Impact of myeloperoxidase-LDL interactions on enzyme activity and subsequent posttranslational oxidative modifications of apoB-100

Cédric Delporte,¹,*¹ Karim Zouaoui Boudjeltia,¹ Caroline Noyon,¹ Paul G. Furtmüller,** Vincent Nuyens,⁸ Marie-Christine Slomianny,†† Philippe Madhoun,⁸ Jean-Marc Desmet,⁸ Pierre Raynal,*** Damien Dufour,⁰ Chintan N. Koyani,††† Florence Reyé,,* Alexandre Rousseau,§ Michel Vanhaeverbeek,¹ Jean Ducobu,§ Jean-Claude Michalski,†† Jean Nève, Luc Vanhamme,§§§ Christian Obinger,²,* Ernst Malle,††† and Pierre Van Antwerpen*†

Laboratory of Pharmaceutical Chemistry* and Analytical Platform of the Faculty of Pharmacy,¹ Faculty of Pharmacy, Université Libre de Bruxelles, Brussels, Belgium; Laboratory of Experimental Medicine (ULB 222 Unit)§ and Unit of Dialysis,§§ CHU de Charleroi, A. Vésale Hospital, Université Libre de Bruxelles, Montigny-le-Tilleul, Belgium; Department of Chemistry, Division of Biochemistry,** Vienna Institute of BioTechnology, BOKU - University of Natural Resources and Life Sciences, Vienna, Austria; Unité Mixte de Recherche CNRS/UCL 8576,†† Glycobiologie Structurale et Fonctionnelle, Université des Sciences et Technologies de Lille 1, Villeneuve d’Ascq, France; Service de Chirurgie Vasculaire,*** ISPCC, CHU de Charleroi, Charleroi, Belgium; Medical University of Graz,††† Center for Molecular Medicine, Institute of Molecular Biology and Biochemistry, Graz, Austria; and Laboratory of Molecular Parasitology,§§§ IBMM, Faculty of Sciences, Université Libre de Bruxelles, Gosselies, Belgium

Abstract  Oxidation of LDL by the myeloperoxidase (MPO)-H₂O₂-chloride system is a key event in the development of atherosclerosis. The present study aimed at investigating the interaction of MPO with native and modified LDL and at revealing posttranslational modifications on apoB-100 (the unique apolipoprotein of LDL) in vitro and in vivo. Using amperometry, we demonstrate that MPO activity increases up to 90% when it is adsorbed at the surface of LDL. This phenomenon is apparently reflected by local structural changes in MPO observed by circular dichroism. Using MS, we further analyzed in vitro modifications of apoB-100 by hypochlorous acid (HOCl) generated by the MPO-H₂O₂-chloride system or added as a reagent. A total of 97 peptides containing modified residues could be identified. Furthermore, differences were observed between LDL oxidized by reagent HOCl or HOCl generated by the MPO-H₂O₂-chloride system. Finally, LDL was isolated from patients with high cardiovascular risk to confirm that our in vitro findings are also relevant in vivo. We show that several HOCl-mediated modifications of apoB-100 identified in vitro were also present on LDL isolated from patients who have increased levels of plasma MPO and MPO-modified LDL. In conclusion, these data emphasize the specificity of MPO to oxidize LDL.—Delporte, C., K. Z. Boudjeltia, C. Noyon, P. G. Furtmüller, V. Nuyens, M-C. Slomianny, P. Madhoun, J-M. Desmet, P. Raynal, D. Dufour, C. N. Koyani, F. Reyé, A. Rousseau, M. Vanhaeverbeek, J. Ducobu, J-C. Michalski, J. Nève, L. Vanhamme, C. Obinger, E. Malle, and P. Van Antwerpen.

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Over the past several years, considerable evidence has been obtained in support of the hypothesis that oxidants generated by the heme enzyme myeloperoxidase (MPO, EC1.11.2.2) play a key role in oxidation reaction of the artery wall. The enzyme, abundantly present in neutrophils and, to a lesser extent, in monocytes, is released during inflammatory activation of immune cells. MPO produces hypochlorous acid (HOCl) by the reaction of hydrogen

Abbreviations:  CD, circular dichroism; Cl-Tyr, 3-chlorotyrosine; di-OxTrp, dioxidized tryptophan; FA, formic acid; HOCl-LDL, MPO-modified LDL; HOCl, hypochlorous acid; HO-Tyr, 3-hydroxytyrosine; MPO, myeloperoxidase; MPO-LDL, LDL modified by the MPO-H₂O₂-chloride system; O-Met, methionine sulfoxide; OxTrp, oxidized tryptophan; PTM, posttranslational modification; QTOF, quadrupole TOF; RRLC, rapid resolution LC; SFI, score peak intensity.

¹To whom correspondence should be addressed. e-mail: cedric.delporte@ulb.ac.be
peroxide (H₂O₂) and chloride ions (Cl⁻), and this potent oxidant contributes to the antimicrobial activity of phagocytes (1, 2). However, evidence has emerged that either chronic or prolonged production of HOCl by the MPO-H₂O₂-Cl⁻ system contributes to tissue damage and the initiation and propagation of vascular diseases (3). HOCl-modified epitopes were present in acute and chronic vascular inflammatory diseases where staining was found to be associated with monocytes/macrophages, smooth muscle cells, and endothelial cells (4–6). As human atherosclerotic lesions contain elevated levels of MPO, the enzyme may act as a catalyst for LDL oxidation (7). Furthermore, the oxidation of LDL/apoB-100, leading to species called “oxidized LDLs (OxLDLs),” plays a crucial role in the pathogenesis of atherosclerosis (8–10). Fingerprinters for in vivo modifications of apoB-100 by the MPO-H₂O₂-Cl⁻ system were observed by immunohistological analyses (4, 11) and GC-MS (12). Observations that an increasing oxidant/LDL molar ratio of HOCl-modified apoB-100 is paralleled by a decreased ligand interaction by the classical LDL receptor (13) suggested that scavenger receptors on macrophages mediate the uptake of HOCl-modified LDL (HOCl-LDL) (14, 15). In addition to its capacity to promote lipid accumulation in monocytes/macrophages (16), HOCl-LDL adversely affects biological properties of smooth muscle cells and endothelial cells (13), thus favoring progression of atherosclerosis. Furthermore, LDL oxidized by the MPO-H₂O₂-Cl⁻ system (MPO-LDL) accumulates in macrophages and exerts proinflammatory effects on monocytes and endothelial cells (17, 18).

Modification by reagent HOCl alters the lipid moiety of LDL but primarily leads to amino acid oxidation favoring posttranslational modification (PTM) of the protein moiety. Lysine (Lys), histidine (His), and the N-terminal ε-amino group may form reactive chloramine species, which may lead to secondary oxidation processes (13). Methionine (Met) can be converted into sulfoxide form while tyrosine (Tyr) may be converted into 3-chlorotyrosine (Cl-Tyr), a specific marker for the MPO-H₂O₂-Cl⁻ system-mediated oxidation in vivo (12, 19, 20) and in vitro (19). Furthermore, it has been reported that MPO, probably due to its charge, can bind LDL (21, 22). That binding, which seems to be mediated via the protein moiety of LDL (23), may result in localized formation of oxidants and hence side-specific damages (22, 24). ApoB-100, the unique protein of LDL, is a highly hydrophobic protein with 4,536 amino acid residues (molecular mass 550 kDa). Furthermore, apoB-100 contains a high number of amino acid residues prone to be modified by HOCl.

In the present work, we have studied the impact of adsorption of MPO on native and HOCl-modified LDL and on its structural and enzymatic features. Using high-resolution MS, we then performed a comprehensive survey of PTMs on apoB-100 treated with HOCl as reagent or generated enzymatically. Numerous modifications were identified including methionine sulfoxide (O-Met), (di)-oxidized tryptophan [(di)-OxTrp], and Cl-Tyr. Finally, we compared these in vitro findings with oxidation patterns of LDL that has been isolated from hemodialysis patients who have increased levels of MPO and MPO-LDL (25).

**EXPERIMENTAL SECTION**

**Materials and reagents**

Ammonium bicarbonate, formic acid (FA), NaCl, K₂HPO₄, methanol, and acetonitrile were from Merck (Darmstadt, Germany). Trichloroacetic acid, butylated hydroxytoluene (BHT), dithio-teritol, iodoacetamide, ¹³C₆-tyrosine, ¹⁵N,Lysine, thioglycolic acid, 2,2,2-trifluoroethanol, tosyl phenylalanyl chloromethyl ketone-treated trypsin (from bovine pancreas), and peptide-N-glycosidase (PNGase) F (from *E. coli*) were from Sigma-Aldrich (St. Louis, MO). NaOCl solution was from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). PD-10 desalting columns were from GE Healthcare (Fairfield, CT). Agilent Si-SCX 1 ml 100 mg solid phase extraction cartridges, HCl, phenol, and benzoic acid were from VWR (Leuven, Belgium). Rapidist SF was from Waters (Milford, MA). Water was purified using a Milli-Q purification system (Millipore, Bedford, MA). MPO isolated from human neutrophils was from Plantag (Vienna, Austria), and human recombinant MPO was expressed in CHO cells (26).

**Isolation and oxidation of LDL**

**Isolation of LDL.** LDL was isolated by ultracentrifugation from healthy blood donors at the A. Vesale Hospital (Charleroi, Belgium) (27). Protein content was measured using the Lowry technique (28).

Chemical oxidation of LDL (HOCl-LDL). LDL (1 mg) was dispersed in 1 ml PBS (150 mM Cl⁻ and 10 mM phosphate, pH 7.4) and incubated for 2 h at 37°C with NaOCl (added as a single addition with gentle vortexing) at oxidant/LDL molar ratios in between 25:1 and 333:1. When needed, 1 mM Met was added to HOCl-LDL preparations to quench the reaction at the end.

**Enzymatic oxidation of LDL (MPO-LDL).** LDL (1 mg) was dispersed in 1 ml PBS (pH 7.4) and incubated with MPO and H₂O₂ for 4 h at 37°C. Two soft oxidations by MPO were performed using 110 nM of MPO and 50 or 100 µM of H₂O₂. Assum-
(10%, v/v) and centrifuged for 10 min at 4,500 g. The supernatant was discarded, and the precipitate was treated one more time with trichloroacetic acid. Pellets were then mixed with 1.1 ml of water-methanol-diethyl ether (1:3:7, v/v/v) to remove lipids. The mixture was sonicated for 30 min and then centrifuged for 10 min at 4,500 g. The supernatant was discarded, and the procedure was repeated.

**Isolation of apoB-100 from patients**

Alternatively, LDL was isolated from patients on chronic maintenance hemodialysis therapy (n = 9) or healthy volunteers (n = 9). Briefly, blood (5 ml) was drawn before dialysis in patients or in volunteers. Four hundred microliters of LDL solution from each patient/volunteer was treated as described previously. This study conforms to the Declaration of Helsinki, and its protocol was approved by the Ethics Committee of the ISPSP (“Intercommunale de Santé Publique du Pays de Charleroi”) Hospital. Finally, all subjects gave their written informed consent.

**Amperometric measurements of MPO activity**

The chlorination activity of MPO (100 nM) in the absence or presence of indicated lipoprotein concentrations was measured by continuously monitoring H$_2$O$_2$ consumption by amperometry using a combined platinum/reference electrode, which was covered with a hydrophilic and a dialysis membrane (cutoff 3,600 Da), fitted to the Amperometric Biosensor Detector 3001 (Universal Sensors Inc.). The applied electrode potential at pH 7.4 was 650 mV, and the electrode filling solution was freshly prepared half-daily (32). The electrode was calibrated against known H$_2$O$_2$ concentrations. In detail, consumption of H$_2$O$_2$ (100 µM) in the presence of 100 mM Cl$^-\text{H}$11002$ and 10 mM PBS (pH 7.4) was followed for 5 min at 25°C. The presence of LDL preparations did not affect the amperometric measurement of H$_2$O$_2$. In the presence of H$_2$O$_2$, Cl$^-\text{H}$11002, and LDL, no H$_2$O$_2$ consumption occurred within the 5 min experiment as long as MPO was absent. Reactions were started by addition of MPO either free or preincubated for 5 min with LDL or HOCl-LDL. One unit of chlorination activity is defined as the consumption of 1 µmol of H$_2$O$_2$ per minute at 25°C in the presence of 100 mM Cl$^-\text{H}$11002.

The peroxidase activity of MPO was measured in 10 mM PBS (pH 7.4) using 100 µM of Tyr as the one-electron donor. One unit of peroxidase activity is defined as the consumption of 1 µmol H$_2$O$_2$ per minute at 25°C in the presence of 100 µM Tyr.

**Circular dichroism spectroscopy**

Circular dichroism (CD) spectra were performed on a Chirascan spectrometer equipped with a thermostatic cell holder (Applied Photophysics, Leatherhead, UK). For recording far-UV spectra (190–260 nm), conditions were as follows: path length, 1 mm; spectral bandwidth, 3 nm; step size, 1 nm; scan time by nanometer, 10 s; protein concentrations, 0.1 mg/ml MPO and 0.1 mg/ml LDL or HOCl-LDL (oxidant/LDL molar ratio of 50:1). In the near-UV visible region (260–480 nm), conditions were as follows: path length, 10 mm; spectral bandwidth, 1 nm; step size, 1 nm; scan time by nanometer, 8 s; protein concentrations, 0.5 mg/ml MPO and 0.5 mg/ml LDL or HOCl-LDL (oxidant/LDL ratio = 50:1). All CD measurements were performed in 5 mM PBS (pH 7.4) at 25°C (33). Each spectrum was automatically corrected with the baseline to remove birefringence of the cell.

**ApoB-100 hydrolysis for amino acid analysis**

ApoB-100 protein pellets (obtained from isolated native or oxidized LDL samples) were hydrolyzed using a StartS microwave oven and a protein hydrolysis reactor according to the manufacturer’s protocol (Milestone, Italy). Briefly, apoB-100 (1 mg) was placed into the vial, 200 µl of acid mixture [6 M HCl supplemented with 10% (v/v) thioglycolic acid, 0.1% (v/v) phenol, and 0.1% (v/v) benzoic acid] and internal standards were added, and hydrolysis was carried out for 40 min at 160°C. $^{13}$C-Tyr and $^{15}$N-Lys were used as internal standards. Samples were thereafter purified using Si-SCX solid phase extraction cartridges. Briefly, columns were flushed twice with 1 ml methanol and then equilibrated with 2 ml FA (0.2 M). After hydrolysis, the samples were loaded and washed with 2 ml FA (0.2 M). Amino acids were eluted with 2 ml methanol containing 5% (v/v) NH$_4$OH. Samples were evaporated to dryness using a vacuum centrifuge and finally dissolved in 50 µl water before injection of 10 µl into the LC-MS system.

The LC system was a rapid resolution LC (RRLC) 1200 series using a Zorbax Eclipse XDB Phenyl RR column [4.6 × 150 mm inner diameter (ID)], 3.5 µM particle size] and coupled to an electrospray ion source (ESI) in positive mode quadrupole TOF (QTOF) 6520 series mass spectrometer from Agilent Technologies. Amino acid residues were separated by an acetonitrile gradient, and amino acids of interest were analyzed by MS/MS using Mass Hunter Acquisition® and Qualitative Analysis® (Agilent Technologies).

**Identification of PTMs of ApoB-100**

**Digestion.** Protein pellets from native or oxidized LDL preparations were treated using an optimized method that ensures an optimal protein recovery (31). Briefly, apoB-100 was unfolded using 250 µl of RapiGest SF (Waters) 0.2% (w/w) in 50 mM ammonium bicarbonate buffer (pH 7.8), reduced using dithiothreitol (20 mM) at 37°C during 30 min, and finally, alkylated with iodoacetamide (60 mM) for 30 min in the dark. The solution was heated at 100°C (5 min), and apoB-100 was then digested by trypsin (enzyme-protein = 1:10, w/w) at 37°C for 24 h. The reaction was then stopped by heating the sample at 100°C for 30 min. Deglycosylation of tryptic peptides was performed with PNGase F (10 U/mg of protein) during 24 h at 37°C. The sample was then adjusted to 0.5% FA, incubated (30 min, 37°C), and centrifuged (10 min, 13,000 g). The supernatant was evaporated to dryness in a centrifugal vacuum evaporator. Finally, peptides were dissolved in 50 µl FA [0.1% (v/v) in water] before analysis.

Additionally, unfolding of protein was performed by 2,2,2-trifluoroethanol. Briefly, to 1 mg protein, 50 µl of 2,2,2-trifluoroethanol, 50 µl of ammonium bicarbonate buffer (100 mM, pH 7.8), and 2 µl of 200 mM dithiothreitol were added. The sample was heated at 60°C (1 h) and then cooled to 20°C. Eight microliters of 200 mM iodoacetamide was added, and the sample was kept in the dark at 20°C (1 h). Finally, 2 µl of 200 mM dithiothreitol was added, and the sample was kept at 20°C (1 h) in the dark before dilution with 600 µl of water and 200 µl of bicarbonate buffer (100 mM). ApoB-100 was then digested at 37°C (24 h) by adding 50 µl of trypsin (enzyme-protein = 1:10, w/w). Trypsin was then inactivated by heating the sample (100°C, 30 min). After cooling to 20°C, deglycosylation was performed at 37°C (24 h) by adding 10 units of PNGase F. Finally, the sample was acidified by addition of 2 µl of FA (100%) and evaporated to dryness in a centrifugal vacuum evaporator. Peptides were dissolved in 50 µl of FA [0.1% (v/v) in water] before analysis.

**LCMS/MS process, data acquisition, and analysis.** Ten microliters of the resulting samples was injected into the LC system and analyzed as described previously (31). Briefly, analyses were performed with the same RRLC 1200 series mentioned previously. Peptides were separated on a Fused Core Ascentis® Express C18 column (100 × 2.1 mm ID, 2.7 µm particle size) from Supelco (Bellefonte, PA) using a 105 min gradient of FA and acetonitrile. The ESI-QTOF mass spectrometer mentioned previously was used for the MS/MS analyses. Auto-MS/MS spectra were

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RESULTS

Activity and structure of MPO at the surface of LDL

Measurement of MPO activity. MPO interacts preferentially with the protein moiety of native LDL (23, 34). It is likely that the enzyme may interact in a similar manner with modified LDL; we therefore studied whether this interaction is paralleled by changes in the activity of MPO. Figure 1A shows that the chlorinating activity of MPO increases as a function of increasing LDL concentration; at the highest LDL concentration, the chlorinating activity of MPO increased by 90%. In order to investigate whether an increased consumption of H₂O₂ could result from scavenging of HOCl by LDL (which would protect MPO from inhibition by its own product), Met was added. However, the presence of this HOCl scavenger had no impact on the observed kinetics when compared with samples containing MPO only.

Second, the effect of HOCl-LDL on H₂O₂ consumption by MPO was investigated. Compared with native LDL (0.3 mg/ml), the chlorinating activity of MPO incubated with HOCl-LDL at identical concentrations was lower (Fig. 1B). Although at low oxidant/lipoprotein molar ratios (83:1 and 166:1) the chlorinating activity of MPO was significantly higher (~20%) compared with MPO alone, a high...
oxidant/lipoprotein molar ratio (333:1) significantly decreased MPO activity by ~20%.

Third, we tested whether the chlorinating activity of MPO is dependent on the concentration of HOCl-LDL at a given oxidant/lipoprotein molar ratio (333:1). Preincubation of MPO with HOCl-LDL did not affect the chlorinating activity at low concentrations of HOCl-LDL, whereas consumption of H$_2$O$_2$ by MPO was decreased by ~35% at the highest lipoprotein concentration (Fig. 1C).

Next, we investigated whether the observed behavior was independent of an electron donor and thus independent of the nature of the oxidation product released by MPO. Peroxidase activity in the presence of Tyr showed similar effects on H$_2$O$_2$ consumption by MPO, namely increased activity in the presence of native LDL but decreased peroxidase activity in the presence of highly modified LDL (Fig. 1D).

These data illustrate that native LDL enhances its ability to be oxidized by MPO apparently due to a specific interaction between MPO and nonmodified apoB-100. In contrast, HOCl-LDL may decrease the activity of MPO, suggesting that oxidation of apoB-100 by HOCl may modulate the MPO-apoB-100 interaction.

**Structural features of MPO adsorbed on LDL.** Next, we investigated whether altered activity of MPO after preincubation with native LDL or HOCl-LDL (Fig. 1) could also be reflected by changes on a spectroscopic basis. Electronic CD spectroscopy was chosen because it allows analysis of both the overall secondary structure and the (asymmetric) environment of the prosthetic heme group. In the far-UV region, the spectra of MPO in the presence of LDL or HOCl-LDL exactly matched the sum of spectra of individual lipoproteins at the same protein concentrations (data not shown). This suggests that the structural changes must be local and that the overall content of the secondary structure of MPO (which is mainly α-helical) is not affected.

However, this observation seems to be in contrast to the Soret region. Native MPO has a Soret minimum at 410 nm, whereas the ellipticity of native LDL and HOCl-LDL is negligible at this wavelength region (Fig. 2). Upon incubation of MPO with native LDL, ellipticity is lost in the Soret region, whereas in the near-UV range (260–350 nm) the difference was very small. This observation is indicative for structural changes in the immediate surroundings of the prosthetic group. The effect on the CD of the heme group was smaller whereas between 300 and 350 nm ellipticity was lost. This indicates that HOCl-LDL has some local impact on the tertiary structure of MPO but not on the architecture of the active site. In any case, the observed changes in the enzymatic activity in the presence of native- or HOCl-LDL are reflected by structural changes at the active site of the peroxidase with native LDL having a stronger impact than HOCl-LDL.

**MS analysis of apoB-100 modified chemically or enzymatically**

**Total hydrolysis of apoB-100.** In order to investigate whether modifications of apoB-100 differed when LDL was oxidized under several conditions, we investigated the formation of Cl-Tyr, O-Met, (di-)OxTrp, and aminoadipic acid, a degradation product of N-chloramine Lys (35, 36). Figure 3 shows that the ratios of aminoadipic acid/Lys and Cl-Tyr/Tyr (Fig. 3A, B, respectively) increased as a function of increasing concentrations of HOCl (added as a reagent), although oxidation of apoB-100 by HOCl (generated by the MPO-H$_2$O$_2$-Cl$^-$ system) resulted in formation of Cl-Tyr only. No increase in the aminoadipic acid/Lys ratio was found when apoB-100 was oxidized enzymatically or when H$_2$O$_2$ and Cl$^-$ were added in the absence of MPO (Fig. 3).

Next, we focused on oxidation of Met and tryptophan (Trp). Independent of whether apoB-100 was native or modified (either chemically or enzymatically), Met was widely oxidized. Formation of OxTrp and di-OxTrp was found in all LDL/apoB-100 samples with no statistical difference even if there was a tendency to increased levels of di-OxTrp under both oxidative conditions (twice the level observed in native LDL).

To confirm that chlorination is specific for HOCl treatment, apoB-100 was oxidized by copper (18) and then subjected to hydrolysis. No Cl-Tyr formation was observed, while low levels of (di-)OxTrp may also occur, data that parallel previous findings (37).

**Mapping of in vitro modifications on apoB-100.** Next, we analyzed the location of PTMs on apoB-100. Instead of RapiGest SF (31), 2,2,2-trifluoroethanol was also used as the unfolding agent to recover apoB-100 with the same results on protein sequence recovery (79%) or even higher.

Analyses revealed that few residues (Met$_4$, Met$_{785}$, Met$_{1873}$, Met$_{2002}$, Trp$_{2468}$, Trp$_{2945}$, and Met$_{1092}$) were already oxidized in native LDL. The latter residues seem thus to be highly sensitive to oxidation.

Oxidation of apoB-100 was first performed by reagent HOCl under soft conditions (oxidant/lipoprotein molar ratio = 25:1 or 50:1). **Table 1** summarizes all PTMs identified.
in the corresponding tryptic peptides. A total of 43 modified peptides have been identified, and modifications (n = 46) include formation of O-Met (n = 33), OxTrp (n = 8), di-OxTrp (n = 3), and Cl-Tyr (n = 2). In most peptides, a single amino acid modification (primarily O-Met but also OxTrp, di-OxTrp, or Cl-Tyr) was found, while in three peptides two modifications were observed (either two O-Met residues or the combination of O-Met and OxTrp).

Next, PTMs generated via the MPO-H$_2$O$_2$-Cl$^-$ system also under soft conditions (oxidant/lipoprotein molar ratio = 25:1 and 50:1 assuming almost equimolar conversion of H$_2$O$_2$ to HOCl) were identified. Under these conditions, several modifications already observed with reagent HOCl were found. However, 13 additional peptides specific for MPO-mediated modification (Table 2) were found. Again, O-Met is the most abundant PTM, while only a single Cl-Tyr-containing peptide was found to be specific for MPO treatment.

Furthermore, oxidation of LDL was performed by the MPO-H$_2$O$_2$-Cl$^-$ system under more drastic conditions (oxidant/lipoprotein molar ratio = 625:1) previously used to raise monoclonal antibodies to detect MPO-LDL in vivo and in vitro (11). In addition to oxidative modifications listed in Table 1, 41 tryptic peptides carrying PTMs (n = 46) were identified (Table 3). These peptides further include 3 residues of 3-hydroxytyrosine (HO-Tyr), 5 O-Met residues, 10 OxTrp residues, 18 di-OxTrp residues, and 10 Cl-Tyr residues, respectively. Again, in most peptides a single amino acid modification (primarily di-OxTrp in this case) was found, while few peptides included either two (O-Met and HO-Tyr or O-Met and Cl-Tyr) or three modifications (two O-Met and HO-Tyr). These findings indicate that a more pronounced enzymatic oxidation of apoB-100 further targets Trp and Tyr residues.

**Mapping of MPO-mediated PTMs of apoB-100 isolated from patients undergoing hemodialysis or healthy volunteers.** To confirm whether our in vitro findings may occur also in vivo, LDL was isolated from patients (n = 9) who have high levels of MPO (38, 39) and circulating MPO-LDL (25) and compared with healthy volunteers (n = 9). As levels of oxidized LDL in patients with hemodialysis are still low when compared with levels of native LDL, apoB-100 was analyzed using a nano-LC-MS/MS with high-resolution analytical column (Polaris CHIP), which is more sensitive than an LC system.

In sum, 90 PTMs were identified over patients and volunteers. Again, O-Met (n = 31) is a prominent modification compared with HO-Tyr (n = 30), OxTrp (n = 16), and di-OxTrp (n = 12), while only one Cl-Tyr was observed. Most of the modifications match those found when apoB-100 was treated in vitro, whereas several were newly detected, such as HO-Tyr (76, 249, 425, 693, 713, 1579, 1753, 1747, 1965, 1972, 2189, 2392, 2400, 2732, 3206, 3258, 3533, 3653, 3744, 4055, 4057, 4184, 4206, 4242, 4244, 4464, 4636), Cl-Tyr (125, 126), O-Met (507, 1022, 1162, 1239, 1560, 2481), OxTrp (936, 4063), and di-OxTrp (936). Among PTMs already detected in vitro, O-Met was described as MPO specific (22). In our experiments, O-Met was observed in apoB-100 of all patients but also all volunteers, showing that this site is highly sensitive and is not relevant as a biomarker. The same results were observed for (di)-OxTrp (1114 and 3536), which were previously described (37).

In contrast, several less prominent PTMs seemed interesting, such as O-Met (499), which was observed only in patients (n = 2); OxTrp (2904), observed in five patients and only one volunteer; OxTrp (3606), observed in four patients and only one volunteer; and finally, Cl-Tyr (125), observed in only one patient. Furthermore, O-Met (125), OxTrp (3606), and OxTrp (3606) are identical to those observed when LDL/apoB-100 was treated with the MPO-H$_2$O$_2$-Cl$^-$ system under conditions using the molar ratio 625:1 (Table 3). Cl-Tyr (125) might also be interesting as it is specific to the MPO-H$_2$O$_2$-Cl$^-$ system and was never detected before.

**DISCUSSION**

Methodologies for detecting and identifying protein modifications have become increasingly important as the roles of modified (lipo)proteins in disease etiology and...
biomarkers are becoming more apparent (40). Because OxLDL impairs the physiological functioning of various cells (13, 17) and plays a causal role in atheroma plaque formation, both the nature of oxidants as well as the modified entity of the lipoprotein particle are of importance. Oxidation of LDL can be carried out by, among others, reactive oxygen species generated by vascular endothelium or transition metals, hemoglobin, lipoxygenases, and reactive nitrogen species (19, 20). HOCl can modify LDL at the lipid and protein moieties in vitro and/or in vivo (13). Modulated phagocytes, and HOCl can modify LDL at the lipid and protein moieties in vitro and/or in vivo (13). Modulated phagocytes, and HOCl can modify LDL at the lipid and protein moieties in vitro and/or in vivo (13).

**TABLE 1. List of apoB-100-derived tryptic peptides carrying modifications generated by reagent HOCl**

| Peptide | Error (ppm) | Peptide Score | % SPI | Z | Sequence with Fragmentation | Modified Amino Acid |
|---------|-------------|---------------|-------|---|-----------------------------|--------------------|
| 1–14    | 1.9         | 17.8          | 91    | 2 | (E)E/E/E m/L/E/N/V/S/L/V/C/P K(D) | O-Met^
| 91–101  | 0.5         | 12.9          | 71    | 2 | (K)N S[E/F/A/A/m/S/R(Y) | O-Met |
| 203–220 | 3.3         | 17.8          | 92    | 2 | (K)G m/T R P l S l T l | S/S Q[S/C/Q]Y(T) | O-Met |
| 249–260 | 2.1         | 18.0          | 85    | 2 | (K)G m/[V][Q][O][Q][T][O][O] l/K(L) | O-Met |
| 279–287 | 2.0         | 13.6          | 93    | 2 | (K)m/[G]l/A/F/E/S T(K) | O-Met |
| 401–407 | 3.0         | 10.2          | 72    | 2 | (R)E/F/N/m/A/R(D) | O-Met |
| 464–480 | 3.0         | 15.2          | 76    | 2 | (R)V/1/G/N m/Q G Q m E Q L/T(G) E/L/K(S) | O-Met |
| 486–498 | 3.5         | 11.6          | 73    | 2 | (K)C/Q[S]T K/P S l m I Q/K(A) | O-Met |
| 532–540 | 1.3         | 15.5          | 85    | 2 | (R)L/A[A]l/m L/m/L/m/R(S) | O-Met |
| 550–563 | 1.9         | 17.5          | 82    | 3 | (K)I V[Q][L]p w E/Q N E Q V/K(N) | OxtTrp |
| 550–563 | 1.2         | 14.0          | 84    | 3 | (K)I V[Q][L]p w E/Q N E Q V/K(N) | OxtTrp |
| 594–604 | 2.3         | 15.7          | 92    | 2 | (K)S/Q[L]p T/V m D/F R(K) | O-Met |
| 691–705 | 0.5         | 14.9          | 80    | 2 | (K)A L/Y|w|V|n G Q/V|P D G V/S K(V) | O-Met |
| 691–705 | 1.1         | 16.3          | 92    | 2 | (K)A L/Y|w|V|n G Q/V|P D G V/S K(V) | O-Met |
| 706–715 | 1.9         | 8.3           | 71    | 3 | (K)V/L[V]/D H F G y T(K) | O-Met |
| 717–771 | 3.0         | 11.5          | 86    | 2 | (K)L I/m/G A/R(T) | O-Met |
| 778–791 | 2.7         | 13.1          | 86    | 2 | (R)L I/[Q]G|l P m Q m G E V I/R(K) | O-Met |
| 837–846 | 1.4         | 10.1          | 89    | 2 | (K)E/V A N m/Q m A/E L(V) | O-Met |
| 870–891 | 3.9         | 19.0          | 83    | 3 | (R)S G[V/Q]m/N/T/N/T/F/H E S G L E A H V/A/L/K(A) | O-Met |
| 1,092–1,051 | 3.4 | 17.9          | 97    | 2 | (R)Q s m T/L/S/E E V/Q[I/P D/F/D/V/D/L/G T I/L/R(V) | O-Met |
| 1,119–1,132 | 0.6 | 20.4          | 70    | 2 | (K)L/L/I/Q m/m D S/S A/T A/Y G S T/V S/K(R) | O-Met |
| 1,162–1,175 | 2.2  | 15.7          | 93    | 2 | (K)m T/S/N/F/P V/D/L/S D/Y P(K) | O-Met |
| 1,176–1,183 | 1.2  | 11.3          | 62    | 2 | (K)s l/h/I/m Y/A N/R(L) | O-Met |
| 1,203–1,213 | 1.1  | 18.2          | 87    | 2 | (K)L/l/I/v[S m/S/W/L/Q/K(A) | O-Met |
| 1,203–1,213 | 2.3  | 12.0          | 81    | 2 | (K)L/I/v[A m S S w/L Q/K(A) | O-Met |
| 1,676–1,697 | 0.4  | 23.7          | 94    | 2 | (K)a a L T/E/L I/S/G/S A/Y/Q A m m I/L G/V/D/S K(S) | O-Met |
| 1,871–1,876 | 1.1  | 8.2           | 91    | 1 | (R)s v[m]a/p[f(T) | O-Met |
| 1,890–1,901 | 3.8  | 14.1          | 91    | 2 | (K)L/a/l/w G E H T G Q executives | O-Met |
| 1,986–2,016 | 3.2  | 18.0          | 82    | 3 | (R)T I/L D /L T/L I/D S P I/K[V P I/L/L S P I/E/P I/N I/L I/D | O-Met |
| 2,214–2,228 | 0.3  | 11.7          | 63    | 2 | (K)G S G T/A/S/w/[I/Q]N V/D/T(K) | O-Met |
| 2,321–2,335 | 0.8  | 19.4          | 81    | 2 | (R)E/V[d]Q/O/Q/[Q/V]I/L/m/D K(D) | O-Met |
| 2,462–2,470 | 10.8  | 12.2          | 74    | 2 | (K)T/I/L I/N w L Q(E) | O-Met |
| 2,565–2,582 | 2.2  | 22.7          | 93    | 3 | (K)T I/L/G/T/m P/A/F/E/V/S/L/Q/A/L/Q(K) | O-Met |
| 2,573–2,593 | 1.6  | 6.6           | 64    | 2 | (K)d P/S I/l w K(T) | O-Met |
| 3,122–3,128 | 1.5  | 6.6           | 64    | 2 | (K)d P/S I/l w K(T) | O-Met |
| 3,257–3,264 | 2.7  | 21.6          | 90    | 2 | (F)T/I E/m/S/A/F/G Y/V/F/P(K) | O-Met |
| 3,257–3,264 | 2.7  | 21.1          | 93    | 2 | (K)a a L/S[p/F/S/F/L/I/G/S D V/R(V) | O-Met |
| 3,423–3,434 | 2.4  | 12.0          | 80    | 3 | (K)S K P T[V]S/S/S m F/E/K(Y) | O-Met |
| 3,926–3,946 | 1.6  | 15.1          | 75    | 3 | (R)d F/S/a/l/E/I/E D G K Y/E/G L/Q e w G K(A) | O-Met |
| 4,080–4,094 | 0.0  | 16.0          | 88    | 2 | (R)n/L/Q N/a/e/w/v/Y/Q/G A/T R(Q) | O-Met |
| 4,080–4,094 | 2.1  | 19.4          | 88    | 2 | (R)n/L/Q N/a/e/w/v/Y/Q/G A/T R(Q) | O-Met |
| 4,187–4,195 | 2.9  | 14.9          | 89    | 2 | (R)E/E/L/C/T/m/F/E/R(E) | O-Met |
| 4,455–4,475 | -0.1 | 11.2          | 80    | 2 | (K)L/D/V/I/S/m/Y R(E) | O-Met |

LDL was modified by HOCl added as a reagent (50 or 100 µM) (oxidant/LDL molar ratio = 25:1 and 50:1). ApoB-100 mapping was carried out by RRLC-MS/MS. Each modified peptide is characterized by its peptide score, % SPI, mass error, fragmentation, and position(s) of modified residue(s).
The pattern of enzymatic modification differs from those reported to act as a specific target for LDL nitration (45). Met, apparently the first target for oxidation, is highly reactive toward HOCl-mediated attack (13), and Met residues may protect proteins from critical oxidative damage (46). In addition to Met$^{3719}$ and Met$^{4192}$, Met$^{3}$ and Cys$^{61,734,4190}$ were reported to be specific MPO-mediated modifications of apoB-100 (22, 24). Our results suggest that O-Met$^{3}$ and O-Met$^{4192}$ are already present on non-in vitro oxidized LDL isolated from healthy volunteers. These data also reflect the high sensitivity of those residues to oxidative damages. Modifications of residues Met$^{3719}$ and Cys$^{61,734,4190}$ were not found under our experimental conditions. Cys residues have been reported to rapidly react with HOCl (47, 48). Despite careful analysis of possibly oxidized Cys residues, none were detected under our experimental conditions.

We further show that oxidation of apoB-100 by HOCl (added as a reagent) leads to a higher content of aminoadipic acid compared with MPO-mediated oxidation. On the other hand, no statistical difference in chemical and enzymatic oxidation was seen for Trp and Met residues, a fact underlining their high sensitivity toward oxidation. So again, oxidation patterns are dependent on the oxidant, suggesting that reagent HOCl does not exactly mimic the respective MPO enzymatic oxidation.

Both the extent of modification as well as the difference in the charge of amino acid side chains may alter the binding properties of MPO to the respective LDL particle in vivo or in vitro. Adsorption of MPO on native LDL increased both chlorinating and peroxidase activities (Fig. 1), and this was reflected by structural changes in the heme cavity using CD spectroscopy (Fig. 2). These findings are in accordance with Sokolov et al. (49) who demonstrated that the addition of LDL (but not HDL) protects MPO from inhibition by ceruloplasmin. This observation favors a specific
interaction between LDL and MPO that differs from MPO activity at the surface of LDL might indicate an interaction with high-density lipoprotein (34). An increase in the activity of MPO could be underestimated in vitro and/or in vivo. On the other hand, MPO activity decreased with increasing HOCl/LDL molar ratios and HOCl-LDL concentrations, diminishing MPO activity to some extent.

Oxidative Trp, or N-chloramine Lys) alter the interaction between LDL and MPO under conditions using the drastic molar ratio 625:1 (11) as performed in the present study. The respective epitope identified by one of the antibodies corresponded to a 66 kDa fragment of apoB-100 starting with Gly1612 (11). Using this oxidation protocol, a total of 41 peptides carrying at least one PTM (e.g., primarily modified Trp and Tyr residues but also O-Met residues) were detected (Table 3). In that particular 66 kDa fragment, six modifications (Met409, Tyr1067, and Trp1095) are probably the likely recognition sites for the antibody.

**TABLE 3.** List of apoB-100-derived tryptic peptides carrying modifications generated by the MPO-H2O2-Cl2 system under drastic conditions (molar ratio = 625:1).

| Peptide | Error (ppm) | Peptide Score | % SPI | Z Position(s) of modified residue(s). |
|---------|-------------|---------------|-------|--------------------------------------|
| 102–105 | 1.9         | 3.1           | 80    | 2 (R) y/E/L K (L)                     |
| 748–751 | 3.7         | 3.0           | 84    | 2 (R) A/y/L R (K)                     |
| 1,108–1,118 | –0.5     | 15.0          | 76    | 2 (R) S/E/I[L]/A/H/w/S P A K (L)      |
| 1,108–1,118 | –0.6     | 12.0          | 81    | 2 (R) S/E/I[L]/A/H/w/S P A K (L)      |
| 1,176–1,183 | 3.2       | 12.6          | 85    | 2 (K)/S/L/A/H/w/S A/N/R (L)          |
| 1,575–1,583 | 1.0       | 14.1          | 71    | 2 (R) S [Q]A/D Y/E/L S/L (R)         |
| 1,682–1,694 | 0.1       | 10.8          | 63    | 2 (L)/S L S A/y Q A m I/L/G/V/D/S K (N) |
| 1,743–1,750 | 2.3       | 6.3           | 82    | 2 (K)/L D/N/I/y S S D K F Y/K (Q)     |
| 1,890–1,901 | 0.4       | 10.2          | 72    | 2 (K)/L A/w/G E H T G Q/L (Y)         |
| 1,890–1,901 | 0.9       | 13.5          | 79    | 2 (K)/L A/w/G E H T G Q/L (Y)         |
| 1,941–1,955 | –0.5      | 10.4          | 70    | 2 (K)/V S A/L[I]/T P A E/T Q T G t/(K) |
| 2,086–2,106 | 1.7       | 18.2          | 94    | 3 (K)/V S A/L[I]/T P A E/T Q T G t/(K) |
| 2,214–2,228 | –2.9     | 15.8          | 74    | 2 (K)/S G S T/A/S/w/ (Q/N/W/D/T/K)    |
| 2,334–2,341 | –1.9      | 11.9          | 73    | 2 (K)/L V/E/L/A/H Q y(K)             |
| 2,402–2,415 | –0.7      | 5.1           | 64    | 2 (K)/S D/y H Q F V/D E T N D K (I)   |
| 2,462–2,478 | 0.3       | 8.5           | 74    | 2 (K)/T L I N/w/L Q E/A/L S/A/S (L/A) |
| 2,462–2,478 | –1.6      | 10.6          | 85    | 2 (K)/T L I N/w/L Q E/A/L S/A/S (L/A) |
| 2,496–2,507 | 0.7       | 12.0          | 80    | 2 (R) m/y/Q/m D I/Q/Q/E L/Q/R (Y)    |
| 2,542–2,548 | 2.0       | 12.5          | 77    | 2 (Y) S/I/Q/D/w/A/K (R)              |
| 2,533–2,548 | –0.1      | 15.0          | 70    | 2 (K)/N L T D/F/A/E/Q/Y I/S/Q/D/w/A/K (R) |
| 2,731–2,736 | 2.0       | 6.2           | 73    | 2 (K)/L y/S/I/L (K)                  |
| 2,889–2,899 | 5.6       | 11.0          | 73    | 2 (K)/A G H J A/w/T S/S/G/K (G)       |
| 3,051–3,065 | 0.1       | 17.9          | 80    | 2 (F)/L/S/P S/A/Q/I/Q/w/Q V/Q S A/R (F) |
| 3,051–3,065 | 1.3       | 13.9          | 73    | 2 (F)/L/S/P S/A/Q/I/Q/w/Q V/Q S A/R (F) |
| 3,122–3,128 | 2.0       | 10.7          | 73    | 2 (K)/D F S I/w/E K (T)              |
| 3,511–3,520 | 0.5       | 18.2          | 82    | 2 (K)/D I[D]/I[w/N/L/E/V/K (E)       |
| 3,511–3,520 | 1.5       | 18.2          | 82    | 2 (K)/D [I[w/N/L/E/V/K (K)          |
| 3,522–3,531 | 0.3       | 10.9          | 77    | 2 (R)/I [S/l/w/E/H/S T/K (N)         |
| 3,532–3,541 | 1.7       | 13.6          | 83    | 2 (K)/Y I/S/L/y/E/H/S T/K (N)        |
| 3,604–3,611 | 1.7       | 4.5           | 68    | 3 (K)/I [R/w/K/N/E/V/R (I)           |
| 3,647–3,655 | –4.4      | 5.0           | 78    | 2 (K)/N I[I]/L/P Y v D K (S)         |
| 3,656–3,662 | 1.3       | 6.8           | 78    | 2 (K)/S I[w/D F L K (L)               |
| 3,656–3,662 | 7.5       | 10.3          | 72    | 2 (K)/S I[w/D F L K (L)               |
| 3,860–3,876 | 0.1       | 18.3          | 85    | 3 (R)/E V D S P/V/Y/n/A/T/w/S A/S L K (N) |
| 3,860–3,876 | 0.7       | 17.2          | 80    | 2 (R)/E V D S P/V/Y/n/A/T/w/S A/S L K (N) |
| 3,926–3,946 | –0.4      | 14.2          | 70    | 3 (R)/D F S [A/I/E/E D E G K Y/E/L/Q/E w E G K (A) |
| 4,106–4,120 | 1.4       | 10.6          | 64    | 3 (K)/A [S/G/T/T/G T Y Q E W K D K (A) |
| 4,106–4,120 | –0.1      | 11.2          | 64    | 3 (K)/A [S/G/T/T/G T Y Q E w/K D K (A) |
| 4,235–4,243 | 0.2       | 10.5          | 87    | 2 (K)/L D[I][V/S/m y R (E)           |
| 4,359–4,372 | 1.2       | 15.3          | 74    | 2 (R)/E Y/E/F/P D [P I/V/G/w/T/V/K (Y) |

LDL was modified by HOCl generated by the MPO-H2O2-Cl2 system using drastic conditions (oxidant/LDL molar ratio = 625:1 considering equimolar formation of HOCl). ApoB-100 mapping was carried out by RRLC-MS/MS, and listed modifications are the ones not recovered with the softest conditions described in Table 1 and Table 2. Each modified peptide is characterized by its peptide score, % SPI, mass error, fragmentation, and position(s) of modified residue(s).
and compared with apoB-100 of healthy volunteers. Among the observed PTMs, O-Met and OxTrp residues were detected, as well as only one CI-Tyr. This suggests that CI-Tyr formation on apoB-100 is rare, but Tyr is an interesting PTM for future studies in MPO. However, only one patient among nine carried this MPO-specific modification. Furthermore, O-Met was only observed in patients and so is an interesting PTM that should be investigated in future studies.

Although we could not find PTM in the close vicinity of the LDL receptor binding domain [between residues 3,346 and 3,369 of apoB-100 (52, 53)], the remaining PTMs, markers of oxidative stress and/or lipoprotein abnormalities (54), could still contribute to impaired binding of MPO-LDL to the LDL receptor favoring scavenger receptor-mediated uptake as reported for nitrated LDL (45). Furthermore, Trp is close to the binding domain and might interact with the latter when oxidized, and OxTrp was observed in more patients (n = 4) than volunteers (n = 1).

Summarizing, using LC-MS/MS we further identified in vitro a series of modified tryptic peptides (n = 97) that are indicative of modification by HOCl, added as reagent or generated enzymatically. Among these modifications, several have been identified in vivo from patients suffering from kidney failure and undergoing hemodiagnosis where high MPO levels are causally linked with LDL modifications and abnormalities in clearance of the modified LDL particles. Our data also highlight that, when studying LDL oxidation, HOCl added as a reagent does not completely mimic enzymatic modification via the MPO-\(\text{H}_2\text{O}_2\)-Cl\(^-\) system and that MPO activity could be misestimated under in vitro or in vivo experiments due to the fact that MPO/apoB-100 interaction is specific and changes the enzymatic activity. Future experiments mapping PTMs of LDL in different cardiovascular diseases are also of importance to link individual oxidative modifications with disease severity.

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