peroxidation products, including H(P)ODEs, H(P)ETEs, F_2-isoprostanes, POxvPC and other PAF-like mimetics, and oxysterols in vivo.

The Reactive Nitrogen Species Formed by MPO-Catalyzed Oxidation of NO_2^- Promotes LDL Lipid Peroxidation under Physiological Conditions. To further assess the potential physiological relevance of lipid peroxidation mediated by the MPO/H_2O_2/NO_2^- system, we used LC/MS/MS to examine whether products of linoleic acid and arachidonic acid peroxidation [i.e., 9-H(P)ODE and 9-H(P)ETE, respectively] were formed during exposure of LDL to the MPO-generated oxidant in the presence of serum constituents (lipoprotein deficient serum, 50%, v/v). In the presence of serum proteins and plasma levels of chloride, incubation of LDL with MPO and a H_2O_2-generating system in Chelex 100-treated buffer supplemented with DTPA failed to produce significant levels of either lipid oxidation product (Figure 7). Addition of NO_2^- (50 μM) to the serum protein-containing reaction mixture produced both 9-H(P)ODE and 9-H(P)ETE in a time-dependent manner (Figure 7). Significant levels of both 9-H(P)ODE and 9-H(P)ETE were formed even at the earliest time point that was examined. Under these conditions, the flux of H_2O_2 generated by the glucose oxidase system was 0.18 μM/min (Experimental Procedures), which corresponds to a total of only ~11 μM H_2O_2 produced over the first hour, significantly less than that generated by circulating levels of stimulated neutrophils (*72*, *73*). In parallel sets of reactions performed in the presence of either methionine (1 mM) or

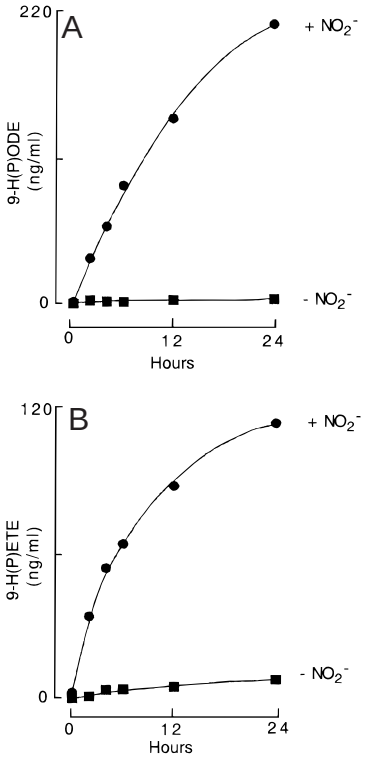


FIGURE 7: LDL oxidation by the MPO/H_2O_2/NO_2^- system in the presence of serum. LDL (0.2 mg of protein/mL) was incubated with lipoprotein deficient serum (50% v/v), isolated human MPO (30 nM), and glucose oxidase (20 ng/mL) in phosphate-buffered saline (20 mM, pH 7.0) supplemented with DTPA (200 μM) for the indicated periods of time in either the presence (+ NO_2^-) or absence (− NO_2^-) of 50 μM NO_2^-. The levels of total (free and esterified) (A) 9-H(P)ODE and (B) 9-H(P)ETE formed were then determined by LC/MS/MS as described in Experimental Procedures. Data represent the means of triplicate determinations.

taurine (10 mM), comparable levels of 9-H(P)ODE and 9-H(P)ETE were formed (data not shown), demonstrating that halogenating intermediates were not involved in the initiation of lipid peroxidation under these conditions.

In another series of experiments, we examined the NO_2^- concentration dependence for MPO-dependent initiation of LDL lipid peroxidation in the presence of lipoprotein deficient serum (50%, v/v). Plasma levels of NO_2^- range between 1 and 5 μM in normal individuals, and can reach levels 10-fold higher under inflammatory conditions (*37*, and references therein). At sites of inflammation, tissue and extracellular fluid levels of NO_2^- may be even higher since a main pathway for NO_2^- removal is via blood where it is oxidized to nitrate (NO_3^-) by reaction with oxyhemoglobin (*74*). Oxidation products of linoleic acid [9-H(P)ODE] and arachidonic acid [9-H(P)ETE] were readily observed by LC/MS/MS following exposure of LDL to MPO in the presence of serum proteins, plasma levels of Cl^-, and a H_2O_2-generating system, even at normal plasma levels of NO_2^- (Figure 8). Further increases in the NO_2^- level over a pathophysiologically relevant range were accompanied by a dose-dependent increase in the extent of lipid peroxidation, monitored as the appearance of 9-H(P)ODE and 9-H(P)ETE by LC/MS/MS analysis (Figure 8).

Endothelial Cell/Neutrophil Coculture Experiments. In a final series of experiments, we sought to determine whether similar lipid peroxidation reactions could be observed using a cellular source of NO (and hence NO_2^-), as might occur

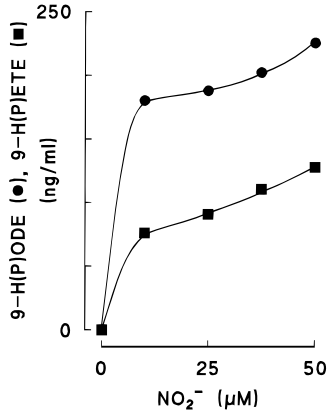


FIGURE 8: NO_2^- concentration dependence of MPO-catalyzed oxidation of LDL in the presence of serum. LDL (0.2 mg of protein/mL) was incubated with lipoprotein deficient serum (50% v/v), isolated human MPO (30 nM), glucose (100 μM), glucose oxidase (20 ng/mL), and the indicated concentrations of NO_2^- in phosphate-buffered saline (20 mM, pH 7.0) supplemented with DTPA (200 μM) for 24 h at 37 °C. The levels of total (free and esterified) 9-H(P)ODE (●) and 9-H(P)ETE (■) formed were then determined by LC/MS/MS as described in Experimental Procedures. Data represent the means of duplicate determinations. Similar results were obtained in three separate experiments.

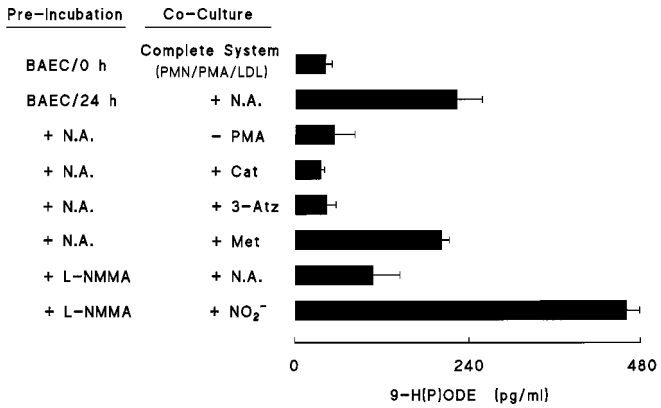


FIGURE 9: Neutrophils promote LDL lipid peroxidation in cocultures with bovine aortic endothelial cells. Confluent bovine aortic endothelial cells (BAEC) were cultured for a 0 or 24 h preincubation period in the absence (N.A., no addition) or presence of 500 μM N^G-monomethyl-L-arginine (L-NMMA) as described in Experimental Procedures. Native LDL (0.2 mg of protein/mL final concentration) and phorbol ester (PMA, 200 nM)-activated human neutrophils (PMN, 2×10^6/mL) were then added to the medium, and the mixture was incubated at 37 °C under 5% CO_2/95% air for an additional 2 h. Reactions were stopped by addition of BHT, and then the level of total 9-H(P)ODE present was determined by LC/MS/MS analysis as described in Experimental Procedures. Where indicated, catalase (Cat, 300 nM), 3-aminotriazole (3-Atz, 10 mM), or nitrite (NO_2^-, 10 μM) was included in the coculture reaction mixtures. Data represent the means ± the standard deviations of triplicate determinations.

in vivo. When activated human neutrophils and LDL were incubated with BAEC that had been previously incubated for 24 h in medium alone [and in which NO_2^- accumulated to a concentration of 7.7 ± 1.8 μM (mean ± standard deviation, $n = 4$)], 9-H(P)ODE levels were significantly increased over those observed with either unstimulated neutrophils or reactions performed in the presence of either catalase or the peroxidase inhibitor 3-aminotriazole (Figure 9). These results suggest that the MPO/H_2O_2 system of neutrophils was involved in the initiation of lipid peroxidation under these conditions. Addition of methionine to the

coculture failed to significantly attenuate 9-H(P)ODE production, indicating that MPO-generated halogenating oxidants were not involved the initiation of lipid peroxidation. A role for NO-derived NO_2^- in promoting lipid peroxidation via the peroxidase/H_2O_2 system of neutrophils was strongly suggested by the results from several additional experiments. (1) When activated neutrophils and LDL were added to BAEC in fresh medium (i.e., without a 24 h preincubation period during which NO_2^- might accumulate), the levels of lipid peroxidation products that were formed were markedly diminished (Figure 9). (2) Addition of activated neutrophils and LDL to BAEC which were preincubated for the preceding 24 h in medium supplemented with the nitric oxide synthase inhibitor L-NMMA [NO_2^- levels were 1.8 ± 1.2 μM (mean $\pm$ standard deviation, $n = 4$)] generated significantly less lipid peroxidation products (Figure 9). (3) Addition of NO_2^- (10 μM) to the medium of L-NMMA-pretreated BAEC reconstituted the capacity of the activated neutrophils to initiate lipid peroxidation (Figure 9). Taken together, these results suggest that NO_2^-, a major end product of NO metabolism, can be oxidized by MPO in a physiological milieu. They also demonstrate that the reactive nitrogen species formed by MPO-catalyzed oxidation of NO_2^- is capable of initiating lipid peroxidation in biological matrices such as serum and plasma.

DISCUSSION

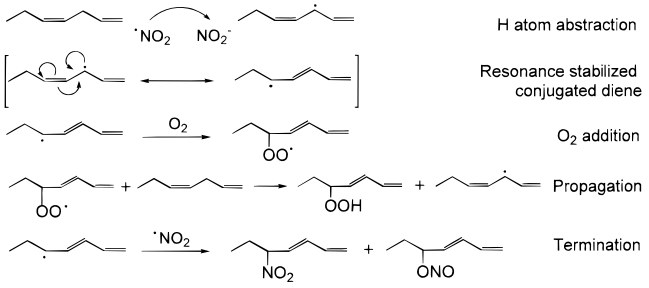
Chance demonstrated that NO_2^- can serve as a substrate for a peroxidase more than four decades ago (*75*). However, only recently has it been appreciated that oxidation of NO_2^- by leukocyte peroxidases such as MPO (*36*−*38*, *43*−*45*) and eosinophil peroxidase (*76*) may contribute to oxidative damage by generating reactive species capable of nitrating free and protein-bound tyrosine residues. The results presented here extend these findings and indicate that the oxidant(s) formed during peroxidase-catalyzed oxidation of NO_2^- is also capable of abstracting an allylic hydrogen atom from polyenoic fatty acids and sterols within complex biological matrices such as serum.

A wealth of evidence links inflammatory and vascular disorders to lipid peroxidation and the formation of biologically active oxidized glycerophospholipids and sterols (*1*−*4*). However, the pathways for generating reactive oxidant species and initiating lipid peroxidation in vivo have not been established. Leukocytes such as neutrophils, monocytes, and macrophages are ideal candidates to serve as a cellular source of reactive species to promote lipid peroxidation in vivo, because they have evolved an arsenal of enzymes that endow them with the capacity to generate oxidants and diffusible radical species (*39*, *77*). The results of the study presented here demonstrate that nitrating intermediates generated during oxidation of NO_2^- by the MPO/H_2O_2 system of neutrophils serve as physiological catalysts for the initiation of lipid peroxidation and the formation of biologically active oxidized glycerophospholipids and sterols. Neutrophil- and MPO-catalyzed initiation of lipid peroxidation was confirmed by multiple independent methods ranging from global indices of lipid oxidation (i.e., TBA reactive products and conjugated dienes) to specific mass spectrometric analyses of multiple structurally distinct fatty acid and sterol oxidation products, including total (free and esterified) 9-H(P)ODE, 9-H(P)ETE, and F_2-isoprostanes, POxvPC, 7-OOH cholesterol, 7-OH cholesterol, and 7-keto cholesterol. Induction of lipid peroxidation by neutrophils and isolated MPO occurred at NO_2^- concentrations approximating those found in inflammatory tissues and fluids. Moreover, lipid peroxidation by the MPO-generated nitrating intermediate(s) was observed using both synthetic vesicles and isolated LDL as targets for oxidation, even in the presence of serum proteins and plasma levels of chloride. Finally, coculture experiments demonstrated that neutrophils can generate NO-derived oxidants capable of initiating lipid peroxidation via the MPO/H_2O_2 system. Thus, activation of neutrophils in vivo is anticipated to promote lipid peroxidation via this pathway.

MPO is one of the most abundant proteins in neutrophils and monocytes, accounting for 3−5% and 1−2% of the total protein content of each cell, respectively (*39*). At sites of inflammation, leukocyte activation is accompanied by MPO secretion and H_2O_2 formation via the respiratory burst. Enhanced NO production and NO_2^- accumulation are also characteristic features of inflammatory processes. Thus, all of the reactants necessary for MPO- and NO_2^--dependent initiation of lipid peroxidation are present at sites of inflammation. Similarly, MPO is enriched with human atherosclerotic lesions (*78*). Mass spectrometric studies demonstrate that MPO promotes oxidative damage in vivo since the level of 3-chlorotyrosine, a specific oxidation product of MPO, is markedly elevated in lipoproteins and proteins recovered from diseased human vascular tissue (*79*). Both immunohistochemical (*31*) and mass spectrometric studies (*32*) have also established that 3-nitrotyrosine is present in human atheroma and LDL recovered from atherosclerotic plaques. Finally, products of lipid peroxidation such as those generated in this study [i.e., H(P)ODEs, H(P)ETEs, F_2-isoprostanes, POxvPC, and oxysterols] are known to be enriched with atherosclerotic lesions (*1*−*3*, *6*−*9*, *53*). For example, recent LC/MS/MS studies demonstrated that elevated levels of 9-HETE, an arachidonic acid oxidation product which cannot be derived from known enzymatic reactions, was selectively enriched within human carotid plaques (*7*). Moreover, the level of 9-HETE in plaques obtained from symptomatic patients was significantly higher than that observed in asymptomatic patients, suggesting that free radical-mediated lipid peroxidation reactions predominate in advanced atherosclerosis and may promote plaque instability. Taken together, the results of the study presented here suggest that phagocyte activation in the presence of NO_2^- may serve as a physiological mechanism for the initiation of lipid peroxidation and the generation of lipid-derived signaling molecules at sites of inflammation and vascular disease.

An intriguing question that has not yet been answered is what the chemical nature of the NO_2^--derived oxidant is. Van der Vleit et al. (*37*) suggested that nitrogen dioxide (NO_2), the one-electron oxidation product of NO_2^-, was the likely intermediate formed during aromatic nitration reactions. Pulse radiolysis studies demonstrate that NO_2 can abstract a hydrogen atom from phenolic compounds and nitrate tyrosine residues via a radical−radical addition reaction between NO_2 and tyrosyl radical (*80*). In the presence of alternative oxidizable targets, phenolic nitration by NO_2 is significantly inhibited (*80*). One such target is lipids. NO_2 is a lipophilic oxidant that partitions into membranes and readily initiates oxidation of polyunsaturated

Scheme 1



fatty acids (*50*, *51*). This potent nitrogen oxide is a component of mainstream tobacco smoke and promotes lipid peroxidation when inhaled (*49*, *52*). Thus, NO_2 generated by the MPO/H_2O_2/NO_2^- system might similarly abstract a hydrogen atom from bis-allylic methylene groups (Scheme 1). The resultant resonance-stabilized pentadienyl radical may react with molecular oxygen to form chain-propagating alkyl peroxyl radicals. Studies with gas phase NO_2 as a lipid peroxidation initiator also demonstrate that radical−radical combinations between a carbon-centered radical intermediate and NO_2 can form nitrated lipids (*81*, *82*). A similar mechanism was recently proposed for conjugated diene formation and nitration of γ-tocopherol mediated by copper−zinc superoxide dismutase in the presence of NO_2^- and H_2O_2 (*83*). Whether or not lipids are nitrated upon exposure to the MPO/H_2O_2/NO_2^- system or are present in vivo has not yet been established.

Lipid peroxidation initiated by NO_2 is distinguished from copper- or iron-dependent oxidation of unsaturated lipids, since it is neither autocatalytic nor enhanced by "seeded" hydroperoxides. In an analogous fashion, lipid oxidation initiated by the MPO/H_2O_2/NO_2^- system was not inhibited by metal chelators such as DTPA or desferroxamine, and demonstrated similar kinetics of lipid oxidation [e.g., as measured by the rate of total 9-H(P)ODE formed] with either native LDL or oxidized LDL as a target (data not shown). These results suggest that like NO_2, the reactive nitrogen species formed by MPO-catalyzed oxidation of NO_2^- is more effective at initiating lipid peroxidation than it is at converting lipid hydroperoxides to alkoxyl or peroxyl radicals. These results are also consistent with the redox potential of the NO_2/NO_2^- pair ($E° = 0.99-1.03$ V; *84*−*87*) which is nearly identical to the redox potential of an alkylperoxyl radical ($E° = 1.00$ V; *85*), as opposed to that of the pentadienyl radical ($E° = 0.60$ V; *85*). Finally, it is interesting to note that initiation of lipid peroxidation by NO_2 would also regenerate NO_2^- (Scheme 1). Thus, if MPO catalyzes the one-electron oxidation of NO_2^- to form NO_2, subsequent interaction of the lipophilic oxidant with polyunsaturated lipids would allow a trace level of the NO metabolite to "cycle" as a substrate for MPO.

Alternatively, Sampson et al. (*44*) recently suggested the possibility that tyrosine nitration mediated by MPO may result from the two-electron oxidation of NO_2^- forming either a protein-bound nitryl cation (NO_2^+)-like intermediate or peroxynitrous acid (ONOOH), which could diffuse away from the peroxidase. By analogy, each of these pathways needs to also be considered as a potential mechanism for initiating lipid oxidation by the MPO/H_2O_2/NO_2^- system. The active site of MPO is sterically hindered, with the heme group buried deep at the base of the substrate binding pocket (*65*). Thus, low-molecular weight intermediates such as HOCl or tyrosyl radical are required to convey oxidizing potential from the heme prosthetic group of MPO to the target for damage. It is therefore not likely that a protein-bound NO_2^+-like intermediate at the active site of MPO will have access to an allylic hydrogen on the fatty acid side chain of membrane phospholipids within lipid vesicles or LDL.

The suggestion that peroxidases make ONOOH as a product of NO_2^- oxidation (*44*) is intriguing. The results presented here do not exclude this possibility. Indeed, MPO adds a hydroxyl group to chloride to form HOCl, suggesting that the enzyme may similarly transfer a hydroxyl group onto the oxygen of NO_2^- forming ONOOH. If ONOOH were formed and diffused away from the active site, protein nitration and initiation of lipid peroxidation would occur, like what is observed. It is interesting to note that under some conditions, the chemical reactivity of ONOOH may be regarded as analogous to that of a caged pair of radicals (i.e., hydroxyl radical and NO_2) (*27*). The overall mechanisms for initiation of lipid peroxidation by ONOOH would be similar to that proposed for NO_2 (Scheme 1), with the first step involving ONOOH-dependent abstraction of a hydrogen atom from a bis-allylic methylene group forming a pentadienyl radical. Taken together, the results of the study presented here suggest that lipid peroxidation mediated by activated neutrophils in the presence of NO_2^- results from the formation of a diffusible oxidant. The chemical nature of the oxidant(s) (NO_2 and/or ONOOH) remains to be established.

Much attention has been focused on the potential role of antioxidants as a therapeutic strategy for preventing free radical-dependent initiation of lipid peroxidation in a variety of disease processes ranging from cancer and cardiovascular disease to the aging process itself (*88*, *89*). The success of these interventions, however, ultimately depends on the ability of the antioxidant to intervene between the oxidant and its target through reactions driven by mass action. Ascorbate significantly inhibited lipid peroxidation mediated by the MPO system of activated neutrophils in medium containing NO_2^- (Figure 2). Lipid peroxidation in human plasma exposed to activated neutrophils is inhibited until ascorbate is depleted (*90*), consistent with a potential role of an aqueous phase oxidant such as an MPO-generated reactive nitrogen intermediate in this system. The results of the study presented here suggest that supplementation with ascorbate should attenuate lipid peroxidation mediated by MPO- and leukocyte-mediated oxidation of NO_2^- in vivo. They also suggest that an alternative rational therapeutic strategy may be the design of selective inhibitors of MPO. Such pharmacological agents would be anticipated to prevent formation of many diffusible oxidants and free radical species at their cellular source.

ACKNOWLEDGMENT

Mass spectrometry experiments were performed at the Cleveland Mass Spectrometry Core Facility.

REFERENCES

1. Chisolm, G. M., and Penn, M. S. (1998) in *Oxidized Lipoproteins and Atherosclerosis* (Fuster, V., Ross, R., and Topol, E. J., Eds.) pp 129−149, Lippincott-Raven Publishers, Philadelphia.

2. Berliner, J. A., Navab, M., Fogelman, A. M., Frank, J. S., Demer, L. L., Edwards, P. A., Watson, A. D., and Lusis, A. J. (1995) *Circulation 91*, 2488−2496.
3. Steinberg, D. (1997) *Circulation 95*, 1062−1071.
4. Liu, T. Z., Stern, A., and Morrow, J. D. (1998) *J. Biomed. Sci. 5*, 415−420.
5. Chisolm, G. M., III, Hazen, S. L., Fox, P. L., and Cathcart, M. K. (1999) *J. Biol. Chem. 274*, 25959−25962.
6. Pratico, D., Iuliano, L., Mauriello, A., Spagnoli, L., Lawson, J. A., Maclouf, J., Violi, F., and Fitzgerald, G. A. (1997) *J. Clin. Invest. 100*, 2028−2034.
7. Mallat, Z., Nakamura, T., Ohan, J., Leseche, G., Tedgui, A., Maclouf, J., and Murphy, R. C. (1999) *J. Clin. Invest. 103*, 421−427.
8. Suarna, C., Dean, R. T., Southwell-Keeley, P. T., Moore, D. E., and Stocker, R. (1997) *Free Radical Res. 27*, 397−408.
9. Watson, A. D., Leitinger, N., Navab, M., Faull, K. F., Horkko, S., Witztum, J. L., Palinski, W., Schwenke, D., Salomon, R. G., Sha, W., Subbanagounder, G., Fogelman, A. M., and Berliner, J. A. (1997) *J. Biol. Chem. 272*, 13597−13607.
10. Morel, D. W., DiCorleto, P. E., and Chisolm, G. M. (1984) *Arteriosclerosis 4*, 357−364.
11. Steinbrecher, U. P., Parthasarathy, S., Leake, D. S., Witztum, J. L., and Steinberg, D. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 3883−3887.
12. Heinecke, J. W., Rosen, H., and Chait, A. (1984) *J. Clin. Invest. 74*, 1890−1894.
13. Cathcart, M. K., Morel, D. W., and Chisolm, G. M., III (1985) *J. Leukocyte Biol. 38*, 341−350.
14. Savenkova, M. L., Mueller, D. M., and Heinecke, J. W. (1994) *J. Biol. Chem. 269*, 20394−20400.
15. Jessup, W., Mohr, D., Gieseg, S. P., Dean, R. T., and Stocker, R. (1992) *Biochim. Biophys. Acta 1180*, 73−82.
16. Hiramatsu, K., Heinecke, J. W., Wolfbauer, G., Chait, A., and Rosen, H. (1987) *Arteriosclerosis 7*, 55−60.
17. Steinbrecher, U. P. (1988) *Biochim. Biophys. Acta 959*, 20−30.
18. Dabbagh, A. J., and Frei, B. (1995) *J. Clin. Invest. 96*, 1958−1966.
19. Mukhopadhyay, C. K., Ehrenwald, E., and Fox, P. L. (1996) *J. Biol. Chem. 271*, 14773−14778.
20. Leeuwenburgh, C., Rasmussen, J. E., Hsu, F. F., Mueller, D. M., Pennathur, S., and Heinecke, J. W. (1997) *J. Biol. Chem. 272*, 3520−3526.
21. Folcik, V. A., Nivar-Aristy, R. A., Krajewski, L. P., and Cathcart, M. K. (1995) *J. Clin. Invest. 96*, 504−510.
22. Kuhn, H., Heydeck, D., Hugou, I., and Gniwotta, C. (1997) *J. Clin. Invest. 99*, 888−893.
23. Moncada, S., and Higgs, A. (1993) *N. Engl. J. Med. 329*, 2002−2012.
24. van der Vliet, A., Eiserich, J. P., Kaur, H., Cross, C. E., and Halliwell, B. (1996) *Methods Enzymol. 269*, 175−184.
25. Lymar, S. V., Jiang, Q., and Hurst, J. K. (1996) *Biochemistry 35*, 7855−7861.
26. Radi, R., Beckman, J. S., Bush, K. M., and Freeman, B. A. (1991) *Arch. Biochem. Biophys. 288*, 481−487.
27. Beckman, J. S., Beckman, T. W., Chen, J., Marshall, P. A., and Freeman, B. A. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 1620−1624.
28. Beckman, J. S., Chen, J., Ischiropoulos, H., and Crow, J. P. (1994) *Methods Enzymol. 233*, 229−240.
29. Murray, H. W., and Nathan, C. F. (1999) *J. Exp. Med. 189*, 741−746.
30. Klebanoff, S. J. (1993) *Free Radical Biol. Med. 14*, 351−360.
31. Beckman, J. S., Ye, Y. Z., Anderson, P. G., Chen, J., Accavitti, M. A., Tarpey, M. M., and White, R. (1993) *Biol. Chem. Hoppe-Seyler 375*, 81−88.
32. Leeuwenburgh, C., Hardy, M. M., Hazen, S. L., Wagner, P., Oh-ishi, S., Steinbrecher, U. P., and Heinecke, J. W. (1997) *J. Biol. Chem. 272*, 1433−1436.
33. Christen, S., Woodall, A. A., Shigenaga, M. K., Southwell-Keely, P. T., Duncan, M. W., and Ames, B. N. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 3217−3222.
34. Hogg, N., Darley-Usmar, V. M., Wilson, M. T., and Moncada, S. (1993) *FEBS Lett. 326*, 199−203.
35. Pfeiffer, S., and Mayer, B. (1998) *J. Biol. Chem. 273*, 27280−27285.
36. Eiserich, J. P., Cross, C. E., Jones, A. D., Halliwell, B., and van der Vleit, A. (1996) *J. Biol. Chem. 271*, 19199−19208.
37. van der Vleit, A., Eiserich, J. P., Halliwell, B., and Cross, C. E. (1997) *J. Biol. Chem. 272*, 7617−7625.
38. Eiserich, J. P., Hristova, M., Cross, C. E., Jones, A. D., Freeman, B. A., and Halliwell, B. (1998) *Nature 391*, 393−397.
39. Klebanoff, S. J., and Clark, R. A. (1978) in *The Neutrophil: Function and Clinical Disorders*, pp 447−451, Elsevier/North Holland Biomedical Press, Amsterdam.
40. Harrison, J. E., and Schultz, J. (1976) *J. Biol. Chem. 251*, 1371−1374.
41. Hazen, S. L., Hsu, F. F., Mueller, D. M., Crowley, J. R., and Heinecke, J. W. (1996) *J. Clin. Invest. 98*, 1283−1289.
42. Heinecke, J. W., Li, W., Francis, G. A., and Goldstein, J. A. (1993) *J. Clin. Invest. 91*, 2866−2872.
43. Jiang, Q., and Hurst, J. K. (1997) *J. Biol. Chem. 272*, 32767−32772.
44. Sampson, J. B., Ye, Y., Rosen, H., and Beckman, J. S. (1998) *Arch. Biochem. Biophys. 356*, 207−213.
45. Podrez, E. A., Schmitt, D., Hoff, H. F., and Hazen, S. L. (1999) *J. Clin. Invest. 103*, 1547−1563.
46. Farrell, A. J., Blake, D. R., Palmer, R. M., and Moncada, S. (1992) *Ann. Rheum. Dis. 51*, 1219−1222.
47. Torre, D., Ferrario, G., Speranza, F., Orani, A., Fiori, G. P., and Zeroli, C. (1996) *J. Clin. Pathol. 49*, 574−576.
48. Gaston, B., Reilly, J., Drazen, J. M., Fackler, J., Famdev, P., Arnelle, D., Mullins, M. E., Sugarbaker, D. J., Chee, C., Singel, D. J., et al. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 10957−10961.
49. Pryor, W. A., and Stone, K. (1993) *Ann. N.Y. Acad. Sci. 686*, 12−28.
50. Pryor, W. A., and Lightsey, J. W. (1981) *Science 214*, 435−437.
51. Huie, R. E. (1994) *Toxicology 89*, 193−216.
52. Thomas, H. V., Mueller, P. K., and Lyman, R. L. (1968) *Science 159*, 532−534.
53. Chisolm, G. M., Ma, G., Irwin, K. C., Martin, L. L., Gunderson, K. G., Linberg, L. F., Morel, D. W., and DiCorleto, P. E. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 11452−11456.
54. Markwell, M. A., Haas, S. M., Bieber, L. L., and Tolbert, N. E. (1978) *Anal. Biochem. 87*, 206−210.
55. Nelson, D. P., and Kiesow, L. A. (1972) *Anal. Biochem. 49*, 474−478.
56. Rakita, R. M., Michel, B. R., and Rosen, H. (1990) *Biochemistry 29*, 1075−1080.
57. van Dalen, C. J., Whitehouse, M. W., Winterbourn, C. C., and Kettle, A. J. (1997) *Biochem. J. 327*, 487−492.
58. Hatch, F. T. (1968) *Adv. Lipid Res. 6*, 1−68.
59. Hazen, S. L., Hsu, F. F., and Heinecke, J. W. (1996) *J. Biol. Chem. 271*, 1861−1867.
60. Thurau, A. M., Schylz, U., Wolf, V., Krug, N., and Schauer, U. (1996) *Cytometry 23*, 150−158.
61. Schmidt, H. H. H. W., and Kelm, M. (1996) in *Determination of nitrite and nitrate by the Griess reaction* (Feelisch, M., and Stamler, J. S., Eds.) pp 491−497, Wiley, New York.
62. Esterbauer, H., Striegl, G., Puhl, H., and Rotheneder, M. (1989) *Free Radical Res. Commun. 6*, 67−75.
63. Ohkawa, H., Ohishi, N., and Yagi, K. (1979) *Anal. Biochem. 95*, 351−358.
64. Heinecke, J. W., Rosen, H., Suzuki, L. A., and Chait, A. (1987) *J. Biol. Chem. 262*, 10098−10103.
65. Kettle, A. J., and Winterbourn, C. C. (1997) *Redox Report 3*, 3−15.
66. Fridovich, I. (1985) in *CRC Handbook of Methods: Oxygen Radical Research* (Greenwald, R. A., Ed.) pp 213−215 CRC Press, Boca Raton, FL.
67. Nelson, S. K., Bose, S. K., and McCord, J. M. (1994) *Free Radical Biol. Med. 16*, 195−200.

68. Heinecke, J. W., Kawamura, M., Suzuki, L., and Chait, A. (1993) *J. Lipid Res. 34*, 2051−2061.
69. Hodis, H. N., Chauhan, A., Hashimoto, S., Crawford, D. W., and Sevanian, A. (1992) *Atherosclerosis 96*, 125−134.
70. Kandutsch, A. A., Chen, H. W., and Heiniger, H. J. (1978) *Science 201*, 498−501.
71. Brown, A. J., and Jessup, W. (1999) *Atherosclerosis 142*, 1−28.
72. Weiss, S. J., Lampert, M. B., and Test, S. T. (1983) *Science 222*, 625−628.
73. Nathan, C. F. (1987) *J. Clin. Invest. 80*, 1550−1560.
74. Parks, N. J., Krohn, K. J., Mathis, C. A., Chasko, J. H., Geiger, K. R., Gregor, M. E., and Peek, N. F. (1981) *Science 212*, 58−60.
75. Chance, B. (1952) *Arch. Biochem. Biophys. 41*, 425−431.
76. Wu, W., Chen, Y., and Hazen, S. L. (1999) *J. Biol. Chem. 274*, 25933−25944.
77. Weiss, S. J. (1989) *N. Engl. J. Med. 320*, 365−376.
78. Daugherty, A., Dunn, J. L., Rateri, D. L., and Heinecke, J. W. (1994) *J. Clin. Invest. 94*, 437−444.
79. Hazen, S. L., and Heinecke, J. W. (1997) *J. Clin. Invest. 99*, 2075−2081.
80. Prutz, W. A., Monig, H., Butler, J., and Land, E. J. (1985) *Arch. Biochem. Biophys. 243*, 125−134.
81. Gallon, A. A., and Pryor, W. A. (1993) *Lipids 28*, 125−133.
82. O'Donnell, V. B., Eiserich, J. P., Chumley, P. H., Jablonsky, M. J., Krishna, N. R., Kirk, M., Barnes, S., Darley-Usmar, V. M., and Freeman, B. A. (1999) *Chem. Res. Toxicol. 12*, 83−92.
83. Singh, R. J., Goss, S. P., Joseph, J., and Kalyanaraman, B. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 12912−12917.
84. Stanbury, D. M. (1989) *Adv. Inorg. Chem. 33*, 69−138.
85. Buettner, G. R. (1993) *Arch. Biochem. Biophys. 300*, 535−543.
86. Jovanovic, S. V., and Simic, M. G. (1986) *J. Phys. Chem. 90*, 974−978.
87. Koppenol, W. H., Moreno, J. J., Pryor, W. A., Ischiropoulos, H., and Beckman, J. S. (1992) *Chem. Res. Toxicol. 5*, 834−842.
88. Diaz, M. N., Frei, B., Vita, J. A., and Keaney, J. F., Jr. (1997) *N. Engl. J. Med. 337*, 408−416.
89. Ames, B. N., Shigenaga, M. K., and Hagen, T. M. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 7915−7922.
90. Frei, B., Stocker, R., and Ames, B. N. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 9748−9752.

BI991623W