Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 337 (2005) 82–88
BBRC
www.elsevier.com/locate/ybbrc

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

Pierre Van Antwerpen [a], Karim Zouaoui Boudjeltia [b], Sajida Babar [c], Ilham Legssyer [c], Patrick Moreau [a], Nicole Moguilevsky [c,1], Michel Vanhaeverbeek [b], Jean Ducobu [d], Jean Nève [a,*]

[a] *Laboratory of Pharmaceutical Chemistry, Institute of Pharmacy, Université Libre de Bruxelles, Brussels, Belgium*
[b] *Laboratory of Experimental Medicine, CHU Charleroi-Vésale, Université Libre de Bruxelles, Montigny-le-Tilleul, Belgium*
[c] *Laboratory of Applied Genetics, Institute of Molecular Biochemistry, Université Libre de Bruxelles, Gosselies, Belgium*
[d] *Department of Internal Medicine, CHU Tivoli, La Louvière, Belgium*

Received 1 September 2005
Available online 13 September 2005

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

* Corresponding author. Fax: +3226505249.
E-mail address: jneve@ulb.ac.be (J. Nève).
[1] Present address: Facultés Universitaires Notre Dame de la Paix, Technology Transfert Office, Namur.

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.09.013

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

1 ml, the reaction mixture contained the following reagents at the final concentrations indicated between brackets: PBS buffer, pH 7.4 (10 mM/140 mM), one of the drugs (600 μM), a gradient of methionine (60–600 μM), MPO (~51 nM), in order to produce 60 μM HOCl during 5 min of incubation at 37 °C with H_2O_2 (100 μM). The reaction was stopped by the addition of 100 μl catalase (4 U/μl). Thiol-containing molecules were detected according to Vanderbist et al. [21] by addition of 1000 μl of a solution of DTNB (4 mM) and 4000 μl water, and measurement of the absorbance at 412 nm. Flufenamic acid was investigated using the liquid chromatographic system described above. The results were expressed as the remaining quantity of drugs (in %) as a function of methionine concentration (means ± SD for $n = 5$).

Accumulation of compound II. In order to study the interaction of thiols and flufenamic acid with the native enzyme and its different oxidized forms, the measurement of compound II lifetime was monitored in three different conditions consisting of the presence of variable concentrations of H_2O_2 and Cl^-.

The method was adapted from Nève et al. [24]. In a 1.5 ml quartz cell, the following reagents were introduced, at a final concentration stated between brackets, for a final volume of 1 ml:200 μl MPO (~595 nM), 400 μl pH 7.4, KH_2PO_4/KOH phosphate buffer (10 mM) with or without NaCl (300 or 0 mM), 250 μl of a drug solution (2 mM), and 50 μl water. The reaction was initiated by addition of 100 μl H_2O_2 (30 or 200 μM) and absorbances were simultaneously monitored with a diode-array spectrophotometer (Agilent 8453, Palo Alto, CA, USA) at wavelengths that were characteristic of compound II (456 nm) and of the native enzyme (430 nm). Compound II lifetime was measured as the time for intersection of absorbance curves at 430 and 456 nm in the presence of a drug inducing an inhibition of MPO chlorinating activity. This time corresponds to the complete conversion of compound II to native enzyme, which is demonstrated by a shift in the enzyme spectrum. The results were expressed as means ± SD of six independent values.

Inhibition of LDL oxidation. The LDL oxidation was carried out at 37 °C in a final volume of 500 μl. The reaction mixture contained the following reagents at the final concentrations indicated between brackets: pH 7.2, PBS buffer, MPO (1 μg/ml), LDL (1000 μg/ml), 2 μl HCl 1 N (4 mM), one of the drugs at different concentrations (5, 15, 30, and 300 μM), and H_2O_2 (100 μM). The reaction was stopped after 5 min by cooling the tubes in ice.

In order to specifically measure the LDL oxidation due to the MPO/H_2O_2/Cl^- system, a recently developed ELISA was used, based on the binding between a mouse monoclonal antibody (Mab AG9) specifically recognizing the oxidized APO B-100 on LDL and an anti-mouse immunoglobulin G (Ig G) coupled with alkaline phosphatase [18]. However, until now, the chemical structure of the epitope recognized by our antibodies has not been elucidated. The amount of oxidized LDL is quantified by measurement of the phosphatase activity and expressed as the absorbance value of the reaction product of the alkaline phosphatase, i.e., *para*-nitrophenol (405 nm).

The assay was performed as described by Moguilevsky et al. [18] in a NUNC maxisorp plate (VWR, Zaventem, Belgium): 200 ng/well of LDL was coated overnight at 4 °C in a sodium bicarbonate pH 9.8 buffer (100 μl). Afterwards, the plate was washed with TBS 80 buffer and then saturated during 1 h at 37 °C with the PBS buffer containing 1% BSA (150 μl/well). After washing the wells twice with the TBS 80 buffer, the monoclonal antibody Mab AG9 (200 ng/well) obtained according to a standard protocol and as previously described [18] was added as a diluted solution in PBS buffer with 0.5% BSA and 0.1% of Polysorbate 20. After incubation for 1 h at 37 °C, the plate was washed four times with the TBS 80 buffer and a 3000 times diluted solution of Ig G anti-mouse Alkaline Phosphatase (Promega, Leiden, The Netherlands) in the same buffer was added (100 μl/well). The wells were washed again four times and a revelation solution (150 μl/well) containing 5 mg of *para*-nitrophenyl phosphate in 5 ml of diethanolamine buffer was added for 30 min at room temperature. The reaction was stopped with 60 μl/well of NaOH 3 N solution. The measurement of the absorbance was performed at 405 nm with a background correction at 655 nm with a Bio-Rad photometer for a 96-well plate (Bio-Rad laboratories, CA, USA). Results were expressed as means ± SD of the percentage of LDL oxidation for six independent measurements.

Results

Inhibition of the MPO/H_2O_2/Cl^- system

In the experimental model used for investigation, a drug which is efficiently oxidized by the MPO system causes an increase in the quantity of methionine required to inhibit this oxidation. As a consequence, a strong inhibitor of the MPO system will have a high IC_{50} value, which corresponds to the concentration of methionine required to inhibit 50% of its oxidation by the MPO system.

The results of Table 1 show that glutathione more intensively inhibited the MPO system than NAL and NAC ($p < 0.01$), which themselves were more efficient than captopril ($p < 0.01$). Flufenamic acid was far more active than the other molecules.

Accumulation of compound II

Fig. 1 shows that MPO is oxidized by H_2O_2 to give compound I, which is able to produce HOCl in the presence of chloride ions (Cl^-) by returning to the native enzyme state. However, in conditions such as the absence of Cl^-, the

Table 1
Determination of the extent of inhibition of the MPO/H_2O_2/Cl^- system by the different drugs investigated

Drug	IC_{50} (10^{-6} M)
Glutathione	290 ± 10
NAC	101 ± 9
NAL	107 ± 6
Captopril	58 ± 1
Flufenamic acid	>4000

Results are expressed as the quantity of methionine required to inhibit 50% of the activity of the system (IC_{50}). Results are the means ± SD for $n = 5$.

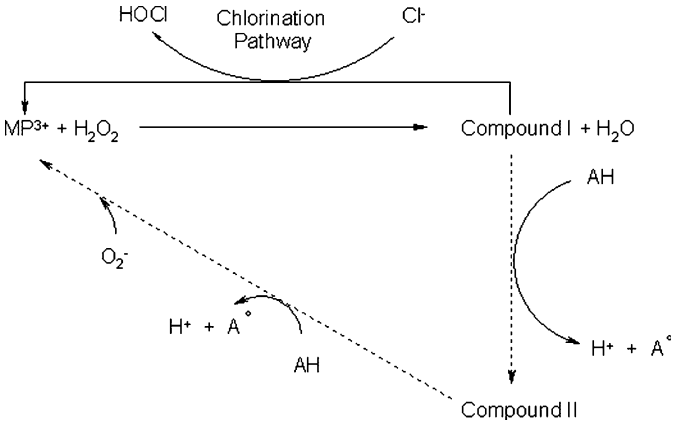

Fig. 1. Representation of MPO redox transformation catalytic pathways. MP^{3+}: native enzyme; compound I: MP^{3+} H_2O_2; compound II: MP^{2+} H_2O_2; AH: reducing agent; O_2^-: superoxide anion. Adapted from Kettle and Winterbourn [33].

presence of an excess of H_2O_2 or the presence of a drug able to inhibit the formation of HOCl, the system evolved to the promotion and the accumulation of compound II, a reduced form of compound I [30–33].

Results of Table 2 reporting the accumulation of compound II as a function of time have to be interpreted knowing that in the first condition (Cl^- 300 mM and H_2O_2 30 μM), the quantity of H_2O_2 is sufficient to induce the synthesis of HOCl and that no compound II is formed (see Table 2, blank). On the other hand, H_2O_2 is in excess in the second condition (Cl^- 300 mM and H_2O_2 200 μM) and the rate of HOCl synthesis is too slow to rapidly consume H_2O_2, a condition which actually leads to the formation of compound II. As a matter of fact, the excess of H_2O_2 was able to reduce compound I to give rise to compound II [30–33]. In the third condition (Cl^- 0 mM and H_2O_2 30 μM), the absence of Cl^- results in the oxidation of MPO in compound I which is directly reduced into compound II by H_2O_2 even in the presence of a relatively small amount of this species (30 μM) [30–33].

Thiol-containing molecules did not cause the formation of compound II in the first condition, proving that they do not inhibit the synthesis of HOCl in physiological conditions although they undoubtedly scavenge this last compound [20,21]. Flufenamic acid, however, inhibited the synthesis of HOCl and caused a transitional formation of compound II. This means that it inhibited the system without interacting with HOCl (see Table 1) [24].

By increasing the concentration of H_2O_2 (from 30 to 200 μM), both glutathione and captopril showed a transitional formation of compound II while this form remained undetectable in the presence of NAC and NAL. As the blank attests to the formation of compound II in the same conditions (see Table 2), these results suggest a duality in the inhibition mechanism of thiols where the scavenging of HOCl is associated with a direct interaction with compounds I and II. In the present study, flufenamic acid only involved an increase of compound II lifetime related to an increasing H_2O_2 concentration.

Finally, the third condition studied reflected the possible direct interaction of drugs with MPO. This non-physiological situation made it possible to rapidly and easily assess and compare the direct interaction of the studied molecules with compound II independently of a potential scavenging effect towards HOCl. Table 2 shows that NAC and NAL interacted with compound II more efficiently than flufenamic acid which itself was more efficient than captopril and glutathione ($p < 0.01$). These results concur perfectly with the values of the rate constants of NAC (4.56×10^2 and 4.76×10^2 M^{-1} s^{-1}) and glutathione (7.21×10^1 and <1 M^{-1} s^{-1}) for the reaction with compounds I and II, respectively [23].

Inhibition of LDL oxidation

Fig. 2 shows the percentage of LDL oxidation related to several drug concentrations: at relatively low concentrations, NAC (5 μM: 38 ± 13%, 15 μM: 17 ± 3%), NAL (5 μM: 47 ± 14%, 15 μM: 17 ± 4%), and (to a lesser extent) captopril (5 μM: 53 ± 5%, 15 μM: 22 ± 4%) inhibited the oxidation of LDL more efficiently than glutathione (5 μM: 67 ± 9%, 15 μM: 41 ± 5%) ($p < 0.01$). At a higher concentration (30 μM), NAC (12 ± 4%) and NAL (10 ± 3%) were still more efficient than glutathione (17 ± 2%) while captopril (14 ± 3%) had a quite similar effect ($p < 0.01$). Flufenamic acid had a weaker antioxidant effect than the thiols at low and medium concentrations (97 ± 4%, 82 ± 14%, and 23 ± 3% for, respectively, 5, 15, and 30 μM, $p < 0.01$). These results demonstrate that the different molecules studied are able to inhibit LDL oxidation in spite of the binding of MPO and LDL.

Discussion

The inhibition of the MPO chlorinating activity could be easily assessed by measuring the chlorinating activity of taurine [24] or of monochlorodimedone [34] in the presence of thiols. However, thiol-containing molecules interfere with such systems, which are therefore unsuitable for this purpose. In order to be able to compare the different studied molecules in a system involving a chemical competition towards the MPO/H_2O_2/Cl^- system, methionine was investigated as a suitable competitor and an original system developed. This methylsulfide-containing derivative has the

Table 2
Accumulation of compound II as assessed by the measurement of the evolution of absorbance at wavelengths typical to the native enzyme (430 nm) and to compound II (456 nm) as a function of time

Drug	Lifetime of compound II (s)		
	[Cl^-] 300 mM [H_2O_2] 30 μM	[Cl^-] 300 mM [H_2O_2] 200 μM	[Cl^-] 0 mM [H_2O_2] 30 μM
Blank	ND	107 ± 13	967 ± 122
Glutathione	ND	3 ± 1	156 ± 16
NAC	ND	ND	4 ± 1
NAL	ND	ND	3 ± 1
Captopril	ND	<1	54 ± 8
Flufenamic acid	18.7 ± 0.5	102 ± 16	20 ± 2

Experiments are performed in three conditions characterized by different amounts of Cl^- and H_2O_2. MPO concentration is about 1.12 μM. Results are means ± SD for $n = 6$; ND, compound II not detected.

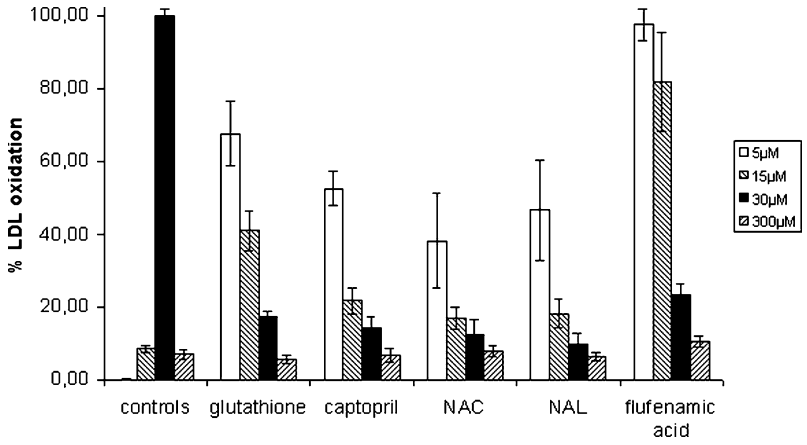

Fig. 2. LDL oxidation (in %) in relation to different concentrations of thiol-containing molecules and flufenamic acid: 5 μM (□), 15 μM (▨), 30 μM (■), and 300 μM (▨). Absorbance of LDL (blank; controls, □) and oxidized LDL without drugs (controls, ■) are respectively considered equal to the 0 and 100%. In the absence of H_2O_2, % = 8.5 ± 0.9% (controls, ▨); in the presence of catalase (400 U/ml) (controls, ▨), % = 7 ± 1%. Results are means ± SD for $n = 6$.

advantage of reacting in the same way as thiols and is not detected by the reagent used in classical systems, i.e., DTNB [21]. By measuring the quantity of thiol-containing molecules or of flufenamic acid remaining after the reaction, a molecule under investigation which reacts with the MPO/H_2O_2/Cl^- system requires a higher amount of methionine to inhibit its oxidation.

Based on this method, the results of Table 1 corroborate and extend the well-known antioxidant properties of the different thiols, particularly towards HOCl. Peskin and Winterbourn [20] already showed that glutathione had a relative rate constant of interaction with HOCl twice as high as NAC. Furthermore, Vanderbist et al. [21] reported that NAC and NAL were more efficient than captopril in the scavenging of HOCl and H_2O_2. However, the rate constant of the interaction of H_2O_2 with MPO ($\sim 2.3 \times 10^7$ M^{-1} s^{-1} [30]) is much higher than that of thiols (0.01–0.03 min^{-1} [21]), suggesting that these last derivatives mostly exert their activity by scavenging HOCl. Finally, Nève et al. [24] recently demonstrated a strong inhibiting effect of flufenamic acid in the MPO system due to the direct interaction of the drug with the enzyme. In this case, flufenamic acid does not directly interact with HOCl but it blocks its synthesis by the MPO/H_2O_2/Cl^- system, which significantly enhances the IC_{50} value (Table 1).

A method based on the accumulation of compound II is very rapid and convenient to investigate the interaction of molecules of interest with MPO and its derived compounds (I and II). This measurement was used to determine the predominant pathway for the reaction of thiols with the MPO/H_2O_2/Cl^- system. Indeed, besides the common direct scavenging of HOCl [20,21], Burner et al. [23] demonstrated that thiols could directly interact with compounds I and II of MPO. In physiological conditions (column 1, Table 2), it appeared that no formation of compound II could be observed, in contrast with what happened with flufenamic acid (Table 2). This supports the theory that thiol-containing molecules do not act by an inhibition of HOCl synthesis but by scavenging the oxidant species. In addition, these results also corroborate those concerning the inhibition of the MPO/H_2O_2/Cl^- system (Table 1) where flufenamic acid showed the strongest inhibiting effect by inducing the formation of compound II. In conditions where the quantity of H_2O_2 was in excess (column 2, Table 2), thiols were able to scavenge both HOCl and compound II. Finally, NAC and NAL, which were less efficient than glutathione for scavenging HOCl produced by MPO (Table 1), rapidly reacted with compound II in the absence of Cl^- (Table 2). This result is in accordance with Burner et al. [23] who emphasized the key role of the net charge and of the size of the molecule in the interaction with the catalytic site of the enzyme. Considering the results of the accumulation of compound II, thiols should act on the MPO/H_2O_2/Cl^- system by a simple scavenging of HOCl in physiological conditions.

The binding of MPO with LDL during LDL oxidation is responsible for the reproducible oxidative modification of apolipoprotein B-100 in plasma [7,18]; however, this event could also somehow block the catalytic site of the enzyme and therefore become unfavourable for antioxidant activity and a protective effect [17]. The heme group of MPO that is responsible for the enzymatic activity is indeed located in a distal hydrophobic cavity with a narrow oval-shaped opening [35]. In the present study, an original ELISA test using a monoclonal antibody was used to specifically measure the oxidative modification of apolipoprotein B-100 caused by the MPO/H_2O_2/Cl^- system and to assess a potential inhibiting effect of the molecules under study [18]. In consideration of the results of Table 1, flufenamic acid and glutathione were expected to be the best inhibitors of this system. Results concerning this system demonstrated that the different studied molecules were able to inhibit LDL oxidation in spite of the binding occurring between MPO and LDL. However, some discrepancy became apparent when comparing the results of the first experiment and the third one (Table 1 and Fig. 2). Indeed,

thiol-containing molecules and, quite unexpectedly, flufenamic acid, showed a totally different inhibiting effect in this last part of the study. As a matter of fact, the significant inhibiting effect of NAC on LDL oxidation seems to be due both to a short lifetime of compound II (4 ± 1 s) in the absence of Cl^- and to a relatively small size. Interestingly, a plot of the lifetime of compound II versus both the percentage of LDL oxidation at 5 μM and the molecular weight of examined thiols gave linear slopes ($r^2 = 0.99$, $p < 0.05$; slope = 0.1553, $p < 0.01$ and $r^2 = 0.99$, $p < 0.05$; slope = 0.1027, $p < 0.01$, respectively) suggesting that the size of the molecule that enters the catalytic site of MPO is a relevant parameter for the inhibition of both the MPO/H_2O_2/Cl^- system and LDL oxidation. This is further supported by the fact that flufenamic acid, for which the compound II lifetime (20 ± 2 s) attests to a moderate interaction with MPO, does not induce inhibition of LDL oxidation (at 5 μM, $97 \pm 4\%$).

In conclusion, the oxidation of several thiol-containing molecules by the MPO/H_2O_2/Cl^- system was mostly due to a simple scavenging of HOCl which was demonstrated by the absence of compound II formation (Table 2, Cl^- 300 mM/H_2O_2 30 μM). By contrast, flufenamic acid inhibited the MPO/H_2O_2/Cl^- system by a direct interaction with the enzyme, blocking the synthesis of HOCl as illustrated by the formation of compound II (Table 2). However, a system allowing the measurement of the extent of LDL oxidation by MPO/H_2O_2/Cl^- indicated that NAC and NAL are the most efficient thiol-containing molecules. Both flufenamic acid and glutathione exhibited a decreased inhibition of LDL oxidation. In this case, the binding of LDL to MPO [17], which is responsible for the presence of LDL close to the site of HOCl production, appeared to be a key parameter making the size of the inhibitors a limiting factor. NAC and NAL were undoubtedly the most efficient inhibiting molecules probably as a consequence of their relatively small size. Therefore, flufenamic acid, an efficient inhibitor of the MPO/H_2O_2/Cl^- system, seems to be devoid of any significant activity in the presence of LDL while thiol-containing molecules significantly inhibit LDL oxidation at pharmacological concentrations. The relevance of such effects should be further documented in in vivo studies.

Acknowledgments

The authors gratefully thank Valérie Delforge and Roger Lema Kisoka for the provision of LDL.

References

[1] P. Barter, The inflammation: lipoprotein cycle, Atheroscler. Suppl. 6 (2005) 15–20.
[2] G.M. Chisolm, D. Steinberg, The oxidative modification hypothesis of atherogenesis: an overview, Free Rad. Biol. Med. 28 (2000) 1815–1826.
[3] J.A. Berliner, J.W. Heinecke, The role of oxidized lipoproteins in atherogenesis, Free Rad. Biol. Med. 20 (1996) 707–727.
[4] P. Holvoet, D. Collen, Oxidized lipoproteins in atherosclerosis and thrombosis, FASEB J. 8 (1994) 1279–1284.
[5] M. Ueda, S. Ehara, Y. Kobayashi, T. Naruko, N. Shirai, E. Hai, Y. Ikura, M. Ohsawa, Plaque instability in human coronary atherosclerotic lesions: roles of oxidized LDL and neutrophils, Int. Congress Ser. 1262 (2004) 75–78.
[6] E. Malle, G. Waeg, R. Schreiber, E.F. Gröne, W. Sattler, H.-J. Gröne, Immunohistochemical evidence for the myeloperoxidase/H_2O_2/halide system in human atherosclerotic lesions, Eur. J. Biochem. 267 (2000) 4495–4503.
[7] K. Zouaoui Boudjeltia, N. Moguilevsky, I. Legssyer, S. Babar, M. Guillaume, P. Delree, M. Vanhaeverbeek, D. Brohee, J. Ducobu, C. Remacle, Oxidation of low density lipoproteins by myeloperoxidase at the surface of endothelial cells: an additional mechanism to subendothelium oxidation, Biochem. Biophys. Res. Commun. 325 (2004) 434–438.
[8] S.J. Klebanoff, Myeloperoxidase, Proc. Assoc. Am. Phys. 111 (1999) 383–389.
[9] E.A. Podrez, H.M. Abu-Soud, S.L. Hazen, Myeloperoxidase-generated oxidants and atherosclerosis, Free Radic. Biol. Med. 28 (2000) 1717–1725.
[10] A.C. Carr, E.A. Decker, Y. Park, B. Frei, Comparison of low-density lipoprotein modification by myeloperoxidase-derived hypochlorous and hypobromous acids, Free Radic. Biol. Med. 31 (2001) 62–72.
[11] A. Jerlich, A.R. Pitt, R.J. Schaur, C.M. Spickett, Pathways of phospholipid oxidation by HOCl in human LDL detected by LC-MS, Free Radic. Biol. Med. 28 (2000) 673–682.
[12] J. Arnhold, A.N. Osipov, H. Spalteholtz, M. Panasenko, J. Schiller, Effects of hypochlorous acid on unsaturated phosphatidylcholines, Free Radic. Biol. Med. 31 (2001) 1111–1119.
[13] L.J. Yan, J.K. Lodge, M.G. Traber, S. Matsugo, L. Packer, Comparison between copper-mediated and hypochlorite-mediated modifications of human low density lipoproteins evaluated by protein carbonyl formation, J. Lipid Res. 38 (1997) 992–1001.
[14] C.C. Winterbourn, A.J. Kettle, Biomarkers of myeloperoxidase-derived hypochlorous acid, Free Radic. Biol. Med. 29 (2000) 403–409.
[15] J.L. Hazell, J.J.M. Vanden Berg, R. Stocker, Oxidation of low density lipoprotein by hypochlorite causes aggregation that is mediated by modification of lysine residues rather than lipid oxidation, Biochem. J. 304 (1994) 297–304.
[16] C. Yang, Z. Gu, M. Yang, S. Lin, A.J. Garcia-Prats, L.K. Rogers, S.E. Welty, C.V. Smith, Selective modification of apoB-100 in the oxidation of low density lipoproteins by myeloperoxidase in vitro, J. Lipid. Res. 40 (1999) 686–698.
[17] A.C. Carr, M.C. Myzak, R. Stocker, M.R. McCall, B. Frei, Myeloperoxidase binds to low-density lipoprotein: potential implications for atherosclerosis, FEBS Lett. 487 (2000) 176–180.
[18] N. Moguilevsky, K. Zouaoui Boudjeltia, S. Babar, P. Delrée, I. Legssyer, Y. Carpentier, M. Vanhaeverbeek, J. Ducobu, Monoclonal antibodies against LDL progressively oxidized by myeloperoxidase react with ApoB-100 protein moiety and human atherosclerotic lesions, Biochem. Biophys. Res. Commun. 323 (2004) 1223–1228.
[19] W. Jantschko, P.G. Furtmuller, M. Zederbauer, K. Neugschwandtner, I. Lehner, C. Jakopitsch, et al., Exploitation of the unusual thermodynamic properties of human myeloperoxidase in inhibitor design, Biochem. Pharmacol. 69 (2005) 1149–1157.
[20] A.V. Peskin, C.C. Winterbourn, Kinetics of the reactions of hypochlorous acid and amino acid chloramines with thiols, methionine, and ascorbate, Free Radic. Biol. Med. 30 (2001) 572–579.
[21] F. Vanderbist, P. Maes, J. Nève, In vitro comparative assessment of the antioxidant activity of Nacystelyn against three reactive oxygen species, Arzneim.-Forsch. Drug Res. 46 (1996) 783–788.
[22] H. Pichorner, D. Metodiewa, C.C. Winterbourn, Generation of superoxide and tyrosine peroxide as a result of tyrosyl radical scavenging by glutathione, Arch. Biochem. Biophys. 323 (1995) 429–437.
[23] U. Burner, W. Jantschko, C. Obinger, Kinetics of oxidation of aliphatic and aromatic thiols by myeloperoxidase compounds I and II, FEBS Lett. 443 (1999) 290–296.

[24] J. Nève, N. Parij, N. Moguilevsky, Inhibition of the myeloperoxidase chlorinating activity by non-steroidal anti-inflammatory drugs investigated with a human recombinant enzyme, Eur. J. Pharmacol. 417 (2001) 37–43.
[25] N. Parij, J. Nève, Nonsteroidal antiinflammatory drugs interact with horseradish peroxidase in an in vitro assay system for hydrogen peroxide scavenging, Eur. J. Pharmacol. 311 (1996) 259–264.
[26] N. Moguilevsky, L. Garcia-Quintana, A. Jacquet, C. Tournay, L. Fabry, L. Pierard, A. Bollen, Structural and biological properties of human recombinant myeloperoxidase produced by Chinese hamster ovary cell lines, Eur. J. Biochem. 197 (1991) 605–614.
[27] W.D. Hewson, L.P. Hager, Mechanism of the chlorination reaction catalyzed by horseradish peroxidase with chloride, J. Biol. Chem. 254 (1979) 3175–3181.
[28] R.J. Havel, H.A. Eder, J.H. Bragdom, The distribution and chemical composition of ultracentrifugally separated lipoprotein in human serum, J. Clin. Invest. 34 (1955) 1345–1353.
[29] G.L. Peterson, A simplification of the protein assay method of Lowry et al. which is more generally applicable, Anal. Biochem. 83 (1977) 346–356.
[30] L.A. Marquez, J.T. Huang, H.B. Dunford, Spectral and kinetic studies on the formation of myeloperoxidase compounds I and II: roles of hydrogen peroxide and superoxide, Biochemistry 33 (1994) 1447–1454.
[31] J. Arnhold, P.G. Furtmüller, C. Obinger, Redox properties of myeloperoxidase, Redox Rep. 8 (2003) 179–186.
[32] P.G. Furtmüller, U. Burner, W. Jantschko, G. Regelsberger, C. Obinger, Two-electron reduction and one-electron oxidation of organic hydroperoxides by human myeloperoxidase, FEBS Lett. 484 (2000) 139–143.
[33] A.J. Kettle, C.C. Winterbourn, Superoxide modulates the activity of myeloperoxidase and optimises the production of hypochlorous acid, Biochem. J. 252 (1988) 529–536.
[34] A. Jerlich, S. Tschabuschnig, J.S. Fabjan, R.J. Schaur, Kinetics of chlorination of monochlorodimedone by myeloperoxidase, Int. J. Clin. Lab. Res. 30 (2000) 33–37.
[35] T.J. Fiedler, C.A. Davey, R.E. Fenna, X-ray crystal structure and characterization of halide-binding sites of human myeloperoxidase at 1.8 Å resolution, J. Biol. Chem. 275 (2000) 11964–11971.