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Thiol-containing molecules interact with the myeloperoxidase/H_2O_2/chloride system to inhibit LDL oxidation

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Abstract

Oxidized low-density lipoproteins (LDL) accumulate in the vascular wall and promote a local inflammatory process contributing to the progression of atheromatous plaque. The key role of myeloperoxidase (MPO) in this process has been documented and the enzyme has been involved in the oxidative modification of apolipoprotein B-100 in the intima and at the surface of endothelial cells. As the inhibition of this last phenomenon could be of relevance in pharmacological interventions, thiol-containing molecules such as glutathione, captopril, and *N*-acetylcysteine (NAC) and its lysinate salt (NAL) were tested in this system and their properties were compared with those of flufenamic acid (control). This last compound already demonstrated an inhibition of the production of HOCl by MPO and a more intense inhibition of MPO activity than glutathione, NAC, NAL, and captopril. However, NAC and NAL inhibited the oxidative modification of LDL more intensively than captopril and glutathione whereas flufenamic acid had no comparable inhibiting effect. This could be related to the presence of LDL close to the catalytic site of the enzyme. NAC and NAL therefore appeared as the most efficient inhibitors probably as a consequence of their relatively small size. The relevance of such effects has to be documented by in vivo studies.

Keywords: Myeloperoxidase; LDL; Compound II; Thiols; Flufenamic acid; Apolipoprotein B-100

The role of lipids, especially low-density lipoproteins (LDL), in the formation and evolution of atheromatous plaque has been largely documented showing that the oxidative modification of LDL is an important step in the process [1,2]. It is generally admitted that oxidized LDL can induce the formation of foam cells and of a number of potentially pro-atherogenic metabolites such as pro-inflammatory cytokines and chemokines in monocytes, endothelial cells, and smooth muscle cells [3,4]. As a consequence, oxidized LDL accumulate in the vascular cell wall and promote a local inflammatory process [3,5,6]. However, the way LDL are oxidized remains unclear as well as the actual contribution of the sub-endothelial oxidation by monocyte-derived macrophages. In this context, some authors also focused on LDL oxidation by endothelial and smooth muscle cells [2,7].

The role of myeloperoxidase (MPO) has been documented for several years, suggesting that the enzyme is not only involved in the inflammatory process [8], but also in the oxidation of LDL. The enzyme is indeed able to produce hypochlorous acid (HOCl), a very reactive species which takes part in both the oxidation of lipids by chlorination and peroxidation [9] and in the oxidation of the

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apolipoprotein B-100 (Apo B-100) [10]. As a matter of fact, various arguments have been put forward by several authors showing that HOCl produced by the MPO/H_2O_2/Cl^- system mainly acts on the protein moiety of LDL. Jerlich et al. [11] demonstrated that the MPO/H_2O_2/Cl^- system is involved in the oxidation of lipids only during a non-physiological severe treatment and they did not observe the production of lipid hydroperoxides in these conditions. Arnhold et al. [12] reported a chlorination of phospholipids in liposomes by the MPO/H_2O_2/Cl^- system but only in the absence of Apo B-100. In addition, Yan et al. [13] showed that the oxidation of LDL by HOCl brought about an increase in the production of carbonyl groups while lipid peroxidation was unaffected, suggesting that the oxidation mainly affects the protein moiety of LDL. Winterbourn and Kettle [14] demonstrated that chlorotyrosines are useful biomarkers of LDL oxidized by HOCl during an oxidative modification of Apo B-100. Carr et al. [10] measured a decrease in the content of amino acids in LDL such as tryptophan, lysine, and cystein, after treatment with the MPO/H_2O_2/Cl^- system. Similarly, Hazell et al. [15] showed that exposure of LDL to exogenously or enzymatically generated HOCl resulted in an immediate and preferential oxidation of amino acid residues of Apo B-100. Finally, Yang et al. [16] were able to demonstrate the oxidation of a cystein residue of Apo B-100 among other modified residues during a selective oxidative modification of Apo B-100.

There is also some evidence that a specific interaction between LDL and MPO is required to initiate the oxidative process. MPO is a highly cationic protein (isoelectric point >10) that easily binds to LDL in vivo, which contributes to a direct oxidation of Apo B-100 [17]. The importance of the phenomenon as a pathogenic factor for atheromatous lesions led some of us to develop a monoclonal antibody and an ELISA test for the specific quantification of this oxidative modification of Apo B-100 [18]. Furthermore, this test already made it possible to demonstrate that the oxidative modification is able to take place at the surface of endothelial cells and that this constitutes an additional mechanism to sub-endothelial oxidation in atheromatous lesions [7].

Considering the well-documented effects of oxidized LDL in atherogenesis and the involvement of MPO in the process, the present study was conducted to further document the role of MPO as a possible target for pharmacological interventions [19]. The hypothesis that thiol-containing molecules such as glutathione, captopril, and *N*-acetylcysteine (NAC) and its lysinate salt (NAL) are able to inhibit the oxidative modifications of apolipoprotein B-100 caused by the MPO/H_2O_2/Cl^- system was presently investigated taking into account a steric hindrance due to the large size of LDL that could completely mask the active site of the enzyme [17]. Such molecules not only have well-documented antioxidant properties against oxygen-derived species including H_2O_2, tyrosyl radical, and HOCl [20–22], but they are also able to directly interact with MPO [23]. The interactions of these molecules with components of the MPO system were first examined in in vitro systems and the effects were compared with those of flufenamic acid, which has a strong inhibiting activity towards MPO in the MPO/H_2O_2/Cl^- system without showing any significant interaction with H_2O_2 or HOCl [24,25]. Thereafter, the inhibitory effects of these molecules on LDL oxidation by MPO were investigated and compared.

Materials and methods

Chemicals. Glutathione (Sigma, St. Louis, USA), captopril (Medichem, Brussels, Belgium), *N*-acetyl-L-cysteine (NAC, Fluka Neu-Ulm, Switzerland), the lysinate salt of NAC (nacystelin or NAL, SMB-Galephar, Brussels, Belgium), and flufenamic acid (Trenker, Brussels, Belgium) were the tested molecules. Hydrogen peroxide (H_2O_2), KI, HCl, EDTA-Na_2H_2, $MgCl_2 \cdot 6H_2O$, $NaHCO_3$, diethanolamine, Polysorbate 80, and NaOH were obtained from VWR (Leuven, Belgium). Bovine serum albumin (BSA), 5,5′-dithio-bis-nitrobenzoic acid (DTNB), NaN_3, paranitrophenyl phosphate, Tris(hydroxymethyl)aminomethane (Tris), methionine, and catalase were purchased from Sigma (St. Louis, USA). A PBS buffer (pH 7.4) was prepared at a final concentration of 10 mM phosphate ions (KH_2PO_4/KOH) and 150 mM NaCl (all from VWR, Leuven, Belgium). The same phosphate buffer (pH 7.4) was also prepared without NaCl. For LDL preparation and oxidation, a PBS buffer at pH 7.2 was prepared with a final concentration of 2.8 mM of EDTANa_2H_2. A pH 7.5 Tris-buffered saline (TBS 80) containing 50 mM Tris, 300 mM NaCl, and 0.1% of Polysorbate 80 was used during the ELISA. Finally, a pH 9.8 diethanolamine buffer was extemporarily made up by dissolving 0.101 g $MgCl_2 \cdot 6H_2O$ and 0.2 g NaN_3 in water with 97 ml of diethanolamine. The pH was adjusted to 9.8 with HCl and the solution was diluted to 1 L. These chemicals were of pro-analysis quality. Ammonium acetate, KOH (VWR International, Leuven, Belgium), and acetonitrile (Fischer Scientific, Loughborough, UK) were LC analytical-grade reagents, used to prepare the mobile phase of the LC system. De-oxygenated milliQ water was used for the preparation of all solutions.

Preparation of the recombinant enzyme and obtaining of LDL. Recombinant MPO was prepared as previously described [26]. Each batch solution is characterized by its protein concentration (mg/ml), its activity (U/ml), and its specific activity (U/mg). The chlorination activity was determined according to Hewson and Hager [27]. Human plasma served for the isolation of LDL by ultracentrifugation according to Havel et al. [28]. Before oxidation, the LDL fraction ($1.019 < d < 1.067$ g/ml) was desalted by two consecutive passages through PD10 gel-filtration columns (Amersham Biosciences, The Netherlands) using PBS buffer. The different steps were carried out in the dark and the protein concentration was measured by the Lowry assay for both MPO and LDL [29].

Two batches of MPO were used in the experiments, the first (0.47 mg/ml, 25 U/ml, and 53 U/mg) for the inhibition of the MPO/H_2O_2/Cl^- system and the oxidation of LDL, and the second (0.25 mg/ml, 13.3 U/ml, and 53 U/mg) for the accumulation of compound II.

Liquid chromatographic system. The interaction of flufenamic acid with the MPO/H_2O_2/Cl^- system was investigated by a liquid chromatography system (Waters, Milford, USA). The mobile phase consisted in a 1:1 mixture of an ammonium acetate solution (20 mM, pH 8.0) and acetonitrile which was used at a flow rate of 1.0 ml/min. The column was an Alltima C18 15 cm × 4.6 mm, 5 μm with a guard column, 5 × 4.6 mm, 5 μm (Alltech, Deerfield, IL) and the detection was performed by monitoring the absorbance at 292 nm with a Waters (Milford, USA) diode array detector.

Inhibition of the MPO/H_2O_2/Cl^- system. The method was adapted from Peskin and Winterbourn [20], who used methionine to monitor the oxidation of thiols by chloramines. The assessment of the inhibition of the MPO/H_2O_2/Cl^- system was based on a simple competition of the different drugs with methionine where the remaining quantity of thiols was measured as function of the concentration of methionine. In a final volume of