

Myeloperoxidase-generated reactive nitrogen species convert LDL into an atherogenic form in vitro

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Oxidized LDL is implicated in atherosclerosis; however, the pathways that convert LDL into an atherogenic form in vivo are not established. Production of reactive nitrogen species may be one important pathway, since LDL recovered from human atherosclerotic aorta is enriched in nitrotyrosine. We now report that reactive nitrogen species generated by the MPO-H₂O₂-NO₂⁻ system of monocytes convert LDL into a form (NO₂-LDL) that is avidly taken up and degraded by macrophages, leading to massive cholesterol deposition and foam cell formation, essential steps in lesion development. Incubation of LDL with isolated MPO, an H₂O₂-generating system, and nitrite (NO₂⁻)—a major end-product of NO metabolism—resulted in nitration of apolipoprotein B 100 tyrosyl residues and initiation of LDL lipid peroxidation. The time course of LDL protein nitration and lipid peroxidation paralleled the acquisition of high-affinity, concentration-dependent, and saturable binding of NO₂-LDL to human monocyte-derived macrophages and mouse peritoneal macrophages. LDL modification and conversion into a high-uptake form occurred in the absence of free metal ions, required NO₂⁻, occurred at physiological levels of Cl⁻, and was inhibited by heme poisons, catalase, and BHT. Macrophage binding of NO₂-LDL was specific and mediated by neither the LDL receptor nor the scavenger receptor class A type I. Exposure of macrophages to NO₂-LDL promoted cholesteryl ester synthesis, intracellular cholesterol and cholesteryl ester accumulation, and foam cell formation. Collectively, these results identify MPO-generated reactive nitrogen species as a physiologically plausible pathway for converting LDL into an atherogenic form.

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Introduction

Oxidative modification of LDL is implicated in atherogenesis (1–5). A key early event is believed to be the conversion of LDL into a form capable of being recognized by “scavenger” or “oxidized LDL” receptors, whose surface expression is not diminished upon exposure to excess cholesterol (1–5). Direct demonstration of the oxidation pathways responsible for rendering LDL atherogenic in vivo, however, has proved difficult because of the evanescent nature of reactive oxidant species and the noninformative products they typically produce (6).

One potential pathway for LDL modification and conversion into a high-uptake form in vivo may involve reactive nitrogen species derived from nitric oxide (nitrogen monoxide, NO), a long-lived free radical that is formed by multiple vascular wall cells (7). NO is relatively unreactive toward most biomolecules and does not promote protein and lipid oxidation at neutral pH. However, a variety of reactive nitrogen species derived from NO are capable of damaging biologic targets (8–14). Antibodies specific for nitrotyrosine, a marker for protein damage by reactive nitrogen species (9, 14, 15), recognize epitopes in human atheroma but not in adjacent normal aortic intima (16). Mass spectrometric studies demonstrated nearly 100-fold-higher levels of nitrotyrosine in LDL recovered from human atherosclerotic aortae compared with LDL isolated from plasma of healthy donors (17).

Thus, multiple lines of evidence suggest that nitrating intermediates promote LDL oxidation in the human artery wall during atherosclerosis; however, the precise nitrating intermediates formed, the pathways responsible for their generation in vivo, and the biologic consequences of LDL nitration are still unknown.

The most well-studied pathway for generating nitrating intermediates from NO involves formation of peroxy-nitrite (ONOO⁻), a product of NO and superoxide (O₂⁻) (12). Bolus addition of reagent ONOO⁻ to biologic targets promotes nitration of protein tyrosyl residues (14), depletes lipid-soluble antioxidants (18, 19), and initiates lipid peroxidation (11, 13). Multiple cellular components of human atherosclerotic lesions produce both O₂⁻ and NO (7), suggesting that ONOO⁻ may contribute to LDL oxidation and nitrotyrosine formation in human atherosclerotic lesions. Recent studies have questioned the significance of ONOO⁻ in mediating protein nitration in vivo, however, because of the inefficient tyrosine nitration observed when this oxidizing agent is formed in situ at physiological pH and low equivalent fluxes of NO and O₂⁻ (20). Moreover, enhanced NO production by macrophages has been shown to have a protective effect on the extent of cell-mediated oxidative damage of LDL (8).

Another potential pathway for generating reactive nitrogen species in vivo has recently been described that involves myeloperoxidase (MPO), an enzyme implicated

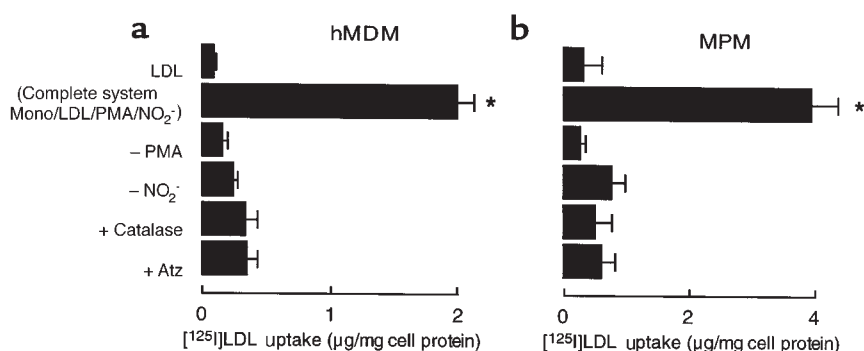


Figure 1

Degradation of [¹²⁵I]LDL by hMDMs and MPMs after modification by activated human monocytes. Isolated human monocytes (10⁶/mL) were incubated at 37°C in HBSS supplemented with DTPA (100 µM), [¹²⁵I]LDL (0.2 mg/mL), and NO₂⁻ (500 µM). Monocytes were activated with phorbol ester (200 nM) and maintained in suspensions by intermittent inversion (complete system) for 8 hours. Additions or deletions to the complete system were as indicated. Reactions were stopped by addition of BHT (40 µM) and catalase (300 nM); the cells were pelleted; and then ¹²⁵I-labeled lipoproteins (5 µg/mL) were incubated with either hMDMs (a) or thioglycollate-elicited MPMs (b) at 37°C for 5 hours in the appropriate media containing additional catalase (300 nM) and BHT (20 µM). Cellular uptake of lipoproteins was subsequently determined as described in Methods. The final concentrations of additions to the complete system were catalase (300 nM) and 3-aminotriazole (1 mM). Data represent the mean ± SD of 3 separate experiments. *P < 0.001 for complete system vs. complete system - NO₂⁻. Atz, 3-aminotriazole; Mono, human peripheral blood monocyte.

in atherosclerosis. MPO is an abundant heme protein that is secreted from activated phagocytes (21). It amplifies the oxidizing potential of H₂O₂ by using it as cosubstrate to generate a variety of cytotoxic oxidants (22–27). Antibodies specific for MPO demonstrate that the enzyme is localized in human atherosclerotic lesions (28). One major and unique oxidant formed by MPO in vivo is hypochlorous acid (HOCl), a potent chlorinating agent generated by the oxidation of chloride (23, 29, 30). Both mass spectrometric and immunohistochemical studies demonstrate that HOCl-generated protein oxidation products are enriched in human atherosclerotic aortic intima and LDL recovered from human atherosclerotic lesions (29, 31). Thus, MPO-generated oxidants are formed in diseased human vascular tissues.

Several recent studies demonstrate that MPO also utilizes H₂O₂ and nitrite (NO₂⁻), the autoxidation product of NO, as cosubstrates to generate a microbicidal oxidant (32) capable of nitrating phenolic compounds and proteins in vitro (25, 26, 33, 34). NO₂⁻ is ubiquitous in biologic tissues and fluids (25). Plasma and extracellular levels of NO₂⁻ are markedly increased in inflammatory and infectious processes in which NO production is enhanced (35–37). Studies with isolated MPO and human neutrophils demonstrate that the enzyme catalyzes aromatic nitration in media containing concentrations of NO₂⁻ approximating those found in biologic fluids, confirming that this major NO metabolite can serve as a physiological substrate for MPO in the presence of plasma levels of chloride (26, 38).

Here, we explore the role of MPO-generated reactive nitrogen species in converting LDL into a novel proatherogenic form. We now report that exposure of LDL to the MPO-H₂O₂-NO₂⁻ system of phagocytes results in concomitant apo B-100 protein nitration, induction of LDL lipid peroxidation, and conversion of the lipoprotein into a form capable of promoting macrophage cholesteryl ester accumulation and foam cell formation, essential steps in the atherosclerotic process.

Methods

Materials. L-[¹³C₆]tyrosine was purchased from Cambridge Isotopes Inc. (Andover, Massachusetts, USA). Tissue culture media and additives were purchased from Life Technologies Inc. (Gaithersburg, Maryland, USA). [¹²⁵I]NaI and [¹⁴C]oleate were supplied by ICN Pharmaceuticals Inc. (Costa Mesa, California, USA). C57BL/6 mice (16–20 weeks of age) were purchased from the Trudeau Institute (Saranac Lake, New York, USA). THP-1 cells were obtained from American Type Culture Collection (Rockville, Maryland, USA). All other reagents were obtained from Sigma Chemical Co. (St. Louis, Missouri, USA) unless otherwise specified.

General procedures. All buffers were passed over a column of Chelex-100 resin (Bio-Rad Laboratories Inc., Hercules, California, USA) and supplemented with diethylenetriamine pentaacetic acid (DTPA) to remove any potential transition metal ions that might catalyze LDL oxidation during incubations. Peroxynitrite was synthesized and quantified as described (14). L-3-[¹³C₆]nitrotyrosine was made from L-[¹³C₆]tyrosine and peroxynitrite and was isolated by reverse-phase HPLC to remove residual NO₂⁻ before use (17). Protein content was determined by the Markwell-modified Lowry protein assay (39), with BSA as standard. Production of H₂O₂ by glucose/glucose oxidase (GGOX) was quantified by oxidation of Fe(II) and formation of an Fe(III)-thiocyanate complex (25). The concentration of reagent H₂O₂ was determined spectrophotometrically (ε₂₄₀ = 39.4 M⁻¹/cm⁻¹; ref. 40). LDL was acetylated with acetic acid anhydride (41). Acetylated lipoproteins were dialyzed against 0.15 M NaCl, 0.3 mM Na₂EDTA, and 100 µM DTPA (pH 7.4). LDL labeling with [¹²⁵I]NaI and agarose gel electrophoresis were performed as described previously (42). The specific activity of labeled LDL preparations was between 100 and 250 dpm/ng protein. Cholesterol and cholesteryl ester content, and cholesteryl [¹⁴C]oleate synthesis by cells incubated with the indicated lipoproteins (50 µg/mL), were determined by published methods (41). α-tocopherol levels were determined as described (43). LDL concentrations are expressed as milligrams of protein per milliliter.

MPO and lipoprotein isolation. MPO (donor: hydrogen peroxide, oxidoreductase, EC 1.11.1.7) was initially purified from detergent extracts of human leukocytes by sequential lectin affinity and gel filtration chromatography as described by Rakita et al. (44). Trace levels of contaminating eosinophil peroxidase were

then removed by passage over a sulfopropyl Sephadex column (45). Purity of isolated MPO was established by demonstrating an *RZ* of 0.87 (A_{430}/A_{280}), SDS-PAGE analysis with Coomassie blue staining, and in-gel tetramethylbenzidine peroxidase staining (46). Enzyme concentration was determined spectrophotometrically utilizing an extinction coefficient of 89,000 M⁻¹/cm⁻¹/heme of MPO (22). The concentration of the MPO dimer was calculated as half the indicated concentration of heme-like chromophore. LDL was isolated from fresh plasma by sequential ultracentrifugation as a 1.019 < *d* < 1.063 g/mL fraction (47). Final preparations were placed in 50 mM sodium phosphate (pH 7.0), 200 μM DTPA (by passage over 10-DG desalting columns; Bio-Rad Laboratories Inc.) and stored under N₂ until use.

Lipoprotein oxidation. NO₂-LDL was prepared by incubating LDL (0.2 mg protein/mL) at 37°C in 50 mM sodium phosphate (pH 7.0), 100 μM DTPA in the presence of 30 nM MPO, 100 μg/mL glucose, 20 ng/mL glucose oxidase (grade II; Boehringer Mannheim Biochemicals, Indianapolis, Indiana, USA), and 0.5 mM NaNO₂ for 8 hours unless otherwise specified. Preliminary studies demonstrated that under these conditions, a constant flux of H₂O₂ (0.18 μM/min) is generated by the GGOx system. Unless otherwise stated, oxidation reactions were terminated by addition of 40 μM butylated hydroxytoluene (BHT; from a 100 mM ethanolic stock) and 300 nM catalase to the reaction mixture. Preliminary studies demonstrated that monocytes isolated by methods that use adhesion/washing steps contained less MPO per cell (presumably because of some premature degranulation during the isolation procedure). Human peripheral blood monocytes used for LDL oxidation were therefore isolated by elutriation (48). Monocytes (1 × 10⁶/mL) were incubated with LDL (0.2 mg/mL) in HBSS (magnesium-, calcium-, phenol-, and bicarbonate-free; pH 7.20) supplemented with DTPA (100 μM) for 8 hours at 37°C in the presence of the additions or deletions indicated. Reactions were terminated by addition of BHT (20 μM) and pelleting of cells. Oxidation of LDL (0.2 mg/mL) by copper was performed by dialysis vs. 5 μM CuSO₄ in PBS for 24 hours. Oxidation was terminated by addition of BHT (40 μM) and dialysis against PBS containing DTPA (100 μM). Aggregated LDL for cytochalasin D experiments was produced by vortexing solutions of LDL (500 μg/mL in 0.15 M NaCl, 0.3 mM Na₂EDTA, 40 μM BHT).

Analysis of lipid peroxidation products. Lipids were extracted by a modified Dole procedure as described by Savenkova et al. (49). Lipids were dried under nitrogen and resuspended in 1% sodium dodecylsulfate, and total lipid hydroperoxides were then measured as described (50). Total (free and esterified) 9-hydroxy-10,12-octadecadienoic acid + 9-hydroperoxy-10,12-octadecadienoic acid [9-H(P)ODE] formed was determined by reverse-phase HPLC of triphenylphosphine-reduced lipid extracts after base hydrolysis as described previously (49). Authentic (*E,Z*)-9-hydroxy-10,12-octadecadienoic acid (9-HODE; Cayman Chemical Co., Ann Arbor, Michigan, USA) was used as standard. Cholesteryl-9-hydroxy-10,12-octadecadienoate + cholesteryl-9-hydroperoxy-10,12-octadecadienoate [9-H(P)ODE] was quantified by reverse-phase HPLC of triphenylphosphine-reduced lipid extracts (not subjected to base hydrolysis) as described (49). (*E,Z*)-cholest-5-en-ol(3β)-9-hydroxy-10,12-octadecadienoate (cholesteryl-9-HODE; Cayman Chemical Co.) was used as standard. The content of thiobarbituric acid (TBA) reactive products in LDL preparations was determined by fluorescence analysis (51) in the presence of 0.05% (wt/vol) BHT. Fluorescence measurements (2-nm slit width, excitation 515 nm, emission 553 nm) were made on an LS-3 Fluorescence Spectrometer (Perkin-Elmer Corp., Norwalk, Connecticut, USA) 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). Total lipid hydroperoxides, total 9-H(P)ODE, cholesteryl-9-H(P)ODE, and TBA reactive products are expressed as nanomole of oxidation product per milligram of LDL protein.

Analysis of LDL protein oxidation products and amino acids. Nitrotyrosine content was determined by stable isotope dilution gas chromatography-mass spectrometry (GC/MS) on a Turbomass Spectrometer (Perkin-Elmer Corp.) equipped with chemical ionization probe. Briefly, reactions were terminated by addition of BHT and catalase as already described here; lipids and salts were removed by extraction with a single-phase solvent mixture of H₂O/water-washed diethyl ether/methanol (1:3:7, vol/vol/vol) (52); and the nitrotyrosine content in amino acid hydrolysates was determined after reduction to amino tyrosine as an *n*-propyl, per heptafluorobutyryl derivative (53). Nitrotyrosine content is normalized to the content of tyrosine determined by stable isotope dilution GC/MS (24).

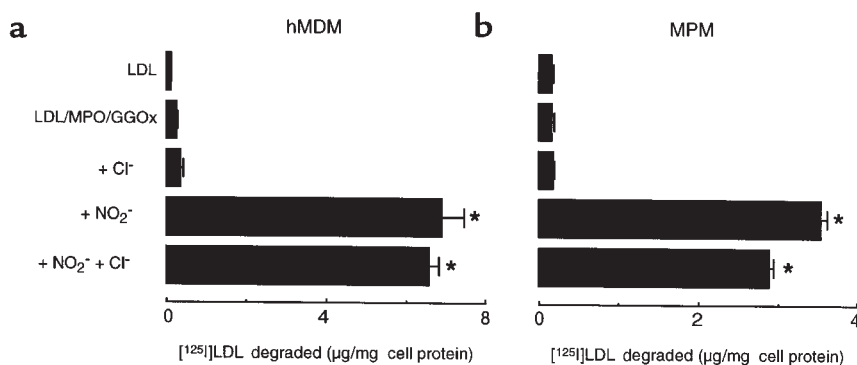


Figure 2

Degradation of [¹²⁵I]LDL by hMDMs and MPMs after modification by MPO-generated chlorinating and nitrating intermediates. [¹²⁵I]LDL (0.2 mg/mL) was incubated with isolated human MPO (30 nM), glucose (100 μM), and glucose oxidase (20 ng/mL) in the presence of the indicated additions in sodium phosphate buffer (50 mM, pH 7.0) supplemented with DTPA (100 μM) overnight at 37°C, as described in Methods. Under these conditions, a constant flux of H₂O₂ (0.18 μM/min) is generated by the GGOx system. Reactions were stopped by addition of BHT (40 μM) and catalase (300 nM), and then [¹²⁵I]-labeled lipoproteins (5 μg/mL) were incubated with either hMDMs (a) or thioglycollate-elicited MPMs (b) at 37°C for 5 hours in the appropriate media containing additional catalase (300 nM) and BHT (20 μM). Cellular uptake of lipoproteins was subsequently determined as described in Methods. When indicated, Cl⁻ (100 mM) or NO₂⁻ (500 μM) were added during LDL modification by MPO. Data represent the mean ± SD of triplicate determinations. Similar results were observed in 3 independent experiments. **P* < 0.001 for comparison vs. LDL modified in the presence of MPO and an H₂O₂-generating system (LDL/MPO/GGOx).

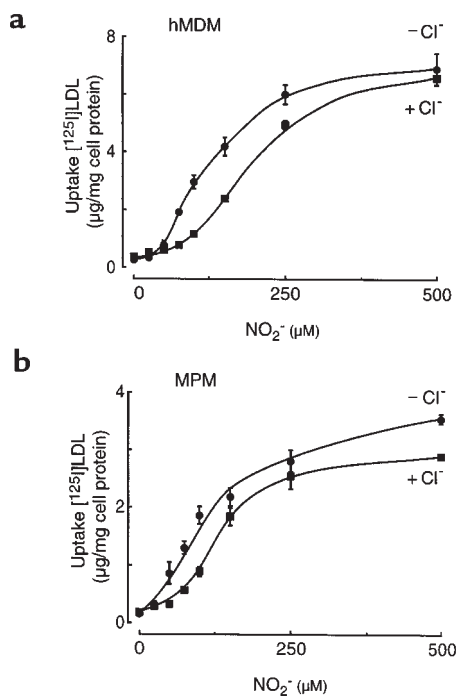


Figure 3 NO_2^- concentration dependence of MPO-mediated modification of LDL and conversion into a high-uptake form for macrophages. [^{125}I]LDL (0.2 mg/mL) was incubated with isolated human MPO (30 nM), glucose (100 μM), glucose oxidase (20 ng/mL), and the indicated concentrations of NO_2^- , in the absence (filled circles) and presence (filled squares) of Cl^- (100 mM) in sodium phosphate buffer (50 mM, pH 7.0) supplemented with DTPA (100 μM) overnight at 37°C, as described in Methods. Uptake of the modified lipoproteins was then assessed using hMDMs (**a**) and thioglycollate-elicited MPMs (**b**) as described in Methods. Data represent the mean \pm SD of triplicate determinations. Similar results were observed in 3 independent experiments.

Samples from time-course experiments quantifying remaining lysine and tryptophan residues were quenched with addition of 200 μM BHT and immediately analyzed. Unmodified lysine residues in LDL were quantified by fluorescamine fluorescence (54). N^α -acetyl lysine was used as standard to construct an external calibration curve. The fluorescence of treated samples was expressed relative to that of native LDL, assuming that the lipoprotein contains 341 lysine residues per particle, the number of lysines present in mature apo B-100 (55). Tryptophan fluorescence of LDL was measured using excitation and emission wavelengths of 280 nm and 335 nm, respectively (54). A linear relationship between loss of fluorescence and number of tryptophan residues oxidized was assumed. Results are expressed relative to those of native LDL; it was assumed that apo B-100 of native LDL contains 37 tryptophan residues (55).

Cells. Elicited mouse peritoneal macrophages (MPMs) were harvested by peritoneal lavage with ice-cold PBS 2–3 days after thioglycollate stimulation of female C57BL/6 mice (42). Resident MPMs were similarly harvested from mice not subjected to thioglycollate stimulation. Primary cultures were prepared at a density of 10^6 cells per 16-mm-diameter well in RPMI-1640 containing 10% FBS and were used 48 hours after plating. Chinese hamster ovary (CHO) cells expressing mouse scavenger receptor class A type I (CHO-mSR-AI) and control vector-transfected parental CHO cells were a generous gift of M. Krieger (Massachusetts Institute of Technology, Boston, Massachusetts, USA) and were cultured as described elsewhere (56). Experiments were

performed on confluent cell monolayers in Ham's F-12 medium containing 3% lipoprotein-deficient FCS, BHT (20 μM), DTPA (100 μM), and catalase (300 nM). THP-1 cells were plated at a density of 0.5×10^6 cells per well and incubated with 64 nM PMA in RPMI-1640 with 10% FBS and 50 μM β -mercaptoethanol for 3 days before use. hMDMs were prepared from isolated human peripheral blood monocytes cultured 10–14 days in macrophage serum-free media (Gibco BRL, Grand Island, New York, USA) supplemented with GM-CSF (20 ng/mL) (48). Experiments with hMDMs were performed on confluent cell monolayers in the same medium containing 200 $\mu\text{g}/\text{mL}$ LDL (to block LDL receptor-mediated recognition of LDL preparations), BHT (20 μM), DTPA (100 μM), and catalase (300 nM).

Lipoprotein uptake, degradation, and binding by cultured cells. BHT (20 μM), catalase (300 nM), and DTPA (100 μM) were included in the media of all uptake, degradation, and binding experiments to inhibit lipoprotein oxidation during incubation with cells. Cells were washed with serum-free medium, and the indicated amounts of ^{125}I -labeled native or modified LDL were added in 250 μL of the appropriate media. After a 5-hour incubation at 37°C, lipoprotein degradation was assessed by removal of media and quantifying TCA-soluble, noniodide degradation products (41, 42). Cells were also washed and assayed for cell-associated label and protein content (41, 42). Cell-free control experiments were performed in parallel and subtracted from counts in cell-containing reactions to calculate cell-dependent lipoprotein degradation (cell-free controls were <10% of the observed TCA-soluble counts in cell-containing reactions). Total uptake was calculated from the sum of degradation and cell-associated label. In competition experiments, ^{125}I -labeled lipoprotein (5 $\mu\text{g}/\text{mL}$) was incubated with cells in the presence of 200 $\mu\text{g}/\text{mL}$ of unlabeled competitor. Lipoprotein binding to macrophages at 4°C was determined after 4-hour incubations of the indicated amounts of modified forms of [^{125}I]LDL, as described (41).

Size exclusion chromatography. [^{125}I]LDL (2 mg in 0.5 mL of 50 mM sodium phosphate buffer [pH 7.0] containing 200 μM DTPA, 5 mg/mL BSA) was applied to a Sephacryl S400-HR column (1.0 \times 45 cm) pre-equilibrated in 50 mM sodium phosphate buffer (pH 7.0) containing 200 μM DTPA. Lipoproteins were eluted at a flow rate 10 mL/h, and radioactivity in 1.1-mL fractions was determined.

Statistics. Data represent the mean \pm SD of the indicated number of samples. Statistical analyses were made using a paired Student's *t* test. For all hypotheses, the significance level was 0.05. When multiple comparisons were made, a Bonferroni correction to the significance criterion for each test was made.

Results

Human monocytes use the MPO- H_2O_2 - NO_2^- system to convert LDL into a high-uptake form. We hypothesized that phagocytes might use the MPO- H_2O_2 - NO_2^- system to generate a nitrating intermediate that can oxidatively modify LDL, rendering it a ligand for macrophage recognition. To test this hypothesis, we incubated LDL with activated human monocytes in the absence and presence of NO_2^- and then determined whether the lipoprotein was converted into a high-uptake form for macrophages. LDL modified by activated human monocytes in the absence of NO_2^- demonstrated only a modest increase in the extent of LDL uptake by macrophages (Figure 1). In contrast, human monocytes activated in the presence of NO_2^- converted the lipoprotein into a form that was readily taken up and degraded by both hMDMs (Figure 1a) and thioglycollate-elicited MPMs (Figure 1b). Conversion of LDL into a high-uptake form by monocytes required cell activation and was inhibited by either the H_2O_2 scavenger

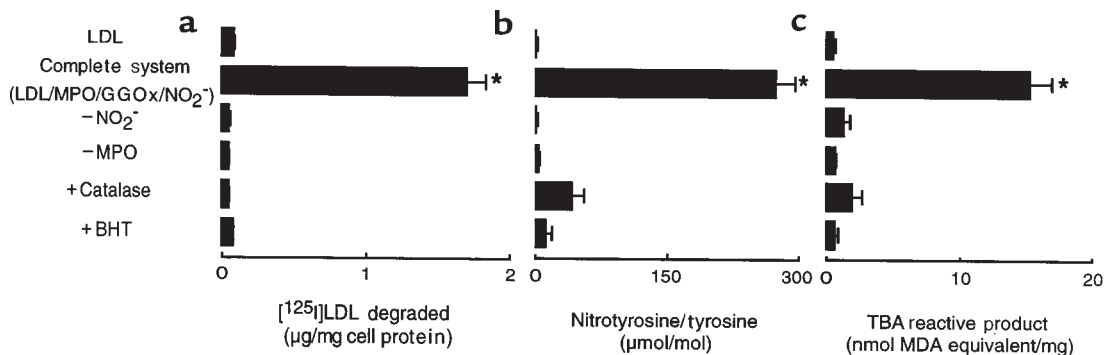


Figure 4

LDL protein nitration, lipid peroxidation, and conversion into a high-uptake form for macrophages mediated by the MPO-H₂O₂-NO₂⁻ system. [¹²⁵I]LDL (0.2 mg/mL) was incubated with isolated human MPO (30 nM), glucose (100 µM), glucose oxidase (20 ng/mL), and NO₂⁻ (500 µM) in sodium phosphate buffer (50 mM, pH 7.0) supplemented with DTPA (100 µM) (complete system) or the indicated additions or deletions. The degradation of lipoproteins by thioglycollate-elicited MPMs (a), the content of nitrotyrosine (b), and TBA reactive products (c) generated were then determined as described in Methods. Data represent the mean ± SD for 3 independent experiments. *P < 0.001 for complete system vs. complete system - NO₂⁻.

catalase or peroxidase inhibitors, e.g., 3-aminotriazole). These results suggest that monocytes may use the MPO-H₂O₂-NO₂⁻ system for oxidative conversion of LDL into an atherogenic form. They also suggest that the MPO-H₂O₂ system of monocytes may use NO₂⁻ as substrate even in the presence of plasma levels of Cl⁻. Finally, macrophage recognition of monocyte-modified LDL appears to involve a mechanism(s) distinct from the LDL receptor, as excess native LDL that was included in the media during exposure of hMDMs to monocyte-modified [¹²⁵I]LDL preparations (see Methods) did not inhibit its uptake of monocyte-modified LDL.

LDL oxidized by MPO, H₂O₂, and physiological levels of NO₂⁻ is converted into a form (NO₂-LDL) that is avidly taken up and degraded by macrophages. Activated monocytes can generate a host of reactive oxidant species, which could have contributed to LDL modification and conversion into a high-uptake form. For example, direct exposure of LDL to additions of either HOCl (54, 57) or HOCl in the presence of NO₂⁻ (58) has been reported to result in lipoprotein aggregation and conversion into a high-uptake form for macrophages. Thus, a role for HOCl production by

the MPO-H₂O₂ system of the cells could not be excluded as a contributor to the cell-dependent conversion of LDL into a form with enhanced macrophage recognition. To explore more fully the mechanism(s) through which the MPO-H₂O₂ system of monocytes might render LDL atherogenic, we used model systems to evaluate the ability of MPO-generated chlorinating and nitrating intermediates to convert LDL into a form that is recognized by macrophages. [¹²⁵I]LDL was incubated with MPO isolated from human leukocytes, an H₂O₂-generating system (GGOx) that produced a low flux of peroxide similar to that which might occur in vivo, and substrate (either Cl⁻ or NO₂⁻, or both). The ability of the modified lipoprotein to be taken up and degraded by either hMDMs or thioglycollate-elicited MPMs was then determined. Incubation of LDL in the presence of an HOCl-generating system (MPO, GGOx, and Cl⁻) failed to convert the lipoprotein into a stable high-uptake form (Figure 2). In contrast, exposure of LDL to MPO and the H₂O₂-generating system, but with NO₂⁻ added, transformed the particle into a form readily taken up and degraded by macrophages (Figure 2). Moreover, addition of plasma

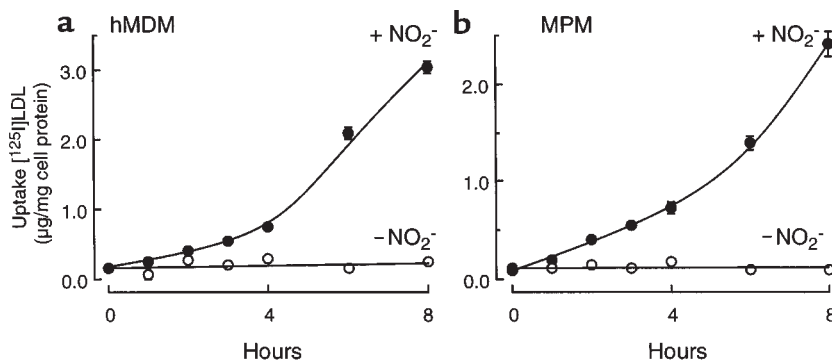


Figure 5

Time course of LDL conversion into a high-uptake form after exposure to the MPO-H₂O₂-NO₂⁻ system. [¹²⁵I]LDL (0.2 mg/mL) was incubated with isolated human MPO (30 nM), glucose (100 µM), and glucose oxidase (20 ng/mL) in sodium phosphate buffer (50 mM, pH 7.0) supplemented with DTPA (100 µM) for the indicated times in either the presence (+ NO₂⁻; filled circles) or absence (- NO₂⁻; open circles) of NO₂⁻ (500 µM) as described in Methods. Uptake of the modified lipoprotein by hMDMs (a) and thioglycollate-elicited MPMs (b) was then determined as described in Methods. Data represent the mean ± SD for triplicate determinations. Similar results were observed in 3 independent experiments.

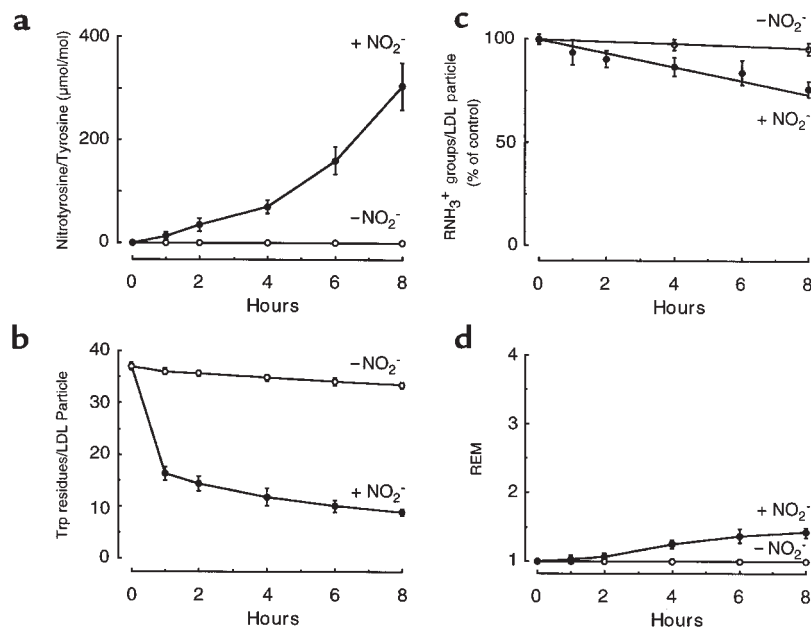


Figure 6

Characterization of multiple protein components of LDL exposed to the MPO- H_2O_2 - NO_2^- system. LDL samples prepared for Figure 5 were analyzed for nitrotyrosine content (a) tryptophan fluorescence (b), unmodified N^ϵ -lysine groups (c), and lipoprotein REM (d), as described in Methods. Under the conditions used, the REM for exhaustively acetylated LDL is 3.8. Data represent the mean \pm SD of 3 independent experiments (1 using ^{125}I -labeled LDL as starting material, and 2 using nonlabeled LDL as starting material). + NO_2^- (filled circles), LDL modified by MPO, an H_2O_2 -generating system (GGOx), and NO_2^- as described in Figure 5; - NO_2^- (open circles), LDL modified by MPO and an H_2O_2 -generating system (GGOx) as described in Figure 5.

levels of Cl^- to NO_2^- -containing reaction mixtures only modestly inhibited conversion of LDL into a high-uptake form. These results suggest that under conditions in which MPO-generated oxidants are formed by a physiological flux of H_2O_2 , the reactive nitrogen species formed by the MPO- H_2O_2 - NO_2^- system (presumably NO_2 ; ref. 25) is more effective than either HOCl (product of the MPO- H_2O_2 - Cl^- system) or NO_2Cl (the presumed nitrating intermediate formed by the reaction of NO_2^- and HOCl; ref. 33) in converting LDL into a stable high-uptake form for macrophages.

The concentration of NO_2^- in aortic intima is unknown. Plasma levels of NO_2^- are normally 1–5 μM but can increase more than 10-fold during inflammation. Because the major pathway for NO_2^- removal is through reaction with oxyhemoglobin-forming nitrate (NO_3^-), levels of NO_2^- in inflamed extracellular fluids and tissues are likely higher (25, 35–37). We therefore evaluated the NO_2^- concentration dependence of LDL modification and conversion into a high-uptake form by MPO in the absence and presence of plasma levels (100 mM) of Cl^- . Incubation of LDL with MPO and an H_2O_2 -generating system in the presence of increasing concentrations of NO_2^- resulted in progressively enhanced association, uptake, and degradation of the modified lipoprotein by all macrophages examined, i.e., both resident and elicited MPMs, THP-1 cells (a human acute monocytic leukemia cell line), and hMDMs. Data for lipoprotein uptake after modification by the MPO- H_2O_2 - NO_2^- system in the absence and presence of Cl^- by hMDMs (Figure 3a) and thioglycollate-elicited MPMs (Figure 3b) are shown. The presence of plasma levels of Cl^- (100 mM) during MPO-dependent modification of

the lipoprotein only modestly inhibited the ability of the peroxidase to utilize NO_2^- as substrate and convert the lipoprotein into a high-uptake form. Half maximal uptake by macrophages was observed at $\sim 100 \mu\text{M}$ NO_2^- for both hMDMs and MPMs (Figure 3), and significant increases in cell recognition were noticeable even when LDL was modified by MPO in the presence of 25 μM NO_2^- , the lowest concentration examined ($P < 0.05$ when comparing LDL modified with 0 vs. 25 μM NO_2^- in both the absence and presence of Cl^- for both cell types). Collectively, these results indicate that concentrations of NO_2^- comparable to those found in inflammatory tissues and fluids are sufficient for MPO-dependent conversion of LDL into a high-uptake form. They also demonstrate that HOCl formation is not obligatory for conversion of LDL into a high-uptake form and that MPO (either isolated or derived from activated monocytes) utilizes NO_2^- as substrate to modify LDL into a form that binds and is taken up and degraded by macrophages, even in the presence of plasma levels of Cl^- . Because MPO-generated reactive chlorinating species can modify protein and lipid components of lipoproteins (29, 31, 52) but are not required for conversion of LDL into a stable high-uptake form by the MPO- H_2O_2 - NO_2^- system of monocytes, subsequent studies characterizing the biochemical and biologic properties of LDL modified by MPO-generated nitrating intermediates were performed under Cl^- -free conditions.

The MPO- H_2O_2 - NO_2^- system promotes LDL protein nitration and induces LDL lipid peroxidation. The nitrating intermediate formed by the MPO- H_2O_2 - NO_2^- system is known to promote nitration of aromatic compounds and soluble proteins (25, 26); however, the products formed by

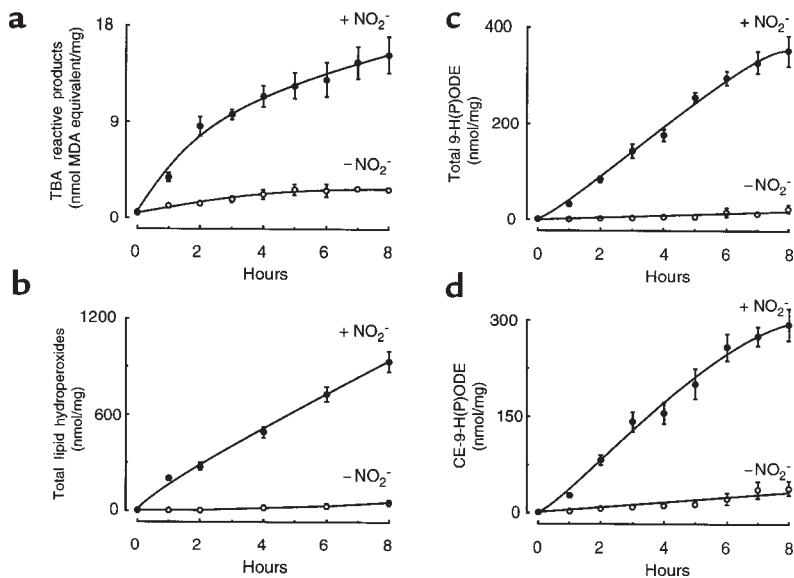


Figure 7

Quantification of multiple lipid oxidation products formed during LDL modification by the MPO-H₂O₂-NO₂⁻ system. LDL samples prepared for Figure 5 were analyzed for TBA reactive products (a), total lipid hydroperoxides (b), total 9-H(P)ODE (c), and cholesteryl-9-H(P)ODE (d) as described in Methods. Data represent the mean ± SD of 3 independent experiments (1 using [¹²⁵I]LDL as starting material and 2 using nonlabeled LDL as starting material). + NO₂⁻ (filled circles), LDL modified by MPO, an H₂O₂-generating system (GGOx), and NO₂⁻ as described in Figure 5; - NO₂⁻ (open circles), LDL modified by MPO and an H₂O₂-generating system (GGOx) as described in Figure 5.

this reactive intermediate after incubation with lipids and lipoproteins have not yet been reported. This is of particular interest because nitrogen dioxide (NO₂), the one electron oxidation product of NO₂⁻, has been suggested to be the reactive nitrogen species formed during peroxidase-catalyzed oxidation of NO₂⁻ (25). NO₂ is known to initiate lipid peroxidation both in vitro and after inhalation (59, 60); thus, exposure of LDL to the MPO-H₂O₂-NO₂⁻ system might also represent a mechanism to promote lipid peroxidation. To test this hypothesis, we determined whether markers of protein and lipid oxidation, such as LDL nitrotyrosine content and TBA reactive products, were increased in LDL preparations after exposure to MPO, NO₂⁻, and an H₂O₂-generating system, using conditions that convert the lipoprotein into a high-uptake form for macrophages. Incubation of LDL with MPO-generated reactive nitrogen species resulted in concomitant conversion of the lipoprotein into a high-uptake form for elicited MPM, nitration of apo B-100 tyrosine residues, and induction of lipid peroxidation (Figure 4). Similar results (enhanced macrophage uptake) were also obtained using cultured hMDMs, resident MPMs, or THP-1 cells (data not shown). Acquisition of macrophage recognition, LDL protein nitration, and lipid peroxidation all demonstrated absolute requirements for MPO and NO₂⁻, were inhibited by catalase and BHT, and occurred in the absence of metal ions (Figure 4). These results are consistent with a role for MPO, H₂O₂, and NO₂⁻ in producing a free radical intermediate that promotes nitration of apo B-100 tyrosyl residues, initiation of lipid peroxidation, and acquisition of macrophage recognition.

Multiple protein and lipid components of LDL are modified by the MPO-H₂O₂-NO₂⁻ system during the acquisition of macrophage recognition. In the next set of experiments, we studied the time course of LDL conversion into a high-uptake form after exposure to MPO, NO₂⁻, and a low steady flux of H₂O₂ (Figure 5). In parallel, we also characterized the extent of lipoprotein modification by a variety of independent indices of protein oxidative dam-

age (Figure 6) and lipid peroxidation (Figure 7). Statistically significant ($P < 0.01$) increases in LDL uptake and degradation were observed with hMDMs and MPMs after a brief (2 hour) incubation of the lipoprotein with the complete MPO-H₂O₂-NO₂⁻ system (Figure 5). Cell recognition of the modified lipoprotein progressively increased in a nonlinear manner without indications of plateau after up to 8 hours of incubation. In stark contrast, no increase in macrophage uptake or degradation was observed in LDL incubated with MPO and H₂O₂ in the absence of NO₂⁻ over the entire time interval examined (Figure 5).

To characterize LDL preparations after exposure to MPO-generated reactive nitrogen species, we first examined several markers of protein modification during the same time interval over which macrophage recognition of the nitrated lipoprotein occurred (Figure 5). Nitrotyrosine was not observed during GC/MS analysis of native LDL; however, after brief exposure to the MPO-H₂O₂-NO₂⁻ system, the nitrated amino acid was detected (Figure 6a). The nitrotyrosine content of NO₂-LDL rose progressively with longer periods of incubation. The curvilinear nature of the plot suggests that alternative targets on LDL may be preferentially oxidized initially by the MPO-generated nitrating intermediate. One such protein target appears to be tryptophan residues, as incubation of LDL with the MPO-H₂O₂-NO₂⁻ system resulted in their rapid initial loss (Figure 6b). It has been demonstrated that marked increases in electrophoretic mobility and loss of N^ε-amino groups of lysine are required for macrophage recognition of other modified forms of LDL, such as acetylated LDL, copper-oxidized LDL, and MDA-modified LDL (61, 62). However, after exposure to the MPO-H₂O₂-NO₂⁻ system, the content of free amine groups on LDL apo B-100 demonstrated only gradual progressive decreases with time (Figure 6c). After 2 hours of modification in the presence of NO₂⁻, <10% of lysine residues were modified (Figure 6c), yet statistically significant increases in LDL uptake and degradation were detected (Figure 5) ($P < 0.01$ and $P < 0.001$ for

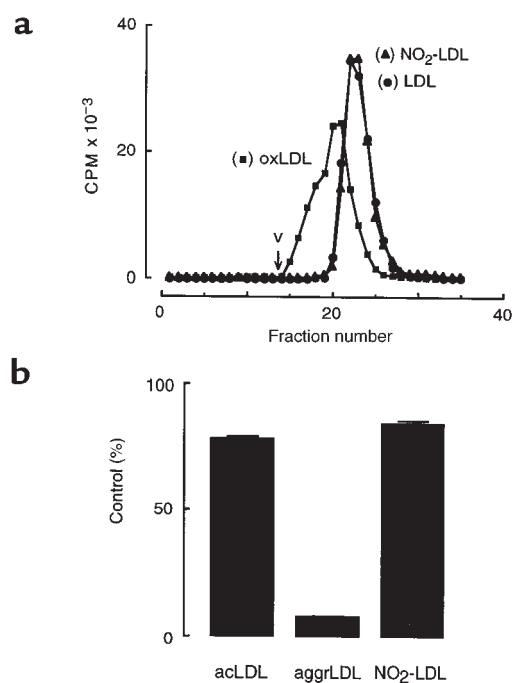


Figure 8

(a) Size exclusion chromatography. (b) The effect of cytochalasin D treatment on macrophage degradation of modified forms of LDL. (a) Native [¹²⁵I]LDL (LDL; filled circles), copper oxidized [¹²⁵I]LDL (oxLDL; filled squares), and [¹²⁵I]LDL modified by the complete MPO-H₂O₂-NO₂⁻ system (NO₂-LDL; filled triangles) were prepared and individually fractionated on a Sephacryl S400-HR column as described in Methods. The elution profiles of native and modified lipoproteins are shown. The void volume (v) of the column is indicated. (b) Acetylated [¹²⁵I]LDL (acLDL), vortex-aggregated [¹²⁵I]LDL (aggrLDL), and [¹²⁵I]LDL modified by the complete MPO-H₂O₂-NO₂⁻ system (NO₂-LDL) were prepared as described in Methods. The [¹²⁵I]-labeled lipoprotein preparations were then individually incubated (5 μg/mL) with thioglycollate-elicited MPMs at 37°C for 5 hours in the presence or absence of cytochalasin D (1 μg/mL) in media supplemented with catalase (300 nM) and BHT (20 μM). Cellular degradation of lipoprotein was subsequently determined as described in Methods. Results are expressed as the percentage of lipoprotein degradation observed in the presence vs. absence of cytochalasin D treatment. Lipoprotein degradation by non-cytochalasin D-treated macrophages (control) exposed to acLDL, aggrLDL, and NO₂-LDL preparations was 3.69 ± 0.14, 1.78 ± 0.03, and 1.49 ± 0.08 μg LDL per milligram of cell protein, respectively. Data represent the mean ± SD of triplicate determinations from a representative experiment performed in duplicate.

0 vs. 2 hours for hMDMs and MPMs, respectively). Consistent with these findings, only a modest progressive increase in the relative electrophoretic mobility (REM) of LDL was observed (Figure 6d), suggesting that only a slight increase in anionic charge of LDL occurred, despite significant macrophage recognition.

Exposure of LDL to MPO, NO₂⁻, and an H₂O₂-generating system resulted in rapid initiation of LDL lipid peroxidation (Figure 7). Even at the earliest time points examined, multiple lipid peroxidation products [TBA reactive products, total lipid hydroperoxides, total 9-H(P)ODE]—including those of core lipids [cholesteryl-9-H(P)ODE]—were formed, suggesting that the oxidant generated by the MPO-H₂O₂-NO₂⁻ system readily promotes hydrogen atom abstraction in unsaturated lipids (Figure 7). As with

the protein markers of oxidation, production of lipid oxidation products was minimal in the absence of NO₂⁻. In a separate set of experiments, we examined α-tocopherol levels vs. time in LDL preparations exposed to the MPO-H₂O₂-NO₂⁻ system, as depicted in Figures 5–7. At 2 hours and 8 hours, 68 ± 6.3% and 93 ± 6.4%, respectively, was consumed (mean ± SD; n = 3 independent experiments). Thus, modification of LDL by the MPO-H₂O₂-NO₂⁻ system is accompanied by oxidation of multiple protein and lipid components of the lipoprotein and by significant depletion of lipid-soluble antioxidant species.

LDL modified by the MPO-H₂O₂-NO₂⁻ system is not aggregated. Aggregation of LDL represents one mechanism for conversion of the lipoprotein into a high-uptake form (63, 64). A number of modifications, including exposure to reagent HOCl and other types of oxidation, result in LDL aggregation (54, 57, 65, 66). Preliminary studies suggested that NO₂-LDL was not aggregated, because only negligible amounts (<0.1%) of the lipoprotein failed to enter 0.5% agarose gels during electrophoresis and because no significant light scattering in NO₂-LDL preparations was observed. To exclude more fully the possibility that small LDL aggregates formed during modification by the MPO-H₂O₂-NO₂⁻ system might contribute to the observed enhanced uptake and degradation by macrophages, additional experiments were performed. No evidence of particle aggregation in NO₂-LDL preparations was observed during analysis by size exclusion chromatography (Figure 8a). The elution profile of NO₂-LDL was identical to that of native LDL, whereas copper-oxidized LDL clearly demonstrated formation of particles of increased size (Figure 8a). We also examined the effect of cytochalasin D, an inhibitor of microfilament formation, on uptake and degradation of modified forms of lipoprotein by MPMs, as cytochalasin D has been effectively used as an inhibitor of phagocytosis of aggregated forms of LDL (66). Macrophage degradation of NO₂-LDL was only modestly affected by cytochalasin D treatment (Figure 8b). Comparable results were seen with acetylated LDL, a prototypic ligand for scavenger receptor-mediated endocytosis (41). In contrast, degradation of vortex-aggregated LDL, which is internalized through a phagocytic mechanism (63), was inhibited by >90% by cytochalasin D treatment (Figure 8b). Collectively, these results suggest that oxidation of LDL by the MPO-H₂O₂-NO₂⁻ system does not promote aggregation of the lipoprotein; moreover, they also suggest that the observed enhanced uptake of NO₂-LDL is not the result of phagocytosis of the modified lipoprotein.

Macrophage binding of LDL modified by the MPO-H₂O₂-NO₂⁻ system is saturable and specific. The lack of lipoprotein aggregation after modification by the MPO-H₂O₂-NO₂⁻ system, and the inability of cytochalasin D to significantly inhibit macrophage uptake and degradation of NO₂-LDL, suggested that cellular recognition was likely mediated through a receptor-mediated process. To test this hypothesis, we examined whether [¹²⁵I]NO₂-LDL binding and degradation by MPMs were saturable and specific. Macrophage binding of LDL that was nitrated by the complete MPO-H₂O₂-NO₂⁻ system was saturable (Figure 9a). Under the conditions used, the half-maximal concentration for [¹²⁵I]NO₂-LDL binding to macrophages was ~10 μg/mL (20 nM lipoprotein),

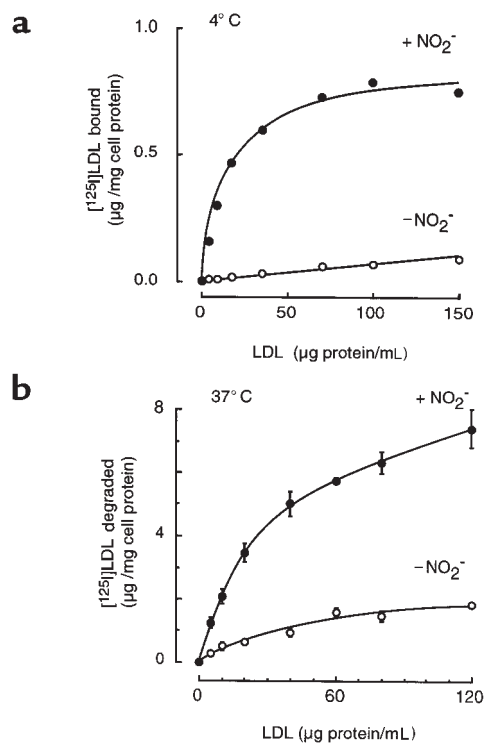


Figure 9 Concentration dependence of macrophage binding (a) and degradation (b) of LDL modified by the MPO-H₂O₂-NO₂⁻ system. (a) [¹²⁵I]LDL was modified in the presence of MPO and an H₂O₂-generating system (GGOx) in either the presence (+NO₂⁻; filled circles) or absence (-NO₂⁻; open circles) of NO₂⁻ in sodium phosphate buffer (50 mM, pH 7.0) supplemented with DTPA (100 µM) as described in Methods. Thioglycollate-elicited MPM binding (a) and degradation (b) of the indicated concentrations of lipoproteins were then assessed as described in Methods. Data represent the mean of duplicate determinations (binding studies) or the mean ± SD of triplicate determinations (degradation studies). Similar results were observed in 3 independent experiments.

confirming that the cells recognize NO₂-LDL with high affinity. Macrophage binding of NO₂-LDL was also specific because binding of LDL exposed to MPO and H₂O₂ in the absence of NO₂⁻ was negligible (Figure 9a). Moreover, coincubation of [¹²⁵I]NO₂-LDL either with a 40-fold excess of nonlabeled native LDL or with LDL modified in the presence of MPO and H₂O₂ without NO₂⁻ failed to significantly block (≤10% inhibition) macrophage recognition of the modified lipoprotein. In contrast, competition studies examining [¹²⁵I]NO₂-LDL binding, uptake, and degradation to macrophages demonstrated that excess nonlabeled NO₂-LDL was an effective competitor (>70% inhibition). The concentration dependence of NO₂-LDL degradation (Figure 9b) by MPMs (at 37°C) was also examined and demonstrated comparable hyperbolic plots, consistent with uptake and degradation of the modified lipoprotein through a saturable process. In contrast, cell-mediated uptake and degradation of LDL modified by MPO and H₂O₂ in the absence of NO₂⁻ were minimal (Figure 9b). Collectively, these results are consistent with macrophage recognition of NO₂-LDL through a specific high-affinity receptor-mediated process.

Macrophage recognition of LDL modified by the MPO-H₂O₂-NO₂⁻ system occurs through a pathway independent of the class A type I scavenger receptor. Increased macrophage binding, uptake, and degradation of [¹²⁵I]LDL modified by the MPO-H₂O₂-NO₂⁻ system, which is saturable and not inhibited by excess native LDL, suggests that the nitrated lipoprotein is being bound and internalized by a receptor that is not the LDL receptor. To characterize the nature of the macrophage receptor mediating recognition of NO₂-LDL, we initially performed competition studies using known inhibitors of scavenger receptor class A. Excess polyanionic inhibitors such as polyinosinic acid, polyguanylate, dextran sulfate, and fucoidan all failed to inhibit MPM uptake of NO₂-LDL by >50% (data not shown). These results suggested that scavenger receptor class A, if involved in NO₂-LDL recognition, could only partially account for the recognition of this modified form of LDL. To assess further whether the classic scavenger receptor participates in the recognition of NO₂-LDL, we examined the uptake and degradation of the nitrated lipoprotein using CHO cells expressing mSR-AI. CHO-mSR-AI demonstrated significant uptake and degradation of both acetylated LDL and copper-oxidized LDL when compared with control vector-transfected CHO cells, as reported previously (56). In contrast, there was only a negligible increase in the uptake and degradation of NO₂-LDL by CHO-mSR-AI cells compared with control vector-transfected cells (Figure 10). Although the difference was significant (P < 0.05), expression of mSR-AI only augmented cell uptake of NO₂-LDL by ~20% over basal levels. Thus, the scavenger receptor SR-AI does not appear to play an important role in macrophage recognition of LDL modified by MPO-generated reactive nitrogen species.

LDL modified by the MPO-H₂O₂-NO₂⁻ system promotes macrophage cholesteryl ester synthesis, lipid loading, and foam cell formation. Protein nitration, induction of lipid peroxidation, and conversion of the lipoprotein into a high-uptake form are all properties consistent with conversion of LDL into a proatherogenic form. To determine whether LDL modification by MPO-generated reactive nitrogen species might contribute to cholesterol accumulation in macrophages, a critical process in lesion development, we first evaluated the ability of NO₂-LDL to stimulate cholesteryl ester biosynthesis by MPMs. NO₂-LDL markedly stimulated [¹⁴C]oleate incorporation into cellular cholesteryl ester pools in MPMs (Figure 11a). In contrast, additions or deletions to the complete system that prevented LDL protein nitration, lipid peroxidation, and macrophage recognition all resulted in ablation of macrophage cholesteryl ester formation from exogenous modified lipoprotein (compare Figures 4 and 11). In a parallel set of experiments, the ability of NO₂-LDL to promote cellular accumulation of intracellular sterol pools was examined. Again, only LDL modified in the presence of each component of the complete MPO-H₂O₂-NO₂⁻ system, but not in the presence of catalase or BHT, promoted significant increases in free and esterified cholesterol content of macrophages (Figure 11).

In a final set of experiments, we examined whether stimulation of cholesteryl ester biosynthesis and cellular lipid accumulation in macrophages incubated with

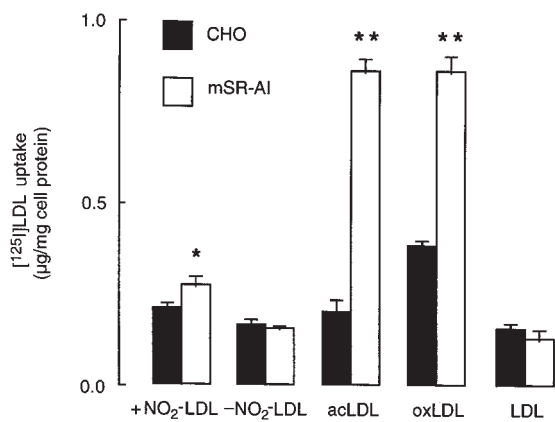


Figure 10

Uptake of ¹²⁵I-labeled lipoproteins by CHO cells transfected with the murine scavenger receptor class A type I. Confluent CHO cells expressing the murine scavenger receptor class A type I (white bars) or control vector-transfected parental CHO cells that lack the LDL receptor (black bars) were incubated for 5 hours at 37°C with 5 µg/mL of the indicated ¹²⁵I-labeled lipoproteins prepared as described in Methods. Cellular uptake of modified lipoproteins was then determined as described in Methods. Data represent the mean ± SD of triplicate determinations. Similar results were observed in 3 independent experiments. **P* < 0.05 vs. control vector-transfected parental CHO cells; ***P* < 0.001 vs. control vector-transfected parental CHO cells. acLDL, acetylated LDL; LDL, native LDL; mSR-AI, CHO cells expressing the murine scavenger receptor class A type I; + NO₂-LDL, LDL modified by the complete MPO-H₂O₂-NO₂⁻ system; - NO₂-LDL, LDL modified by the complete system in the absence of NO₂⁻; oxLDL, copper oxidized (5 hours) LDL.

NO₂-LDL resulted in the formation of lipid-laden foam cells, an early cellular event in the atherosclerotic process. Thioglycollate-elicited MPMs were incubated for 72 hours with either native LDL (negative control), acetylated LDL (positive control), NO₂-LDL, or LDL exposed to MPO and H₂O₂ in the absence of NO₂⁻, and then microscopically examined after neutral lipid staining with oil red O. LDL nitrated by MPO promoted massive cholesterol deposition in macrophages (Figure 12). Moreover, the oil red O-positive staining of NO₂-LDL demonstrated comparable intensity to that observed in macrophages exposed to an equivalent level of exhaustively acetylated LDL (Figure 12). The total cholesterol content (cholesteryl ester + free cholesterol) of macrophages exposed to equivalent levels of NO₂-LDL and exhaustively acetylated LDL was 189 ± 13 µg/mg cell protein and 333 ± 100 µg/mg cell protein, respectively (mean ± SD; *n* = 3). Interestingly, the lipid droplets within the cytoplasm of the NO₂-LDL-treated cells were more punctate and diffusely spread than those observed with acetylated LDL (Figure 13), suggesting that the intracellular processing of nitrated and acetylated lipoproteins differs. In contrast, cells incubated with either native LDL or with LDL exposed to MPO and H₂O₂ in the absence of NO₂⁻ were devoid of oil red O-positive droplets (Figure 12). Collectively, these results demonstrate that LDL modification by MPO-generated reactive nitrogen species converts the lipoprotein into a form that promotes extensive lipid loading of macrophages and foam cell formation.

Discussion

Reactive nitrogen species have been implicated in promoting oxidative damage of LDL and tissues in atherosclerosis (16, 17). However, neither the pathways for nitrating LDL in vivo nor the biologic consequences of these reactions are established. The results of the current study demonstrate that nitrating intermediates formed by the MPO-H₂O₂-NO₂⁻ system of monocytes mediate LDL protein nitration, initiate LDL lipid peroxidation, and convert the lipoprotein into a form that promotes cholesterol deposition in macrophages and foam cell formation.

Despite intense interest in the role of reactive nitrogen species in inflammatory injury and vascular disease, few studies have examined the effects of nitrating intermediates on LDL recognition by macrophages. LDL incubated with SIN-1, a compound that spontaneously decomposes to generate comparable rates of O₂⁻ and NO production, results in increased levels of lipid peroxidation products but does not generate a high-uptake form of LDL (8, 13). Studies examining the effects of modulators of NO synthesis on phorbol ester-stimulated MPMs are consistent with NO playing a protective role, as increased NO production inhibited, and decreased NO production promoted, LDL oxidation and conversion into a high-uptake form (8). LDL exposed to reagent ONOO⁻ has been shown to promote macrophage cholesteryl ester biosynthesis (13). However, the mechanism(s) of cell recognition of ONOO⁻-modified lipoproteins has not yet been examined. Several observations suggest that macrophage recognition of ONOO⁻-modified LDL occurs through mechanisms distinct from those involved with LDL modified by the MPO-H₂O₂-NO₂⁻ system. Stimulation of macrophage cholesteryl oleate synthesis by ONOO⁻-treated LDL requires millimolar quantities of oxidant and extensive LDL modification (~60% lysine modification, REM > 2) (13). In contrast, modification into a high-uptake form by MPO-generated nitrating intermediates requires only low levels of H₂O₂, results in minimal depletion of free lysine residues, and only modestly enhances lipoprotein REM (Figure 6). Moreover, modification of LDL with high levels of ONOO⁻ results in lipoprotein aggregation, as judged by light scattering and size exclusion chromatography (D. Schmitt and S.L. Hazen, unpublished study); thus, cellular uptake mechanisms of ONOO⁻-modified LDL may be via phagocytosis of aggregates. Similarly, LDL aggregation (and hence, phagocytosis by macrophages) has been demonstrated after exposure of LDL to an atmosphere containing NO₂ gas (67), vortexed mixtures of HOCl and NO₂⁻ (58), and millimolar levels of NO₂⁻ for prolonged periods (68).

In contrast, multiple lines of evidence suggest that macrophage uptake of LDL modified by MPO, H₂O₂, and NO₂⁻ occurs via a pathway involving receptor-mediated endocytosis rather than phagocytosis. Binding of NO₂-LDL to macrophages occurred through a high-affinity, saturable, and specific interaction — characteristics of a receptor-mediated process. Moreover, uptake and degradation of the modified lipoprotein were saturable, unlike bulk-phase phagocytosis of lipoprotein aggregates (63, 64). Competition studies examining [¹²⁵I]NO₂-LDL binding, uptake, and degradation by macrophages further confirm

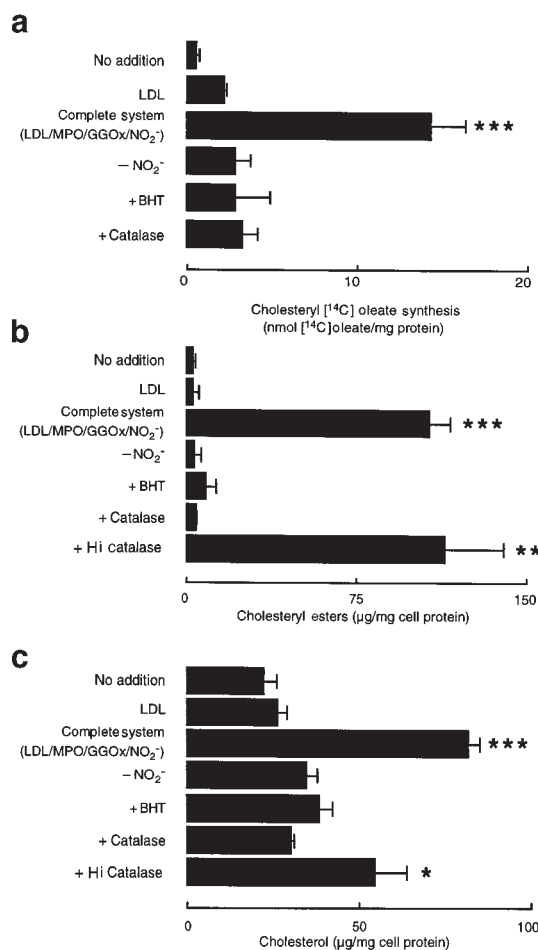


Figure 11 Cholesteryl oleate synthesis (a), cholesteryl ester mass (b), and cholesterol mass (c) of cells exposed to LDL modified by the MPO-H₂O₂-NO₂⁻ system. [¹²⁵I]LDL (0.2 mg/mL) was incubated with isolated human MPO (30 nM), glucose (100 μM), glucose oxidase (20 ng/mL), and NO₂⁻ (500 μM) in sodium phosphate buffer (50 mM, pH 7.0) supplemented with DTPA (100 μM) (complete system) or the indicated additions or deletions. Lipoproteins were then incubated with thioglycollate-elicited MPMs, and the extent of cholesteryl [¹⁴C]oleate formation (a), cellular cholesteryl ester mass (b), and free cholesterol mass (c) was determined as described in Methods. Data represent the mean ± SD of triplicate determinations. Similar results were observed in 3 independent experiments. **P* < 0.05; ***P* < 0.01; ****P* < 0.001 for comparison vs. LDL modified by MPO and an H₂O₂-generating system only (-NO₂⁻). No significant cholesteryl [¹⁴C]oleate formation or increase in cholesteryl ester or free cholesterol mass above basal values was observed in LDL preparations modified by the complete system in the absence of either MPO or GGOx. Hi catalase, heat-inactivated catalase.

the specificity of the interaction because excess nonlabeled NO₂-LDL was an effective competitor, but native LDL and LDL modified in the presence of MPO and H₂O₂ without NO₂⁻ were not. Finally, lipoprotein aggregation during oxidative modification by the MPO-H₂O₂-NO₂⁻ system was excluded by a variety of methods including size exclusion chromatography, light scattering, gel electrophoresis, and cytochalasin D treatment of macrophages.

The distinction between macrophage uptake and degradation of an aggregated lipoprotein compared with a monomeric/soluble oxidized lipoprotein also underscores

the unexpected findings observed when examining the potential role of MPO-generated chlorinating and nitrating intermediates in conversion of LDL into a high-uptake form. Previous studies reported by Hazell et al. (57) noted that LDL treatment with reagent HOCl becomes aggregated and, like other aggregated forms of lipoproteins, promotes intracellular lipid accumulation in macrophages (54). However, in the present study, LDL incubated with either activated human monocytes in Cl⁻-containing media (Figure 1) or an HOCl-generating system (MPO, GGOx, and Cl⁻; Figure 2) was not converted into a stable high-uptake form. These results suggest that direct addition of reagent HOCl and in situ formation of HOCl by the MPO-H₂O₂-Cl⁻ system of phagocytes modify LDL in different ways. A similar conclusion was reached in a recent mass spectrometric study examining peptide oxidation products in tryptic digests of apo B-100 from LDL exposed to either reagent HOCl or the MPO-H₂O₂-Cl⁻ system (69). Such a difference might be observed if MPO binds directly to LDL and locally delivers oxidant to select sites on the lipoprotein particle. Alternatively, certain targets for oxidation on LDL may only be modified in the presence of high local concentrations of oxidants produced during bolus addition in vitro, but that otherwise might not be observed under more physiological conditions. Indeed, under the conditions of a low steady flux of H₂O₂ used in the present study, no significant LDL aggregation was observed during exposure of the lipoprotein to the MPO-H₂O₂-Cl⁻ system. In contrast, LDL aggregation and conversion into a particle ingested by phagocytosis was observed after addition of reagent HOCl or after bolus addition of H₂O₂ to LDL incubated in the presence of MPO and Cl⁻ (E. Podrez et al., unpublished study). The results of the present study do not rule out formation of a labile oxidation product from the MPO-H₂O₂-Cl⁻ system (e.g., *N*-monochloramines) that is subsequently repaired (57) during incubations with cells. Whether such a labile modified form of LDL is a ligand for macrophages, and might thus play a role in LDL uptake in vivo in circumstances in which repair processes might become depleted, cannot be excluded. Taken together, the results of the present study suggest that under conditions in which only a low flux of H₂O₂ is formed, MPO-generated nitrating intermediates are more effective than chlorinating intermediates in promoting oxidative conversion of LDL into a stable high-uptake form.

Because of the high concentrations of Cl⁻ in biologic matrices, reactive chlorinating species have routinely been thought to be the predominant oxidant formed by the MPO-H₂O₂ system of phagocytes in vivo (21–24). However, the results of the present study clearly demonstrate that MPO (either isolated or derived from activated monocytes) utilizes NO₂⁻ to convert LDL into a form that binds to, and is taken up and degraded by, macrophages, even in the presence of plasma levels of Cl⁻. Furthermore, these results also demonstrate that HOCl formation is not obligatory for MPO- and NO₂⁻-dependent conversion of LDL into a high-uptake form. Consequently, many of the experiments described in the present study were performed with no Cl⁻ present during the oxidation reaction because from a mechanistic standpoint, it is then possible to demonstrate that both the alterations in the biologic

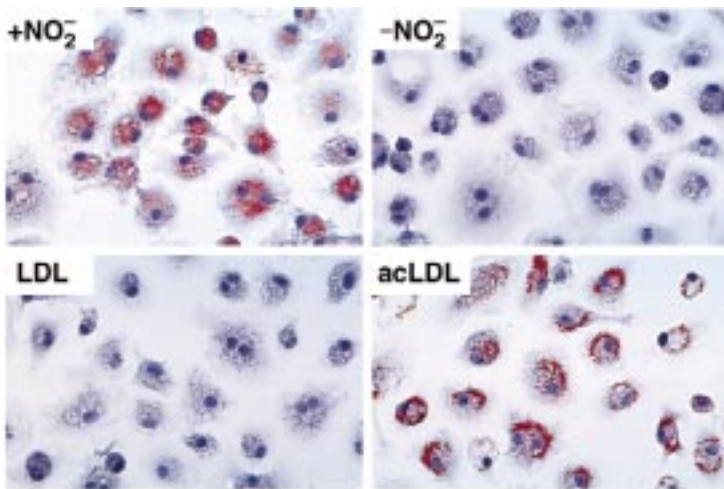


Figure 12

Macrophage foam cell formation by exposure to LDL modified by the MPO-H₂O₂-NO₂⁻ system. Thioglycollate-elicited MPMs were grown for 48 hours in RPMI-1640 containing 10% FBS. Cells were then incubated for 72 hours in the same media containing catalase (100 nM), BHT (20 μM), and vitamin E (20 μM) in the presence of the following lipoprotein preparations (50 μg/mL): LDL (0.2 mg/mL) previously incubated with isolated human MPO (30 nM), glucose (100 μM), and glucose oxidase (20 ng/mL) in sodium phosphate buffer (50 mM, pH 7.0) supplemented with DTPA (100 μM) at 37°C for 8 hours in the presence (+ NO₂⁻) and absence (- NO₂⁻) of NO₂⁻ (500 μM), native LDL (LDL), and acetylated LDL (acLDL). Media containing the appropriate modified lipoproteins were exchanged after 24-hour incubation. Cells were fixed with 4% formaldehyde and stained with hematoxylin and oil red O. Original magnification: ×300.

end points examined (e.g., binding, cell association, uptake, degradation, sterol accumulation) and the oxidation products formed (e.g., nitrotyrosine, various lipid peroxidation products) are the result of an oxidant species derived from MPO-dependent oxidation of NO₂⁻. Although the nitrating intermediate(s) formed by the reaction of NO₂⁻ with the MPO-H₂O₂ system has not been identified, NO₂ (the one electron oxidation product of NO₂⁻) and NO₂Cl (formed by reaction of HOCl with NO₂⁻) have been implicated (25, 26, 33). Thus, under physiological conditions in which both Cl⁻ and NO₂⁻ are present, a variety of halogenating and nitrating intermediates may be formed by MPO (24, 25, 30, 33, 52). The product(s) of the MPO-H₂O₂-NO₂⁻ system is anticipated to be particularly effective at converting LDL into a high-uptake form for intimal macrophages.

Despite evidence suggesting that a receptor-mediated pathway is involved in macrophage recognition of NO₂-LDL, the identity of the receptor and ligand mediating the interaction is unknown. Experiments using CHO cells expressing mSR-AI demonstrated no difference in NO₂-LDL binding, uptake, or degradation when compared with control vector-transfected CHO cells, strongly suggesting that class A type I scavenger receptor does not participate in recognition of the nitrated lipoprotein. This lack of recognition is consistent with the relatively low level of apo B-100 lysine blockage and REM observed (Fig-

ure 6) compared with that required for recognition of other modified forms of LDL (61, 62). Future studies identifying the nature of the ligand(s) and receptor(s) mediating macrophage recognition of NO₂-LDL are warranted.

The ability of modified forms of LDL to promote accumulation of cellular cholesteryl esters has been widely accepted as an index of the atherogenic potential of a lipoprotein. While our studies show that incubation of LDL with activated monocytes in the presence NO₂⁻ transforms the lipoprotein into a high-uptake form, the biologic significance of this type of modification in vivo is not yet established. All of the in vitro cell experiments in the present study were performed under serum-free conditions. Whether or not the MPO-H₂O₂-NO₂⁻ system of monocytes effectively modifies LDL in a more physiological, serum/plasma-containing milieu remains to be explored. However, considerable evidence suggests that MPO may contribute to nitration of LDL in human atheroma because all of the components of the MPO-H₂O₂-NO₂⁻ system are present and available for lipoprotein and tissue oxidative damage in human lesions, and products of this pathway – e.g., nitrotyrosine (16, 17), dityrosine (70), and lipid hydroperoxides (43, 71) – are enriched in human lesions. Indeed, immunohistochemical staining demonstrates that MPO colocalizes with lipid-laden macrophages (28), and our recent mass spectrometric studies demonstrate that chlorotyrosine, a spe-

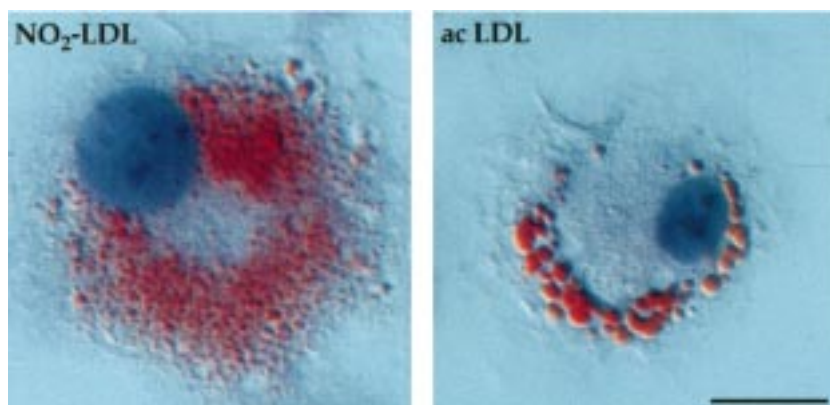


Figure 13

High-power magnification view of macrophage foam cells formed by exposure to LDL modified by the MPO-H₂O₂-NO₂⁻ system and acetylated LDL (acLDL). Differential interference contrast microscopy of hematoxylin and oil red O-stained thioglycollate-elicited MPMs exposed to NO₂-LDL and acLDL as in Figure 12. Scale bar: 10 μm.

cific marker for MPO-dependent protein damage by chlorinating intermediates (24, 72, 73), is significantly enriched in LDL recovered from human aortic lesions and atherosclerotic intima (29). Thus, catalytically active MPO and sufficient levels of H₂O₂ to generate reactive oxidants with the enzyme are present in human vascular tissues. Moreover, the low rate of H₂O₂ flux used for lipoprotein modification in this study is less than that generated by circulating levels of leukocytes activated in suspension (74) or on biologic surfaces (75). Furthermore, although the actual concentration of NO₂⁻ in arterial intima is not known, the concentration of NO₂⁻ required for MPO-dependent conversion of LDL into a high-uptake form is well within the range of that observed in other inflammatory fluids and tissues (35–37). Finally, the levels of nitrotyrosine observed in apo B-containing LDL-like particles recovered from human atherosclerotic lesions (17) are several-fold greater than those observed in NO₂-LDL preparations, which are avidly taken up and degraded by macrophages. Thus, MPO-generated nitrating intermediates may represent a physiologically plausible mechanism for promoting protein nitration, lipid peroxidation, and cholesterol deposition in macrophage-rich human atherosclerotic tissues.

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