Nitrogen dioxide radical generated by the myeloperoxidase-hydrogen peroxide-nitrite system promotes lipid peroxidation of low density lipoprotein

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Abstract Myeloperoxidase, a heme protein secreted by activated phagocytes, is present and enzymatically active in human atherosclerotic lesions. In the current studies, we explored the possibility that reactive nitrogen species generated by myeloperoxidase promote lipid peroxidation of low density lipoprotein (LDL) – a modification that may render the lipoprotein atherogenic. We found that myeloperoxidase, an H_2O_2-generating system and nitrite (NO_2^-) peroxidized LDL lipids. The process required NO_2^- and each component of the enzymatic system; it was inhibited by catalase, cyanide and ascorbate, a potent scavenger of aqueous phase radicals. LDL peroxidation did not require chloride ion, and it was little affected by the hypochlorous acid scavenger taurine. Collectively, these results suggest that lipid peroxidation is promoted by a nitrogen dioxide radical-like species. These observations indicate that myeloperoxidase, by virtue of its ability to form reactive nitrogen intermediates, may promote lipid peroxidation and atherogenesis.

Key words: Atherosclerosis; Oxidized LDL; Nitryl chloride; Peroxynitrite; Nitric oxide; Nitrotyrosine

1. Introduction

An elevated level of low density lipoprotein (LDL), the major carrier of blood cholesterol, is an important risk factor for atherosclerotic vascular disease [1]. Many lines of evidence suggest that LDL lipids must be oxidatively modified to promote atherogenesis [2–4]. Whereas LDL oxidation has been widely studied in vitro, the mechanisms that promote it in vivo remain poorly understood [5]. Early studies demonstrated that cultured arterial cells oxidize LDL lipids by reactions that require iron or copper [6–8]. However, it is uncertain whether the requisite free (or low molecular weight chelate) metal ions exist in plasma or tissue [5,9].

We have described a pathway for LDL oxidation that does not require free metal ions [10–13]. It involves oxidants generated by myeloperoxidase, an enzyme secreted by phagocytes. Myeloperoxidase uses H_2O_2 to generate cytotoxic oxidants that help defend the host against invading pathogens [14]; these oxidants also have the potential to damage host macromolecules such as LDL [5]. Because the enzyme's active site is buried in a hydrophobic cleft [15], it cannot directly oxidize large molecules. Instead, it relies on low molecular weight intermediates to convey oxidizing equivalents from its heme group to its target [10–14].

Another pathway for LDL oxidation that is independent of free metal ions involves nitric oxide (nitrogen monoxide; NO) produced by the constitutive nitric oxide synthase of endothelial cells and the inducible nitric oxide synthase of inflammatory cells [16]. When released from the endothelium, NO plays a critical role in normal physiology by regulating vasomotor tone. In striking contrast, the larger amounts produced by inflammatory cells are cytotoxic [16,17]. Though NO is a relatively stable free radical that fails to oxidize biological targets, reactive nitrogen species that are derived from it under pathological conditions may promote LDL oxidation [17,18].

Myeloperoxidase may provide a different mechanism for generating reactive nitrogen species. At plasma concentrations of halide ion, the enzyme uses chloride together with H_2O_2 to generate hypochlorous acid (HOCl) [19,20]. This potent oxidant has been proposed to react with nitrite (NO_2^-), a major decomposition product of NO, to yield a reactive intermediate similar to nitryl chloride, NO_2Cl [21–23]:

$$H_2O_2 + Cl^- + H^+ \rightarrow HOCl + H_2O$$

$$HOCl + NO_2^- \rightarrow NO_2Cl + HO^-$$

Myeloperoxidase also oxidizes NO_2^- to a nitrogen dioxide ($NO_2^\bullet$)-like radical that nitrates tyrosine and other aromatic compounds [21–27]:

$$H_2O_2 + NO_2^- + H^+ \rightarrow NO_2^\bullet + HO^- + H_2O$$

Polyunsaturated fatty acids exposed to $NO_2^\bullet$ in vitro undergo oxidation and nitration [9,28,29]. However, the relative physiological importance of NO_2Cl and $NO_2^\bullet$ remain poorly understood.

In the current studies, we show that reactive nitrogen species generated by myeloperoxidase promote LDL lipid peroxidation. The reaction requires NO_2^- but is independent of chloride ion, implicating a $NO_2^\bullet$-like species. Our results therefore suggest that myeloperoxidase may provide one pathway

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Abbreviations: DTPA, diethylenetriaminepentaacetic acid; HODE, hydroxyoctadecadienoic acid; LDL, low density lipoprotein

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for promoting LDL lipid peroxidation and nitration in the artery wall.

2. Materials and methods

Fisher Scientific supplied organic solvents (HPLC grade). Unless otherwise indicated, all other materials were obtained from Sigma Chemical Company.

2.1. Myeloperoxidase and LDL preparation

Myeloperoxidase was isolated from HL60 cells by lectin affinity chromatography and size exclusion chromatography [30]. Myeloperoxidase (A_{430}/A_{280} ratio >0.70) concentration was determined spectrophotometrically ($\varepsilon_{430}=170$ mM^{-1} cm^{-1}; [31]). LDL ($d=1.019$–1.063 g/ml) was prepared from pooled plasma (EDTA 4 mM) of healthy volunteers by discontinuous density gradient ultracentrifugation [32] and stored under nitrogen at 4°C in the dark up to 1 week. LDL protein was determined spectrophotometrically ($\varepsilon_{280}=1.05$ ml m^{-1} mg^{-1}).

2.2. Lipoprotein oxidation

Reactions with myeloperoxidase were carried out at 37°C in buffer A (50 mM sodium phosphate, 100 μM diethylenetriaminepentaacetic acid (DTPA), pH 7.4) under conditions where LDL oxidation was a linear function of enzyme concentration. Buffer A was prepared with deionized glass-distilled water and passed over a Chelex 100 resin (Bio-Rad) column to remove transition metal ions potentially able to catalyze oxidation reactions. The reaction mixture contained a final concentration of 0.2 mg/ml LDL protein, 50 ng/ml glucose oxidase (Boehringer Mannheim) and 100 μg/ml D-glucose.

2.3. Analysis of lipid peroxidation products

LDL oxidation was terminated by lipid extraction using a modified Dole procedure [12]. Lipid hydroperoxides were reduced to alcohols with triphenylphosphine to prevent breakdown during analysis [33]. To release free fatty acids from LDL lipids, the heptane phase was subjected to gentle base hydrolysis (methanol:5 N potassium hydroxide; 4:1; v:v) for 20 min at 60°C [33]. Hydrolysis was terminated by cooling and then acidifying with acetic acid. The acidified solution was injected directly into the HPLC system for analysis. Recovery of 13-hydroxyoctadecadienoic acid (HODE) subjected to this procedure was greater than 85%. Analyses for HODE were performed on a Beckman high performance liquid chromatography (HPLC) system equipped with a reverse phase column (Ultrasphere; 250 mm×4.6 mm; particle size 5 μm; Beckman). Oxidized lipids were detected by monitoring absorbance at 234 nm [3,23]. HODEs were separated using a solvent system of methanol:water:acetic acid (85:15:0.1; v:v:v) and a flow rate of 1 ml/min [12,33]. Authentic 13-HODE was prepared from linoleic acid (Nu Chek Prep Inc.) with soybean lipoxygenase [34] and reduced with triphenylphosphine prior to analysis. Peaks I and II (Fig. 1) were isolated by HPLC and subjected to positive ion electrospray ionization-mass spectrometry as described [37].

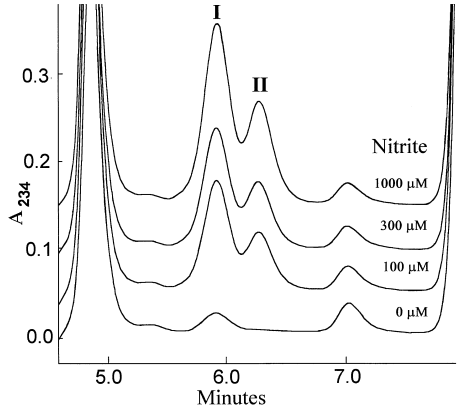

Fig. 1. Reverse phase HPLC analysis of LDL exposed to the myeloperoxidase-H_2O_2-NO_2^- system. The complete system consisted of 0.2 mg/ml LDL protein, 40 nM myeloperoxidase, 100 μg/ml D-glucose, 50 ng/ml glucose oxidase in buffer A (50 mM sodium phosphate buffer, 100 μM DTPA, pH 7.4). Where indicated, NO_2^- was included in the reaction mixture. Reactions were initiated by addition of glucose oxidase. After a 90 min incubation at 37°C, the reaction mixture was subjected to lipid extraction, reduction, hydrolysis, and HPLC analysis. Authentic 13-HODE co-migrated with peak I. Electrospray ionization-mass spectrometric analysis demonstrated that hydroxylated C18 fatty acids were present in both peak I and peak II, indicating that these materials are likely to represent isomers of HODE.

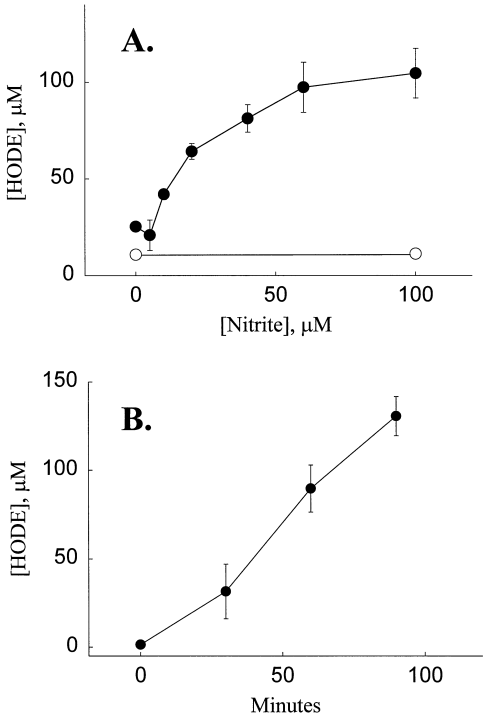

Fig. 2. Influence of NO_2^- (A) and progress curve (B) of LDL oxidation by the myeloperoxidase-H_2O_2 system. LDL was oxidized by the myeloperoxidase-H_2O_2 system containing (A) the indicated concentration of NO_2^- or (B) 100 μM NO_2^-. Where indicated, catalase was present at 300 nM (○). After (A) a 90 min incubation or (B) at the indicated time, the reaction mixture was subjected to HPLC analysis. Results represent (A) mean ± S.E.M. of two independent experiments with triplicate determinations (similar results were observed in five independent experiments) and (B) mean ± S.E.M. of four independent experiments.

3. Results

3.1. Myeloperoxidase stimulates lipid peroxidation by a pathway that requires H_2O_2 and nitrite

To explore the potential role of myeloperoxidase-derived oxidants in lipid peroxidation, we exposed LDL to myeloperoxidase and a peroxide-generating system in chloride-free sodium phosphate buffer (pH 7.4). To mimic more closely the cellular production of oxidant, glucose oxidase was used to continuously produce H_2O_2. In the absence of NO_2^-, we detected only low levels of lipid peroxidation products, monitored by reverse phase HPLC analysis as the appearance of HODEs (Fig. 1). Adding NO_2^- to the buffer markedly increased lipid peroxidation of LDL. The extent depended on the concentration of NO_2^- (Fig. 2A). Little peroxidation was apparent at $[NO_2^-]<10$ μM. At higher concentrations of NO_2^-, lipid peroxidation rapidly increased and was maximal at 100 μM.

Lipid peroxidation by the myeloperoxidase-H_2O_2-NO_2^- system exhibited a lag phase, then increased and gradually slowed (Fig. 2B). All components of the reaction mixture were required for oxidation (data not shown). Lipid peroxidation was inhibited by the peroxide scavenger catalase (300 nM) and the heme poison cyanide (10 mM), implicating H_2O_2 and myeloperoxidase in the reaction.

3.2. Inhibition of LDL lipid peroxidation by antioxidants

Probucol and vitamin E are hydrophobic antioxidants that block chain-propagating radical reactions in the lipid phase [3,35,36]. In contrast, ascorbic acid prevents the initiation of lipid peroxidation by scavenging radicals in the aqueous phase [9,37]. It can also interrupt the propagation phase of lipid peroxidation by converting oxidized vitamin E back to its active form [3,36,37]. When LDL was exposed to reactive nitrogen species generated by the myeloperoxidase-H_2O_2-NO_2^- system, we found that 50 μM ascorbate – a well-known scavenger of $NO_2^\bullet$ – almost completely blocked lipid peroxidation, whereas the same concentration of vitamin E or probucol was less effective (40% and 60% inhibition, respectively). Mannitol, a hydroxyl radical scavenger, failed to affect lipid peroxidation by myeloperoxidase.

3.3. The myeloperoxidase-H_2O_2-NO_2^- system stimulates LDL lipid peroxidation in the presence of the HOCl scavenger taurine

The major substrate for myeloperoxidase is thought to be chloride ion [14,19,20]. Recent studies suggest that the enzyme mediates certain nitration and chlorination reactions via an NO_2Cl-like species derived from HOCl and NO_2^- [22,23,25]. However, there was no detectable increase in lipid peroxidation when LDL was incubated for 30 min at 37°C with 100 μM NO_2^- and 100 μM HOCl. HOCl or NO_2^- alone also failed to promote peroxidation.

To further investigate the potential role of NO_2Cl in mediating LDL lipid peroxidation, we determined the effect of chloride and taurine on lipid peroxidation by the myeloperoxidase-H_2O_2-NO_2^- system. LDL oxidation by myeloperoxidase, H_2O_2 and NO_2^- was increased ~20% by plasma concentrations (100 mM) of chloride (Fig. 3). This increase might reflect non-specific ionic effects or generation of HOCl. To distinguish between these possibilities, we determined whether lipid peroxidation was inhibited by taurine, a potent scavenger of HOCl [38]. Taurine (Fig. 3) at up to 1000 μM (10 times the concentration of NO_2^-) failed to affect LDL lipid peroxidation induced by the complete myeloperoxidase-H_2O_2-NO_2^- system (with or without chloride ion). Collectively, these observations indicate that HOCl generated by myeloperoxidase is unlikely to promote LDL lipid peroxidation.

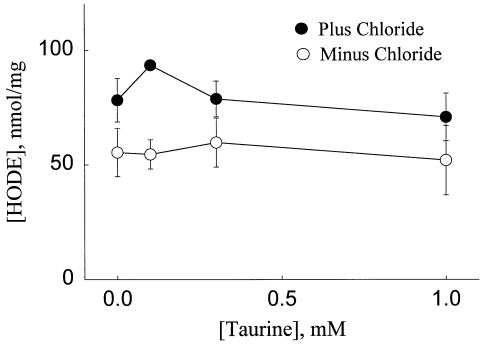

Fig. 3. Influence of chloride ion and taurine on LDL oxidation by the myeloperoxidase-H_2O_2-NO_2^- system. LDL was oxidized by the myeloperoxidase-H_2O_2 system containing 100 μM NO_2^-. Where indicated, 100 mM sodium chloride or taurine was included. After a 90 min incubation at 37°C, the reaction mixture was subjected to HPLC analysis. Results represent the mean ± S.E.M. of four independent experiments.

4. Discussion

Our observations indicate that myeloperoxidase and H_2O_2 convert NO_2^- into reactive nitrogen species that promote the peroxidation of LDL lipid. In contrast to generally studied reactions [2–8], this mechanism for LDL oxidation is independent of free metal ions.

Previous studies have suggested that myeloperoxidase can generate reactive nitrogen intermediates from NO_2^- by two distinct pathways [21–27]. One involves the one-electron oxidation of NO_2^- to $NO_2^\bullet$ by compound I, a complex of myeloperoxidase and H_2O_2. This pathway should not require chloride ion. The other involves the initial generation of HOCl by compound I, which subsequently reacts with NO_2^- to yield NO_2Cl, a nitrating and chlorinating intermediate [22,23,25]. This pathway should be inhibited by compounds that react with HOCl. We found that myeloperoxidase's ability to initiate the peroxidation of LDL lipid did not require chloride ion. Moreover, LDL lipids were not peroxidized by NO_2^- and reagent HOCl, and lipid peroxidation by the myeloperoxidase-H_2O_2-NO_2^- system was insensitive to taurine, a potent scavenger of HOCl [38]. The reduction potentials of NO_2^- and chloride ion are −0.99 V and −1.36 V [39,40], respectively, suggesting that myeloperoxidase should preferentially oxidize NO_2^-. These observations indicate that the myeloperoxidase pathway that promotes lipid peroxidation likely involves an $NO_2^\bullet$-like species.

Antioxidants might inhibit LDL oxidation by scavenging myeloperoxidase-derived reactive nitrogen species, thereby preventing them from initiating lipid peroxidation [9,37], and by blocking chain-propagating reactions in the lipid phase [3,35,36]. Ascorbate at a physiologically relevant concentration (50 μM) almost completely inhibited lipid peroxidation by the myeloperoxidase-H_2O_2-NO_2^- system. In contrast, this concentration of vitamin E or probucol only partially inhibited lipid peroxidation. The difference in potency between lipid and water soluble antioxidants is consistent with the proposed role of $NO_2^\bullet$ as the oxidizing intermediate, and suggests that ascorbate inhibits LDL lipid peroxidation by myeloperoxidase through direct scavenging of reactive nitrogen species.

A key question is whether reactive nitrogen intermediates produced by myeloperoxidase could oxidize LDL in the artery wall. In healthy humans, the level of NO_2^- in plasma is < 5 μM [41], though concentrations as high as 100 μM are found in saliva, respiratory fluid and gastric juice as well as in plasma during inflammation [42]. The higher concentrations are similar to those required for LDL lipid peroxidation by myeloperoxidase in vitro, suggesting that reactive nitrogen species generated by myeloperoxidase may execute oxidative reactions at sites of inflammation in vivo.

Our previous studies have implicated myeloperoxidase in artery wall damage [10–13,43]. LDL isolated from human atherosclerotic lesions contains high levels of 3-chlorotyrosine,

a specific marker of protein oxidation by myeloperoxidase-generated HOCl [43], indicating that the enzyme chlorinates lipoproteins in the artery wall. Moreover, human neutrophils employ the myeloperoxidase system to generate 3-nitrotyrosine and 3-chlorotyrosine in vitro [25,43], and LDL isolated from human atherosclerotic lesions contains markedly elevated levels of 3-nitrotyrosine [44]. Collectively, these observations raise the possibility that myeloperoxidase both nitrates and chlorinates host tissues in vivo. Myeloperoxidase promotes peroxidation of LDL lipids at pathophysiological levels of NO_2^-. The enzyme may therefore promote atherogenesis by virtue of its ability to form reactive intermediates, including $NO_2^{\bullet}$, counteracting the well-established anti-atherogenic effects of low levels of NO.

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