FEBS Letters 487 (2000) 176–180 FEBS 24328

Myeloperoxidase binds to low-density lipoprotein: potential implications for atherosclerosis

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Received 23 October 2000; accepted 31 October 2000

First published online 10 November 2000

Edited by Shozo Yamamoto

Abstract Myeloperoxidase (MPO), an abundant heme enzyme released by activated phagocytes, catalyzes the formation of a number of reactive species that can modify low-density lipoprotein (LDL) to a form that converts macrophages into lipid-laden or 'foam' cells, the hallmark of atherosclerotic lesions. Since MPO has been shown to bind to a number of different cell types, we investigated binding of MPO to LDL. Using the precipitation reagents phosphotungstate or isopropanol, MPO co-precipitated with LDL, retaining its catalytic activity. The association of MPO with LDL was confirmed using native gel electrophoresis. MPO was also found to co-precipitate with apolipoprotein B-100-containing lipoproteins in whole plasma. No precipitation of MPO was observed in lipoprotein-deficient plasma, and there was a dose-dependent increase in precipitation following addition of LDL to lipoprotein-deficient plasma. Binding of MPO to LDL could potentially enhance site-directed oxidation of the lipoprotein and limit scavenging of reactive oxygen species by antioxidants.

Key words: Atherosclerosis; Low-density lipoprotein; Myeloperoxidase; Plasma

1. Introduction

Oxidative modification of low-density lipoprotein (LDL) trapped within the arterial subendothelial space has been strongly implicated in the development and progression of atherosclerosis [1]. Oxidized LDL has a number of potentially atherogenic effects on vascular cells in culture, including uncontrolled uptake by the scavenger receptors of macrophages, resulting in the formation of lipid-laden or 'foam' cells, the hallmark of fatty streaks [1]. Although the in vivo mechanisms of LDL oxidation are not yet fully elucidated, in vitro studies have shown that various vascular cells, including neutrophils, monocytes, macrophages, endothelial cells and smooth muscle cells, can oxidize LDL [1]. These cells contain a number of enzymes that produce reactive oxygen species [2,3]. The plasma membrane-associated NADPH oxidase reduces extracellular oxygen to superoxide anion radical [2], which dismutates to form hydrogen peroxide [2]. Nitric oxide synthase isozymes generate nitric oxide [3], which reacts rapidly with superoxide to form peroxynitrite [1]. These reactive oxygen and nitrogen species, bar nitric oxide [4], have been implicated in cell-mediated LDL oxidation [1,4].

Leukocytes also contain the abundant heme enzyme myeloperoxidase (MPO), which is released from storage granules following activation of the cells by inflammatory stimuli [2,5]. MPO catalyzes the reaction of hydrogen peroxide with chloride ions to produce the strong oxidant hypochlorous acid (HOCl) [5]. Studies have shown that HOCl can modify LDL to a form that is accumulated by macrophages [6]. Furthermore, HOCl-modified LDL has a number of other detrimental effects on vascular cells, including stimulating increased generation of reactive oxygen species [7–9]. Recent studies have shown that MPO can also utilize nitrite, the major aerobic metabolite of nitric oxide, to generate reactive nitrogen species, most likely nitrogen dioxide radicals [10,11], and that these reactive nitrogen species can modify LDL to a form that is recognized by the scavenger receptors of macrophages [12].

MPO is a highly cationic protein ($pI > 10$ [13]) and can adhere to endothelial cells [13,14], leukocytes [15,16], certain bacteria [17,18] and yeast [19], with retention of peroxidase activity. Thus, it is possible that association of MPO with LDL could potentially enhance site-directed oxidation of the LDL and limit the scavenging of reactive oxygen and nitrogen species by antioxidants. Therefore, we have investigated the binding of MPO to isolated LDL and apolipoprotein B-100 (apoB)-containing lipoproteins in plasma.

2. Materials and methods

2.1. Materials

Human leukocyte MPO was purchased from Calbiochem, La Jolla, CA, USA. PD-10 gel filtration columns were from Pharmacia Biotech, Uppsala, Sweden. Native Tris–glycine gels were from Novex, San Diego, CA, USA. All other reagents were from Sigma Chemical, St. Louis, MO, USA. Phosphate-buffered saline (PBS) was comprised of 10 mM phosphate buffer, 140 mM NaCl, pH 7.4.

2.2. Preparation of plasma, lipoproteins and lipoprotein-deficient plasma

Peripheral blood was drawn from healthy volunteers into vacutainers containing EDTA, heparin, or no addition for serum samples. To obtain plasma, the blood was centrifuged at $1125 \times g$ for 20 min at 4°C. LDL and very low-density lipoprotein (VLDL) were isolated from EDTA-anticoagulated plasma by a sequential centrifugation method [20], modified as described previously [21]. The isolated LDL ($1.019 < d < 1.067$ g/ml fraction) and VLDL ($d < 1.019$ g/ml

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Abbreviations: ApoB, apolipoprotein B-100; HRP, horseradish peroxidase; LDL, low-density lipoprotein; LPO, lactoperoxidase; MPO, myeloperoxidase; TMB, tetramethylbenzidine; VLDL, very low-density lipoprotein

PII: S0014-5793(00)02227-4

fraction) were desalted by two sequential passages through PD-10 gel filtration columns using PBS. The total protein content was determined using the Lowry Micro Method kit (Sigma P5656). Lipoprotein-deficient plasma was prepared from the $d > 1.21$ g/ml fraction of plasma and was dialyzed at 4°C against three changes of Chelex-treated PBS.

2.3. Incubation conditions

MPO, horseradish peroxidase (HRP, type VI-A) and lactoperoxidase (LPO, from bovine milk) were incubated at 50 nM concentrations with LDL (0.5 mg of protein/ml PBS, ≈1 μM) before addition of precipitating reagents. Potential inhibitors of complex formation, such as mannan (from *Saccharomyces cerevisiae*; 1 mM) and mixed alkyltrimethylammonium bromide (Cetrimide; 0.5%), were pre-incubated with both MPO and LDL. MPO was also added to whole plasma and lipoprotein-deficient plasma at ambient temperature. Known amounts of LDL were added back into the lipoprotein-deficient plasma using appropriate dilution controls.

2.4. Precipitation techniques

The samples (100 μl) containing LDL and the peroxidases were treated with $MgCl_2$ (10 μl of 0.5 M) and phosphotungstate (10 μl of a 4% stock solution in 0.19 M NaOH) to precipitate the LDL [22]. The samples were centrifuged and peroxidase activity was measured in the supernatants and the pellets, following resolubilization of the pellets with 0.1% Tween-20. For the supernatants, numerical values are presented as a percentage of the control activity in the supernatant, and for the pellets, values are presented as a percentage of the activity of control MPO added to resolubilized LDL. Samples were also treated with an equal volume of isopropanol [23] and peroxidase activity determined in the supernatants.

2.5. Peroxidase assay

Peroxidase activity was determined using tetramethylbenzidine (TMB) oxidation [24]. Briefly, aliquots of the phosphotungstate or isopropanol supernatants were diluted into 140 μl of 100 mM acetate buffer, pH 5.4. Samples containing HRP were diluted 100-fold before addition to the TMB assay. TMB (140 μl of 2.8 mM, diluted from 5 mg/ml stock in DMSO) and H_2O_2 (120 μl of 1 mM) were added and the samples incubated for 1 min at ambient temperature. Stop buffer (100 μl of 10 mM azide in 4 N acetic acid) was added and the absorbance measured at 655 nm.

2.6. Native gel electrophoresis

Samples were run on Tris–glycine native gels with in-gel protein and peroxidase staining [25]. Briefly, 20 μl samples containing either MPO (200 nM), LDL (0.2 mg/ml), or both were loaded onto 4–20% Tris–glycine gels and run for 3 h at 100 V. LDL was detected by protein staining using Coomassie blue and MPO was detected using in-gel peroxidase staining. For the latter, the gel was incubated for 10 min in buffer A (comprising 10 mM each of citrate and EDTA, pH 5.0), 15 min in buffer B (containing 10% dextran sulfate in buffer A), then washed three times with buffer A for 5 min each. The gel was then incubated in buffer C (containing 0.4 ml of 5 mg/ml TMB and 4.7 μl of 3% H_2O_2 per 20 ml of buffer A) to detect peroxidase activity.

3. Results

ApoB-containing lipoproteins can be precipitated from plasma using sulfated polysaccharides such as heparin and dextran sulfate [26]. However, since both MPO and LDL bind to sulfated polysaccharides [14,27], precipitation of LDL with phosphotungstate [22] and isopropanol [23] was used instead. MPO (50 nM) was incubated at ambient temperature with and without LDL (0.5 mg protein/ml PBS; ≈1 μM) and enzyme activity was measured in the supernatant following treatment of the sample with phosphotungstate. MPO in the absence of LDL was not precipitated by phosphotungstate, however, following incubation with LDL, only 5% of the added MPO activity remained in the supernatant (Fig. 1), suggesting that most of the MPO had co-precipitated with LDL. When the pellet was resuspended in 0.1% Tween-20, there was almost complete recovery of MPO activity (Fig. 1), indicating that LDL-associated MPO remains catalytically active. Control experiments showed that 0.1% Tween-20 did not inhibit or reverse MPO binding to LDL (data not shown). Time course experiments showed that the binding of MPO to LDL occurred very rapidly, within the time it took to add the precipitation reagents (data not shown).

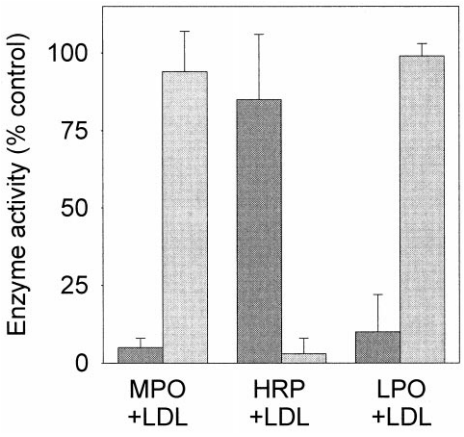

Fig. 1. Co-precipitation of MPO with LDL. MPO, HRP and LPO (50 nM each) were incubated with LDL (0.5 mg protein/ml PBS, ≈1 μM) and treated with phosphotungstate to precipitate the LDL. Peroxidase activity was then measured in the supernatant (dark bars) and the pellet resuspended in 0.1% Tween-20 (light bars), using TMB oxidation as described in Section 2. Results are presented as percent of control MPO activity and are the mean ± S.D., $n = 4$.

Two other peroxidases, the plant-derived HRP and the mammalian enzyme LPO, were also incubated at equivalent concentrations (50 nM) with LDL (Fig. 1). Following precipitation of the LDL with phosphotungstate, most of the HRP activity (85%) remained in the supernatant, whereas very little of the LPO activity (10%) was detected (Fig. 1). HRP activity in the pellet was negligible, whereas LPO activity was completely recovered from the pellet (Fig. 1). Phosphotungstate did not precipitate HRP or LPO in the absence of LDL (data not shown).

In agreement with the above findings, precipitation of LDL with isopropanol and measurement of peroxidase activity in the supernatants gave MPO, HRP and LPO activities of 2 ± 1,

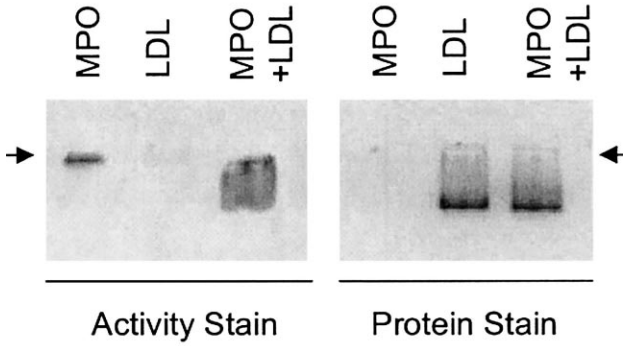

Fig. 2. Co-migration of MPO with LDL. MPO (200 nM) was incubated with LDL (0.2 mg protein/ml PBS) and run on a 4–20% Tris–glycine native gel. LDL was detected using Coomassie blue protein staining, and MPO activity was determined using in-gel peroxidase-mediated oxidation of TMB as described in Section 2. Arrows indicate the top of the gel lanes.

83 ± 21 and 7 ± 10% of controls (mean ± S.D., $n = 4$), respectively. Comparable results with the three enzymes were observed following phosphotungstate and isopropanol precipitation of the $d > 1.019$ g/ml fraction of plasma containing VLDL (data not shown).

To confirm that MPO was binding to LDL, the samples were run on native Tris–glycine gels and peroxidase activity was measured in the gel using TMB oxidation (Fig. 2). In the absence of LDL, the MPO did not enter the gel, presumably due to its charge characteristics (Fig. 2). However, in the presence of LDL, peroxidase activity was detected in the gel. There was incomplete co-migration of MPO with LDL (Fig. 2, TMB oxidation versus apoB staining), likely due to the opposing electromotive forces of the two proteins.

Since albumin is the major protein in plasma, the effect of albumin on binding of MPO to LDL was investigated. Addition of human albumin to MPO and LDL at concentrations that mimic the situation of human plasma did not inhibit co-precipitation of MPO with LDL (Fig. 3a), suggesting a specific or strong interaction between MPO and LDL. Decreasing the concentration of LDL (in the presence of albumin) resulted in a dose-dependent decrease in the amount of MPO precipitated (Fig. 3a). Approximately 30% of the MPO (15 nM) co-precipitated with 10 nM LDL (Fig. 3a, 0.01 LDL), suggesting a ratio of MPO:LDL of about 1.5 in the presence of physiological concentrations of albumin.

When MPO was added to EDTA-anticoagulated plasma, approximately 70% of the enzyme activity was precipitated by phosphotungstate (Fig. 3b); the non-precipitated MPO (30%) possibly bound to non-apoB proteins in plasma. Comparable enzyme activities were observed in precipitates of heparin-plasma and serum (35 ± 1 and 24 ± 10% of control in the supernatant, and 15 ± 5 and 25 ± 14% in the pellet, respectively, mean ± S.D., $n = 3$). Interestingly, although about 70% of the MPO activity in EDTA-plasma was lost, only 30% of the MPO activity was recovered in the pellet (Fig. 3a), suggesting partial inactivation of the added MPO or interference by plasma component(s) in the TMB assay. Lipoproteins were not isolated by ultracentrifugation from plasma to which MPO had been added because the high salt concentrations and/or *g*-forces would have likely disrupted the association between the lipoproteins and the enzyme.

MPO was next added to lipoprotein-deficient plasma. As shown in Fig. 3b, there was no significant loss of enzyme activity following addition of the precipitating reagent. When LDL was added back to the lipoprotein-deficient plasma, there was a concentration-dependent loss of MPO activity from the supernatant and concomitant recovery of the enzyme activity in the pellet (Fig. 3b). Similar results were observed when VLDL was added back into the lipoprotein-deficient plasma (data not shown). When 1.5 mg protein/ml (≈3 μM) of apoB-containing lipoprotein was added to lipoprotein-deficient plasma, a comparable amount of MPO precipitation was observed as with whole plasma (Fig. 3b, LDP+3LDL vs. plasma). Normal human plasma contains approximately 0.8–1.2 mg/ml (≈1.6–2.4 μM) apoB [28].

In order to determine the nature of the MPO/LDL complex, several compounds were pre-incubated with both MPO and LDL to determine if they could inhibit binding. Mannan from yeast cell wall has been shown to inhibit the binding of MPO to both prokaryotic and eukaryotic cells [18,19]. However, incubation of mannan (1 mM) with MPO and LDL did not inhibit their subsequent binding (data not shown). Since MPO is highly cationic ($pI > 10$), it is possible that the enzyme is binding to LDL via charge interactions [13,18]. We attempted to examine the effects of high salt concentrations on the interaction, however, these concentrations of salt interfered with the precipitation techniques used, so the data were inconclusive. In contrast, pre-incubation with the cationic detergent Cetrimide (0.5%) inhibited co-precipitation of MPO with LDL and also reversed the binding (75 ± 1% and 81 ± 8% MPO activity in the phosphotungstate supernatant, respectively, mean ± S.D., $n = 4$). These data suggest that the interaction between MPO and LDL is of ionic nature.

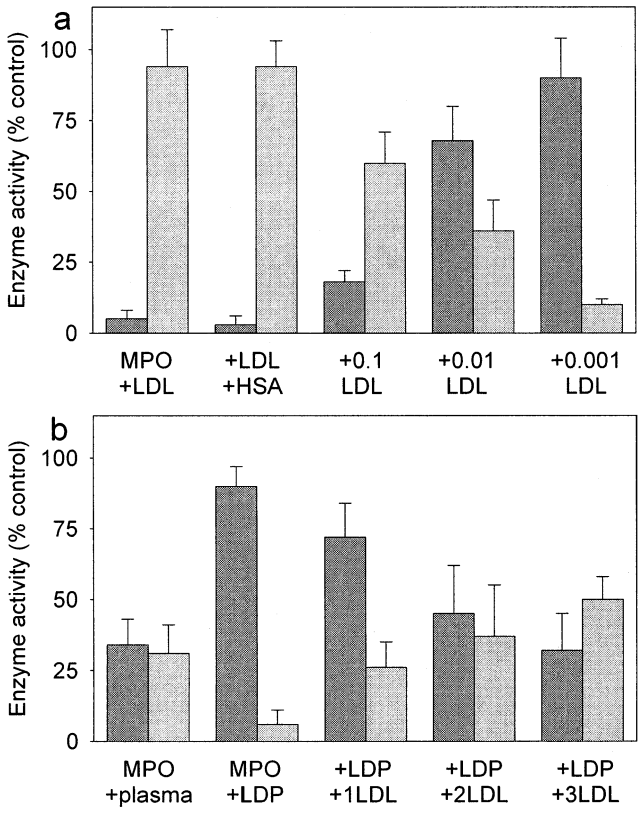

Fig. 3. (a) Precipitation of MPO in the presence of albumin. MPO (50 nM) was incubated in the absence and presence of human serum albumin (HSA, 40 mg/ml) with decreasing amounts of LDL (0.1–0.001 μM). (b) Precipitation of MPO in whole and lipoprotein-deficient plasma. MPO (50 nM) was incubated with whole plasma and lipoprotein-deficient plasma (LDP); LDL (1.0–3.0 μM) was added to LPD. For both (a) and (b), the samples were treated with phosphotungstate to precipitate apoB-containing lipoproteins. MPO activity was then measured in the supernatant (dark bars) and the pellet resuspended in 0.1% Tween-20 (light bars), using TMB oxidation as described in Section 2. Results are presented as percent of control MPO activity and are the mean ± S.D., $n = 3$.

4. Discussion

This is the first study to show that MPO binds to apoB-containing lipoproteins, including LDL, in isolation and in human plasma. We found that MPO co-precipitated with LDL using the precipitating reagents phosphotungstate [22] and isopropanol [23]. These reagents were used instead of the sulfated polysaccharides [26] because the latter have been shown to bind both MPO and LDL [14,27]. Another

mammalian peroxidase, LPO, also co-precipitated with LDL, in contrast to the plant-derived enzyme, HRP, suggesting specificity of the mammalian peroxidases for LDL. Association of MPO with LDL was confirmed using native gel electrophoresis. Similar results were observed for the other apoB-containing lipoprotein, VLDL. Physiological concentrations of albumin, the major plasma protein, did not inhibit co-precipitation of MPO with LDL.

Addition of MPO to plasma resulted in precipitation of approximately 70% of the enzyme activity following addition of precipitating reagents, whereas no decrease in enzyme activity was observed in lipoprotein-deficient plasma. That MPO was binding to apoB-containing lipoproteins was confirmed by adding LDL back into lipoprotein-deficient plasma. When MPO was added to plasma, approximately 30% of the enzyme was not precipitated, suggesting that some MPO was binding to plasma proteins other than the apoB-containing lipoproteins. Several studies have shown that MPO binds to the plasma metalloenzyme ceruloplasmin [29,30], anti-MPO immunoglobulins [13,29], and complement fragments [30]. Although not determined, it is possible that MPO is also able to bind to high-density lipoprotein. Interestingly, binding to ceruloplasmin inhibits the catalytic activity of MPO [29], which could account for the less than complete recovery of MPO activity in the present study [30]. However, interference by plasma component(s), such as ascorbate, in the TMB assay cannot be ruled out.

A number of studies have shown that MPO readily adheres to both prokaryotic and eukaryotic cells in culture [13–19]. Similar binding was observed for the mammalian peroxidases eosinophil peroxidase (EPO) [31] and LPO [32], but not for HRP [15], consistent with the findings of the present study. LPO shares greater than 50% amino acid homology with the other cationic peroxidases MPO and EPO [33]. HRP, in contrast, exists as a number of isozymes with different net charges [34]; the HRP isozyme used in this study, type A, has a p$I \approx 4$ [34]. Furthermore, all of the above studies [13–19] reported retention of peroxidase activity upon binding of the MPO to cells, which we also observed with MPO bound to LDL.

The nature of the interaction between MPO and cells is most likely of ionic nature [13,18,19]. Studies have shown that MPO binds to yeast cell wall mannan by interaction with the phosphate groups of the mannan [35]. Although mannan was able to inhibit binding of MPO to yeast and bacteria [18,19], it was unable to inhibit binding of MPO to leukocytes [15,35] or LDL (this study). Other studies have shown that cationic compounds can inhibit the binding of MPO to cells [13,18]. Consistent with this, we found that the cationic detergent Cetrimide (used in the isolation of MPO from leukocytes [36]) inhibited the binding of MPO to LDL. While this suggests that the association is charge-mediated, disruption of the LDL particle by the detergent cannot be ruled out.

Although LDL is comprised of approximately equivalent amounts (by weight) of protein and phospholipid [37], it is not certain whether MPO associates with the protein and/or lipid component(s) of LDL. ApoB is also a glycoprotein consisting of 8–10% carbohydrate (primarily mannose, galactose, glucosamine and sialic acid residues) [37], thus it is possible that MPO is associating with the carbohydrate component of apoB [38]. Specific interactions between MPO and other glycoproteins, such as adhesion molecules, as well as cell surface receptors, have been reported [15,16]. Binding of MPO to neutrophils and monocytes was found to occur via the $\alpha M\beta_2$ integrin [15], and was proposed to mediate leukocyte adhesion to endothelial cells in culture. MPO was also shown to be taken up by the mannose receptor of macrophages [16], suggesting a possible mechanism for clearance of the enzyme. We attempted to determine the role of the carbohydrate component of apoB in binding of MPO using agarose-conjugated neuraminidase (data not shown), however, since it is difficult to get consistent and/or complete desialylation using this technique, the data were inconclusive.

Recent studies have implicated both MPO and MPO-modified LDL in atherosclerosis [39–41]. Immunohistochemical analysis of human lesions showed the presence of HOCl-modified proteins [39], and gas chromatography-mass spectrometry (GC-MS) analysis of LDL isolated from human lesions showed significantly increased levels of 3-chlorotyrosine, a specific marker of HOCl, compared to plasma LDL [40]. Furthermore, 3-nitrotyrosine has been detected in lesions using immunohistochemistry [42] and GC-MS [43]. While these results were interpreted initially as evidence for the presence of peroxynitrite, more recent findings have shown that MPO too can generate reactive nitrogen species capable of nitrating tyrosine residues [44,45]. Catalytically active MPO has been detected in human atherosclerotic lesions [41] and was found to be predominantly associated with macrophages in transient lesions and adjacent to cholesterol clefts in advanced lesions [41]. Since a majority of the MPO appears to be localized intracellularly, it is uncertain whether this is due to the enzyme not being released, or secreted enzyme being endocytosed. In contrast to MPO, HOCl-modified proteins have been reported to be localized extracellularly in human atherosclerotic lesions [39]. This has been confirmed recently by Malle et al. [46] who reported co-localization of immunoreactive MPO and HOCl-modified LDL epitopes in the extracellular matrix and both inside and outside monocyte/macrophages and endothelial cells. The possibility of co-uptake of MPO complexed with LDL has not yet been explored.

A recent study investigating the oxidation of LDL by MPO versus reagent HOCl found differences in the extent to which cysteine residues were oxidized [47]. The selective modification of apoB by MPO reported in [47] was proposed to be due to direct interaction of the enzyme with the LDL particle. However, differences in reactivity due to bolus addition of HOCl versus continuous production of the oxidant by MPO cannot be ruled out. Another recent study reported increased oxidation of LDL by MPO in the presence of heparin [48]. These findings suggest that either interaction of heparin with LDL modifies the lipoprotein structure in a way that increases its oxidizability, or the negatively charged polysaccharide is enhancing association of MPO with the LDL particle. Another possibility is that heparin-induced alteration in LDL structure could increase the binding of MPO to LDL. The effects of other glycosaminoglycans, present in the subendothelial space, on the association of MPO with LDL are an area for further investigation.

In this study we have shown that MPO binds to LDL, even in the absence of sulfated polysaccharides such as heparin. Binding of MPO to LDL is likely ionic in nature and the major plasma protein albumin does not inhibit the binding. The association of MPO with LDL has potentially important implications with respect to the oxidative modification of

LDL, especially in the presence of competing substrates such as the small molecule antioxidants ascorbate and urate [21,49], as well as proteins like albumin [50].

Acknowledgements: This work was supported by Grants from the American Heart Association Northwest Affiliate (9920420Z to A.C.) and the U.S. National Institutes of Health (HL-56170 to B.F.).

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