The Nitric Oxide Congener Nitrite Inhibits Myeloperoxidase/H$_2$O$_2$/Cl$^-$-mediated Modification of Low Density Lipoprotein*

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Nitric oxide, a pivotal molecule in vascular homeostasis, is converted under aerobic conditions to nitrite. Recent studies have shown that myeloperoxidase (MPO), an abundant heme protein released by activated leukocytes, can oxidize nitrite (NO$_2^-$) to a radical species, most likely nitrogen dioxide. Furthermore, hypochlorous acid (HOCl), the major strong oxidant generated by MPO in the presence of physiological concentrations of chloride ions, can also react with nitrite, forming the reactive intermediate nitryl chloride. Since MPO and MPO-derived HOCl, as well as reactive nitrogen species, have been implicated in the pathogenesis of atherosclerosis through oxidative modification of low density lipoprotein (LDL), we investigated the effects of physiological concentrations of nitrite (12.5–200 μM) on MPO-mediated modification of LDL in the absence and presence of physiological chloride concentrations. Interestingly, nitrite concentrations as low as 12.5 and 25 μM significantly decreased MPO/H$_2$O$_2$/Cl$^-$-induced modification of apoB lysine residues, formation of N-chloramines, and increases in the relative electrophoretic mobility of LDL. In contrast, none of these markers of LDL atherogenic modification were affected by the MPO/H$_2$O$_2$/NO$_2^-$ system. Furthermore, experiments using ascorbate (12.5–200 μM) and the tyrosine analogue 4-hydroxyphenylacetic acid (12.5–200 μM), which are both substrates of MPO, indicated that nitrite inhibits MPO-mediated LDL modifications by trapping the enzyme in its inactive compound II form. These data offer a novel mechanism for a potential antiatherogenic effect of the nitric oxide congener nitrite.

Nitric oxide (nitrogen monoxide, NO$^\cdot$) is synthesized in vivo by a family of inducible and constitutively expressed nitric-oxide synthases (NOS)$^1$ (1, 2). Nitric oxide generated by the NOS isoform present in endothelial cells (eNOS) is critically involved in normal vascular function through regulation of smooth muscle cell relaxation and vasodilation as well as modulation of platelet, leukocyte, and endothelial cell adhesion (1, 2). The inducible NOS isoform present in phagocytes (iNOS) is thought to be involved in their antimicrobial activity, whereas up-regulation of iNOS during chronic inflammation has been implicated in vascular pathology (1, 3). Since nitric oxide does not readily react with biological macromolecules, the tissue damage associated with increased nitric oxide levels has been attributed to the generation of peroxynitrite (3), which is formed by rapid reaction of nitric oxide with superoxide ($k_2 = 1.9 \times 10^{10} \text{ M}^{-1} \text{s}^{-1}$) (4). Under aerobic conditions, nitric oxide also reacts with molecular oxygen ($k_2 = 6 \times 10^6 \text{ M}^{-2} \text{s}^{-1}$) (5) to form a dinitrogen trioxide intermediate that hydrolyzes to nitrite (Reaction 1) (5–7). Nitrite is found in biological fluids at concentrations between 0.5 and 210 μM (8–10). In human plasma, levels of nitrite are typically low, between 0.5 and 3.3 μM (8, 9), due to oxidation of nitrite to nitrate by oxyhemoglobin (3). In inflammatory conditions, however, plasma nitrite levels can significantly increase, e.g. up to 36 μM in patients with human immunodeficiency virus infection (11).

$$\text{H}_2\text{O} + \frac{1}{2}\text{O}_2 \rightarrow \text{N}_2\text{O}_3 \rightarrow \text{NO}_2^- + 2\text{H}^+$$

**REACTION 1**

Leukocytes such as neutrophils, monocytes, and macrophages, as well as endothelial cells, can synthesize both nitric oxide and superoxide (1, 12). Thus, it is likely that peroxynitrite is formed in vivo by these cells. 3-Nitrotyrosine, a product of the reaction of peroxynitrite with either free tyrosine or tyrosine residues in (lipi)proteins has been used as a biomarker for the generation of peroxynitrite in vivo (13). Elevated 3-nitrotyrosine levels have been detected, e.g. in atherosclerotic lesions (14, 15), as has increased expression of the inducible NOS isoform (16, 17). Leukocytes, however, also release the abundant heme protein myeloperoxidase (MPO) upon activation by inflammatory stimuli (12, 18), and recent studies show that mammalian peroxidases can oxidize nitrite to a radical species, most likely nitrogen dioxide (Reaction 2) (19, 20). Furthermore, hypochlorous acid (HOCl), the major strong oxidant generated by MPO in the presence of physiological concentrations of chloride ions (Reaction 3) (12, 18), can also react with nitrite, forming the reactive intermediate nitryl chloride (Reaction 4) (21, 22). Both nitrogen dioxide and nitryl chloride, like peroxynitrite, can nitrate tyrosine residues (21, 23, 24), calling into question the specificity of 3-nitrotyrosine as a marker of peroxynitrite generation in vivo (25, 26).

$$2\text{NO}_2^- + \text{H}_2\text{O}_2 + 2\text{H}^+ \rightarrow 2\text{NO}_3^- + 2\text{H}_2\text{O}$$

**REACTION 2**

$$\text{MPO} + \text{Cl}^- + \text{H}_2\text{O}_2 + \text{H}^+ \rightarrow \text{HOCl} + \text{H}_2\text{O}$$

**REACTION 3**
Several recent studies implicate MPO and MPO-derived HOCl in the pathogenesis of atherosclerosis (27–31). Catalytically active MPO (27) and epitopes recognized by antibodies against HOCl-modified proteins have been detected in human atherosclerotic lesions (28); these were found to colocalize with monocye/macrophages, endothelial cells, and the extracellular matrix (29). Dityrosine and 3-chlorotyrosine, biomarkers of MPO- and HOCl-mediated protein modification, have also been detected in atherosclerotic lesions (30, 31). Oxidative modification of low density lipoprotein (LDL) in vitro by MPO or HOCl primarily involves chlorination of the ε-amino groups of lysine residues of apolipoprotein B-100 (apoB), the major protein component of LDL, resulting in the formation of N-chloramines (32–34). LDL-associated N-chloramines have been implicated in the altered electrophoretic migration, aggregation, and subsequent uncontrolled uptake of HOCl-modified LDL by macrophages (32, 33, 35). Since 3-nitrotyrosine has also been detected in atherosclerotic lesions (14, 15), it is possible that MPO-derived reactive nitrogen species are involved in atherogenesis in addition to HOCl. In contrast to HOCl, however, MPO-derived reactive nitrogen species primarily cause lipid peroxidation in LDL (36–38). Thus, increased macrophage uptake of LDL modified by reactive nitrogen species is most likely due to increased levels of lipid oxidation products (36, 39).

In this study we investigated the effects of physiological concentrations of nitrite (12.5–200 μM) on MPO-mediated modification of LDL in the absence and presence of physiological chloride concentrations (140 mM). In addition, we examined whether the physiological antioxidant ascorbate can inhibit LDL modification under these conditions. Our data reveal a novel mechanism by which nitrite inhibits MPO-mediated atherogenic modification of LDL.

EXPERIMENTAL PROCEDURES

Materials—Human leukocyte MPO and anti-nitrotyrosine monoclonal antibodies were procured from Calbiochem, HOCl was from Aldrich, and 7-fluorobenz-2-oxa-1,3-diazole-4-sulfonamide (ABD-F) was from Molecular Probes, Eugene, OR. Alkaline phosphatase-conjugated anti-mouse IgG antibody and BCIP/TNTB color reagent were from Chemicon International, Temecula, CA. All other reagents were obtained from Sigma. Phosphate-buffered saline (PBS) was composed of 10 mM sodium phosphate buffer, 140 mM NaCl, pH 7.4, and contained the metal chelator diethylthiocarbamiminetetraacetic acid (DTPA, 100 μM). Tris-buffered saline was composed of 10 mM Tris-HCl, 140 mM NaCl, pH 7.4, and contained 0.1% Tween 20. Griess reagent was composed of 1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2.5% H3PO4. Thionitrobenzoic acid (TNB) was prepared from 5,5'-sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 36–38% nitrite (12.5–200 μM) residues; formation of RNCHCl, (RNHCl) and increase in REM of LDL (7) were determined as described under “Experimental Procedures.” Percent N-chloramines was estimated using 356 lysine residues per LDL (52). Results represent the mean ± S.D. (n = 3–4).

Oxidation of LDL by MPO/H2O2/Cl−—Freshly isolated human LDL (0.5 mg of protein/ml of PBS containing 100 μM DTPA) was incubated for 30 min at 37 °C with MPO (50 nM) and increasing concentrations of H2O2 (25–200 μM), added in 25 or 50 μM aliquots over the incubation period. a, modification of apoB cysteine (●), tryptophan (■), and lysine (□) residues; b, formation of N-chloramines (RNCHCl, ●) and increase in REM of LDL (□) were determined as described under “Experimental Procedures.” Percent N-chloramines was estimated using 356 lysine residues per LDL (52). Results represent the mean ± S.D.

RESULTS

Modification of LDL by MPO/H2O2/Cl−—We (34) and others (32, 33) have shown that bolus addition of reagent HOCl (25–200 μM) to human LDL results in a number of modifications to apoB, e.g. oxidation of cysteine, tryptophan, and lysine residues, formation of N-chloramines, and an increase in REM. In the present study, we investigated the physiologically more relevant system of HOCl generation by MPO/H2O2/Cl−. The addition of increasing amounts of H2O2 (25–200 μM) to human LDL (0.5 mg protein/ml; ~1 μM) in the presence of MPO (50 nM) caused basically identical modifications to LDL (Fig. 1) as those seen with reagent HOCl (34). In contrast, 200 μM H2O2 alone did not cause oxidation of LDL amino acid residues or an
increase in REM (data not shown). Using the MPO/H2O2/Cl\textsuperscript{−} system, all of the added H2O2 was converted into HOCl as determined by TNB oxidation using taurine as a trap (40). Cysteine residues were the most sensitive target on LDL and were completely oxidized in the presence of 75 μM H2O2 (Fig. 1a). Lysine and tryptophan residues were modified at equal rates, with 39 and 43% of the residues modified, respectively, after the addition of 200 μM H2O2 (Fig. 1a). Since LDL contains ~3–5 free cysteine residues (50, 51), 356 lysine residues, and 37 tryptophan residues (52), lysine residues can be calculated to be quantitatively the major target of HOCl generated by the MPO system. The decrease in lysine residues was mirrored by the formation of N-chloramines, which accounted for approximately one-third of the added H2O2 (Fig. 1b). A dose-dependent increase in REM was also observed with increasing concentrations of H2O2 (Fig. 1b).

Modification of LDL by MPO/H2O2/NO\textsubscript{2}—Previous studies have investigated the modification of LDL by MPO/H2O2/NO\textsubscript{2} in the absence of chloride (36, 37). We found that this system caused relatively few changes to apoB under the conditions used in the present study (Fig. 2). No significant modification of lysine residues occurred, and only a small dose-dependent decrease of tryptophan residues was observed with increasing nitrite concentrations (Fig. 2a); ~25% of the tryptophan residues in LDL were oxidized in the presence of 200 μM nitrite. Analysis of cysteine residues was confounded by the fact that in the absence of nitrite ~50% of the residues were already oxidized (Fig. 2a). Since 200 μM H2O2 alone did not cause cysteine oxidation (data not shown), this finding is most likely due to a small amount of halide contamination in the buffers used. Nevertheless, nitrite did not exert any dose-dependent effect on oxidation of apoB cysteine residues (Fig. 2a). In agreement with the lack of lysine oxidation by the MPO/H2O2/NO\textsubscript{2} system, there was no increase in the REM or TNB reactivity of LDL (Fig. 2c). However, dot blot analysis using an anti-nitrotyrosine monoclonal antibody showed dose-dependent formation of 3-nitrotyrosine (Fig. 2b). Interestingly, measurement of nitrite using the Griess assay indicated that approximately twice as much of the added nitrite was consumed in the absence of chloride than in its presence (e.g. 124 ± 4 μM nitrite versus 56 ± 7 μM nitrite, respectively, after the addition of 200 μM nitrite) (data not shown). It should be noted, however, that reaction of nitrogen dioxide with substrates that can donate hydrogens regenerates nitrite, and thus, the Griess assay may underestimate the amount of nitrite utilized by MPO.

Modification of LDL by MPO/H2O2/Cl\textsuperscript{−} and NO\textsubscript{2}—Since nitrite is a substrate for MPO (20), the effect of physiological concentrations of nitrite on LDL modification in the presence of 200 μM H2O2 and physiological chloride concentrations was investigated. The addition of increasing concentrations of nitrite (12.5–200 μM) affected specific MPO-mediated modifications of LDL (Fig. 3). Low concentrations of nitrite (≤12.5 μM) significantly decreased modification of apoB lysine residues induced by MPO/H2O2/Cl\textsuperscript{−} (Fig. 3a). A concurrent, although less extensive, decrease in modification of tryptophan residues was observed at 25–50 μM nitrite, but this effect was lost at higher nitrite concentrations (Fig. 3a). Since the cysteine residues were almost fully oxidized with 200 μM H2O2 (Fig. 3a), LDL was treated with sufficient H2O2 (25 μM) to oxidize approximately one-third of the cysteine residues; the addition of nitrite, however, did not have a significant effect on thiol oxidation at this concentration of H2O2 (data not shown). In agreement with the decrease in lysine oxidation by low concentrations of nitrite (Fig. 3a), formation of N-chloramines and increase in REM were also inhibited (Fig. 3b). Interestingly, at higher concentrations of nitrite (150 and 200 μM), the REM increased again, although not to control levels seen in the absence of nitrite (Fig. 3b). This increase in REM could be a result of increased nitrite-dependent lipid peroxidation and subsequent derivatization of apoB lysine residues to carbonyls by lipid hydroperoxide breakdown products. In support of this hypothesis, we found that nitrite dose-dependently increased apoB carbonyl levels (e.g. 14 ± 1 μM carbonyls without nitrite versus 60 ± 2 μM carbonyls at 200 μM nitrite) but not until the concentrations of added nitrite reached ≥50 μM (data not shown).

Modification of LDL by Reagent HOCl in the Presence of NO\textsubscript{2}—A number of mechanisms could account for the marked decrease in lysine oxidation, N-chloramine formation, and increase in REM by MPO/H2O2/Cl\textsuperscript{−} in the presence of low concentrations of nitrite (Figs. 3, a and b). For example, nitrite could “scavenge” MPO-derived HOCl (21, 22) or react with preformed N-chloramines. To investigate the former mechanism, LDL was exposed to reagent HOCl (200 μM) in the presence of increasing concentrations of nitrite. As shown in Table 1, nitrite had little effect on the oxidation of LDL by HOCl, with only a small dose-dependent decrease in lysine oxidation and N-chloramine formation. Thus, nitrite does not appear to protect LDL to a significant extent by scavenging HOCl. Similarly, when LDL was pretreated with HOCl and subsequently incubated with nitrite, no reversal of N-chloramine formation or lysine modification was observed (data not shown).

Effects of Ascorbate on LDL Modification by MPO/H2O2/Cl\textsuperscript{−}

Fig. 2. Modification of LDL by MPO/H2O2/NO\textsubscript{2} LDL (0.5 mg of protein/ml of phosphate buffer containing 100 μM DTPA) was incubated for 30 min at 37 °C with MPO (50 μM) and H2O2 (200 μM; added in 50 μM aliquots over the incubation period) in the presence of increasing concentrations of nitrite (12.5–200 μM). a, modification of apoB cysteine (●), tryptophan (○), and lysine (◇) residues; b, formation of 3-nitrotyrosine (3-NT); c, formation of N-chloramines (RNHCl) (●) and increase in REM of LDL (◇) were determined as described under “Experimental Procedures.” Percent N-chloramines was estimated using 356 lysine residues per LDL (52). Results represent the mean ± S.D. (n = 3).

Fig. 3. Modification of LDL by MPO/H2O2/Cl\textsuperscript{−} and NO\textsubscript{2} LDL (0.5 mg of protein/ml of PBS containing 100 μM DTPA) was incubated for 30 min at 37 °C with MPO (50 μM) and H2O2 (200 μM; added in 50 μM aliquots over the incubation period) in the presence of increasing concentrations of nitrite (12.5–200 μM). a, modification of apoB cysteine (●), tryptophan (○), and lysine (◇) residues; b, formation of N-chloramines (RNHCl) (●) and increase in REM of LDL (◇) were determined as described under “Experimental Procedures.” Percent N-chloramines was estimated using 356 lysine residues per LDL (52). Results represent the mean ± S.D. (n = 3).
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Modification of LDL by 200 μM HOCl in the presence of increasing concentrations of nitrite

| %     | Lys μM | Trp μM | Cys μM | RNHCl μM | REM μM | P value |
|-------|--------|--------|--------|----------|--------|---------|
| 0     | 72 ± 2 | 73 ± 6 | 5 ± 3  | 19 ± 5   | 61 ± 12| 12.5    |
| 12.5  | 74 ± 1 | 72 ± 12| 1 ± 1  | 19 ± 3   | 61 ± 12| 25      |
| 25    | 76 ± 2 | 71 ± 8 | 1 ± 1  | 16 ± 2   | 61 ± 12| 50      |
| 50    | 78 ± 5 | 70 ± 7 | 1 ± 1  | 14 ± 1   | 61 ± 12| 100     |
| 100   | 80 ± 7 | 69 ± 8 | 1 ± 1  | 13 ± 2   | 61 ± 12| 150     |
| 200   | 79 ± 4 | 69 ± 9 | 1 ± 1  | 15 ± 3   |       |         |

Nitrite and NO₂—Another potential mechanism for low concentrations of nitrite decreasing MPO/H₂O₂/Cl⁻-mediated modification of LDL is inhibition of enzyme activity. This is conceivable because nitrite has a relatively high reaction rate with compound I and a low reaction rate with compound II of MPO (20), thus effectively trapping the enzyme in a form incapable of generating HOCl (Scheme 1). To investigate this possibility, the complete system (MPO/H₂O₂/Cl⁻/NO₂) was incubated with ascorbate, a “cosubstrate” of MPO that has a relatively high reaction rate with compound II (53) (54) and, thus, can cycle the enzyme (Scheme 1). Indeed, the addition of ascorbate to LDL in the presence of the complete MPO system strongly affected LDL modification (Fig. 4). Low concentrations of ascorbate (12.5–50 μM) abrogated the inhibitory effect of the equivalent concentrations of nitrite on lysine oxidation by MPO (Fig. 4a). In contrast to the complete system (Fig. 3a), the addition of ascorbate provided almost stoichiometric protection against lysine and tryptophan oxidation (Fig. 4a). Another noticeable difference between the complete system in the absence (Fig. 3a) and the presence of ascorbate (Fig. 4a) was the significant inhibition of cysteine oxidation at high concentrations of ascorbate (150 and 200 μM). In agreement with the dose-dependent protection against lysine modification in the presence of ascorbate, dose-dependent inhibition of N-chloramines formation and an increase in REM was also observed under these conditions (Fig. 4b), with complete protection by 150 and 200 μM ascorbate.

**Effects of Ascorbate on LDL Modification by MPO/H₂O₂/Cl⁻ without NO₂**—In the absence of nitrite, ascorbate provided identical protection as in the presence of nitrite against MPO-mediated oxidation of lysine, tryptophan, and cysteine residues, formation of N-chloramines, and increases in REM (compare Figs. 5, a and b with Figs. 4, a and b, respectively). Since low μM concentrations of ascorbate have been shown to stimulate HOCl production by MPO (53), LDL was treated with sufficient H₂O₂ (25 μM) to oxidize approximately one-third of the cysteine residues, and low concentrations (0.5–5 μM) of ascorbate were added. These concentrations of ascorbate, however, failed to enhance thiol oxidation, whereas slightly higher concentrations (5–50 μM) dose-dependently inhibited thiol oxidation (data not shown). The above findings (Figs. 5, a and b) agree with our previous data using bolus addition of HOCl to LDL in the presence of increasing concentrations of ascorbate (34). The one exception, however, is protection against modification of cysteine residues; 200 μM ascorbate gave approximately 2-fold greater protection in the MPO/H₂O₂/Cl⁻ system compared with oxidation by 200 μM reagent HOCl (80 ± 15% versus 39 ± 6% protection, respectively). Interestingly, HPLC analysis showed that all of the added ascorbate (200 μM) was consumed in the MPO/H₂O₂/Cl⁻ system, whereas 36 ± 12 μM (n = 3) ascorbate remained in the presence of HOCl (data not shown). This difference is likely due to more efficient scavenging by ascorbate of HOCl continuously generated by MPO rather than added as a bolus.

**Effects of HPA on LDL Modification by MPO/H₂O₂/Cl⁻ and NO₂**—Ascorbate not only cycles MPO by reducing compound II to the native enzyme (54) but also scavenges HOCl in a stoichiometric manner (43) and regenerates amines from N-chloramines (34). To avoid these “confounding” reactions, the tyrosine analogue HPA was used as an alternative substrate to

**Table I**

| %     | Lys value | Trp value | Cys value | RNHCl value | REM value |
|-------|-----------|-----------|-----------|-------------|-----------|
| 0     | 72 ± 2    | 73 ± 6    | 5 ± 3     | 19 ± 5      | 61 ± 12   |
| 12.5  | 74 ± 1    | 72 ± 12   | 1 ± 1     | 19 ± 3      | 61 ± 12   |
| 25    | 76 ± 2    | 71 ± 8    | 1 ± 1     | 16 ± 2      | 61 ± 12   |
| 50    | 78 ± 5    | 70 ± 7    | 1 ± 1     | 14 ± 1      | 61 ± 12   |
| 100   | 80 ± 7    | 69 ± 8    | 1 ± 1     | 13 ± 2      | 61 ± 12   |
| 150   | 79 ± 4    | 69 ± 9    | 1 ± 1     | 15 ± 3      |           |
| 200   |           |           |           |             |           |

**Fig. 4.** Effect of ascorbate on LDL modification by MPO/H₂O₂/Cl⁻ and NO₂. LDL (0.5 mg of protein/ml of PBS containing 100 μM DTPA) was incubated for 30 min at 37 °C with MPO (50 nm) and H₂O₂ (200 μM; added in 50 μM aliquots over the incubation period) in the presence of increasing concentrations of ascorbate (12.5–200 μM) and nitrite (12.5–200 μM). a, alteration of apoB cysteine residues (Δ); formation of N-chloramines (RNHCl); and increase in REM of LDL (Δ) were determined as described under “Experimental Procedures.” Percent N-chloramines was estimated using 356 lysine residues per LDL (52). Results for Lys, Trp, and Cys are expressed as percent control and for RNHCl and REM as percent increase and represent the mean ± S.D. (n = 3). NS, not significant.
cycle MPO since it has only minimal reactivity with HOCl and N-chloramines. Like ascorbate, the addition of low concentrations of HPA (12.5 and 25 μM) to the complete MPO/ H2O2/Cl-/NO2 system abrogated the inhibitory effect of nitrite on lysine oxidation (compare Figs. 3a and 6a). However, unlike ascorbate, higher concentrations of HPA (50–200 μM) did not dose-dependently protect against modification of lysine, tryptophan, and cysteine residues (compare Figs. 4a and 6a). Modification of tryptophan residues was actually increased in the presence of HPA (Fig. 6a), most likely due to enhanced turnover of the enzyme. The small decrease in N-chloramines and change in REM (Fig. 6b) and modification of lysine residues (Fig. 6a) could be due to increased competition for reaction of chloride with compound I of MPO by the additional substrates nitrite and HPA (see Scheme 1). In the absence of nitrite, HPA had little effect on the modification of apoB by the MPO/ H2O2/Cl- system (data not shown). The lack of an effect of HPA on tryptophan oxidation in the latter system (data not shown) suggests that it is nitrogen dioxide radicals rather than HPA-derived phenoxy radicals that are causing tryptophan oxidation in the complete system (Fig. 6c; see also Fig. 2a).

DISCUSSION

The present study shows for the first time that the nitric oxide congener nitrite inhibits MPO-mediated modification of LDL. Modification of LDL by MPO in the presence of physiologically relevant concentrations of H2O2 (25–200 μM) and chloride ions (140 mM) results in a number of potentially atherogenic alterations to LDL. These include modification of lysine residues to form N-chloramines and an increase in REM of LDL, which reflects an increase in its net negative charge. We have shown that modification of LDL with 200 μM HOCl (34) or with MPO, 200 μM H2O2, and chloride ions (this study) causes modification of 26 and 39% of LDL lysine residues, respectively. Previous studies using chemically modified LDL show that modification of as little as 16% of the apoB lysine residues results in recognition of LDL by the scavenger receptor(s) of macrophages (55, 56). LDL modified with HOCl also exhibits increased uptake by macrophages (32, 35), likely due to modification of the apoB lysine residues by HOCl (32). In the presence of physiologically relevant concentrations of nitrite (12.5–50 μM) (8, 11), a major oxidation product of nitric oxide, a dose-dependent decrease in the MPO/H2O2/Cl- -mediated oxidation of LDL lysine residues was observed. A similar decrease in N-chloramines and change in REM was also observed.

There are a number of mechanisms that could account for the marked decrease in MPO/H2O2/Cl- -induced lysine oxidation, N-chloramine formation, and increase in REM by low concentrations of nitrite. HOCl reacts with nitrite in a stoichiometric manner to form intermediates such as nitryl chloride (21, 22), which may have decreased reactivity with amines. However, we found that exposure of LDL to HOCl (200 μM) in the presence of increasing concentrations of nitrite (25–200 μM) only minimally decreased oxidation of lysine residues and formation of N-chloramines. Others have shown that the ratio of nitrite to HOCl needs to be greater than unity to afford protection against HOCl-mediated cytotoxicity (57–59). The protective effects of nitrite would also be highly dependent on other available targets. HOCl reacts with nitrite with a second order rate constant of 7.4 × 10⁻¹⁰ m⁻¹ s⁻¹ (22); in contrast, the reaction of HOCl with amines is almost 2 orders of magnitude faster (k2 = 4.8 × 10⁻⁸ m⁻¹ s⁻¹), and with thiols, approximately 4 orders of magnitude faster (k2 > 10⁻⁶ m⁻¹ s⁻¹) (60). Thus, 20 mM nitrite would be required to completely protect LDL amines from oxidation by 200 μM HOCl. Furthermore, since preformed LDL-associated N-chloramines did not react with nitrite, as has been observed previously for taurine chloramine (59, 61), this mechanism is unlikely to account for the decreased levels of N-chloramines and lysine modification observed in the present study.

An alternative inhibitory mechanism of nitrite could involve competition between nitrite and chloride ions for reaction with compound I of MPO (see Scheme 1). In the absence of chloride ions, the MPO/H2O2/NO2 system failed to significantly modify lysine residues or increase REM of LDL, implying that the MPO-derived products of nitrite are unable to cause significant oxidation of amines. The rate constant for the reaction of nitrite with compound I (kJ = 2.0 × 10⁻⁹ m⁻¹ s⁻¹) (20) is 2 orders of magnitude greater than that for chloride with compound I (k2 = 2.5 × 10⁻⁴ m⁻¹ s⁻¹) (62). However, chloride was present in our experiments at a concentration approximately 4 orders of magnitude higher than that of nitrite (140 mM and 12.5 μM, respectively). Furthermore, the activity of MPO (compound I) is not saturated at plasma concentrations of chloride (63). Therefore, it is unlikely that competition between these physiological concentrations of nitrite and chloride for compound I are the major mechanism.

The most likely mechanism by which low concentrations of nitrite decrease MPO/H2O2/Cl- -mediated modifications of LDL is inhibition of MPO enzyme activity. This could occur if nitrite has a low reaction rate with compound II of MPO (20), thus effectively trapping the enzyme in a form incapable of gener-
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ating HOCl (see Scheme 1). To determine whether nitrite is inhibiting MPO by this mechanism, a cosubstrate with a relatively high reaction rate with compound II can be used to cycle the enzyme. One such substrate is ascorbate, which has been shown to increase the chlorinating activity of MPO (53) by reducing compound II back to the native enzyme (54). The second order rate constant for the reaction of ascorbate with compound II of MPO is $1.1 \times 10^3$ M$^{-1}$ s$^{-1}$ (64), which is approximately 2 orders of magnitude higher than the reaction of nitrite with compound II ($k_2 = 5.5 \times 10^2$ M$^{-1}$ s$^{-1}$) (20). As such, ascorbate should be able to effectively cycle the enzyme back to its native ferric form. We found that the addition of low concentrations of ascorbate (12.5–50 μM) significantly attenuated the inhibition of lysine oxidation, N-chloramine formation, and change in REM by equivalent concentrations of nitrite, whereas higher concentrations of ascorbate dose-dependently inhibited MPO-mediated modifications of apoB. Thus, although ascorbate protects against modification of LDL by HOCl (34) and MPO/HOCl/Cl$^-$ (this study), nitrite appears to be a better “antioxidant” than ascorbate at low μM concentrations due to inhibition of the chlorinating activity of MPO. However, at higher concentrations, ascorbate was a better antioxidant than nitrite, as indicated by dose-dependent protection against tryptophan and cysteine oxidation and an increase in REM, most likely due to direct scavenging of HOCl.

Since ascorbate not only cycles MPO by reducing compound II to the native enzyme (54) but also scavenges HOCl in a stoichiometric manner (43) and regenerates amines from N-chloramines (34), we sought to compare the effects of ascorbate with an alternative substrate. Others have shown that MPO-mediated nitration of tyrosine residues can be enhanced by addition of free tyrosine (65). Since the rate constant for the reaction of tyrosine with compound II of MPO is even greater than that of ascorbate (i.e. $k_3 = 1.6 \times 10^9$ M$^{-1}$ s$^{-1}$) (66), tyrosine also effectively cycles the enzyme. We used HPA instead of tyrosine to avoid interfering reactions of MPO-derived oxidants with the amino group of tyrosine and found a significant decrease in the nitrite-dependent inhibition of LDL lysine oxidation, N-chloramine formation, and change in REM. These results agree with those observed in the presence of ascorbate (see above) and support our hypothesis that nitrite inhibits MPO-mediated modification of LDL by trapping the enzyme in its “inactive” compound II form.

Several groups have investigated the reaction of HOCl-, MPO-, and leukocyte-derived-nitrating species with LDL (22, 36–39). The reactive nitrogen species results in enhanced macrophage cholesterol accumulation (22, 36, 39), likely due to increased levels of lipid oxidation products (36, 39). MPO-derived HOCl, in contrast, causes very little LDL lipid peroxidation (32, 33) but still converts LDL into a high uptake form for macrophages (32, 35), likely due to direct modification of lysine residues.

A number of the above studies also measured formation of 3-nitrotirosine (22, 36, 38, 39). Chloride does not appear to inhibit nitrite-dependent 3-nitrotirosine formation in LDL (36, 38), likely due to incomplete saturation of the activity of MPO (compound I) at physiological concentrations of chloride (63). Although a potentially useful biomarker of nitration reactions, 3-nitrotirosine is only a minor product, e.g., ~0.3 mmol/mol of tyrosine formed in the presence of 50 to 500 μM nitrite (36, 38), and the (patho)physiological significance of this type of modification for atherosclerosis is unknown (39).

In this study we observed a protective effect of low concentrations of nitrite against MPO-dependent modification of LDL lysine residues, most likely due to inhibition of the chlorinating activity of MPO. The nitrite-dependent decrease in oxidative modification of LDL may translate into decreased LDL uptake by macrophages, thus attenuating the formation of lipid-laden foam cells, the hallmark of atherosclerotic lesions. Since NO itself has been shown to inhibit leukocyte-dependent modification of LDL by reacting with lipid radicals (67), this study provides a novel mechanism by which metabolites of NO may exert an antiatherogenic effect.

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