

Kinetics of tryptophan oxidation in plasma lipoproteins by myeloperoxidase-generated HOCl

Andreas Jerlich¹, Michal Hammel², Fabienne Nigon³, M. John Chapman³, R. Jörg Schaur¹

¹Institute of Molecular Biology, Biochemistry and Microbiology, University of Graz, Austria; ²Department of Physical Chemistry, Faculty of Pharmacy, Comenius University, Bratislava, Slovak Republic; ³National Institute of Health and Medical Research (INSERM) Unit 321, Lipoproteins and Atherogenesis, Hôpital de la Pitié, Paris, France

The relative susceptibility of the apoprotein components of human lipoproteins [high-density lipoprotein (HDL) and low-density lipoprotein (LDL)] and their subclasses to oxidation by the myeloperoxidase/H₂O₂/Cl⁻ system *in vitro* was studied by measuring the decrease in rate of tryptophan fluorescence. Whereas the lipoprotein-modification rate showed a saturation type of dependence on the concentration of myeloperoxidase, a biphasic dependence on the concentration of the lipoproteins was found. High concentrations of H₂O₂ were also found to inhibit tryptophan oxidation in LDL but to a lesser extent in HDL. The optimal rate of LDL and HDL modification was observed at pH 6.0. HDL was modified much more rapidly than LDL, which may be due to differences in size and different relative contents of protein and lipids per particle. No differences in rates of modification of LDL subclasses were observed, when the assays were standardized to equal LDL protein concentrations, but, when standardized to equal particle mass, an optimum at subclass 8 was found, which is probably due to differences in apolipoprotein B-100 conformation. It was concluded that HDL may have a beneficial effect in retarding LDL modification in inflammatory processes.

Keywords: high-density lipoprotein; hypochlorous acid; low-density lipoprotein; myeloperoxidase; tryptophan fluorescence.

Oxidative modification of low-density lipoprotein (LDL) is generally thought to be a crucially important process in atherogenesis [1], while high-density lipoprotein (HDL) is believed to protect against vascular diseases by removing excess cholesteryl esters from cells of the artery wall [2].

HDL apparently inhibits LDL oxidation [3–6] but is itself susceptible to Cu²⁺-stimulated lipid peroxidation [5,7], thus losing its ability to promote cholesterol efflux from cultured macrophage foam cells, suggesting that oxidation may favor the formation of vascular lesions [7].

Myeloperoxidase (MPO), an enzyme present in polymorphonuclear neutrophil granulocytes and monocytes, has been found in human atherosclerotic tissue [8], and specific products of MPO have been detected during all stages of the development of atherosclerosis [9]. This enzyme catalyzes several redox reactions resulting in efficient modification of both the lipid and the protein moiety of plasma lipoproteins. A major product is HOCl, which is formed by the reaction of Cl⁻ ions with H₂O₂. HOCl, which is in equilibrium with its anion and molecular chlorine [10], is produced in micromolar concentrations and is rapidly consumed by LDL in a concentration-dependent manner [11].

For HOCl, various protein chlorination/oxidation reactions have been shown: inactivation of thiol groups, chlorination of protein amines [12,13], modification of proteins including enzymes with antioxidant function [14–16], and conversion of protein-bound or free tyrosine to a tyrosyl radical by removal of a hydrogen atom [17,18].

According to the findings of Hazell *et al.* [19], exposure of LDL to reagent or enzymatically generated HOCl causes aggregation which is mediated by modification of lysine residues rather than lipid oxidation. Similarly, Jerlich *et al.* [11] observed a low rate of formation of thiobarbituric acid-reactive substances in LDL by the MPO/H₂O₂/Cl⁻ system, but a rapid oxidation of apolipoprotein B-100 (apo B-100)-bound tryptophan under the same conditions using a similar approach to that described here. Indeed all oxidizable groups are subject to oxidation by HOCl, and if these groups are required for the integrity of the target, damage ensues. For a recent review, see Schaur *et al.* [20]. The issue is not what it can attack, but what it does attack and where [21].

The oxidizability of LDL is size-dependent: compared with individuals with a predominance of large more buoyant LDL, subjects with a predominance of small dense LDL exhibit a greater risk of coronary artery disease [22], because small dense LDL is more susceptible to oxidation [23]. HDL was found to be even more easily oxidized than LDL [24].

Because of their relevance in the pathogenesis of atherosclerosis, we have chosen human LDL and HDL and their subclasses to study the oxidation of their fairly well-characterized apoprotein components by the MPO/H₂O₂/Cl⁻ system *in vitro*. The modification of apo B-100 (the most abundant protein within the LDL particle) and apolipoprotein A-I (apo A-I, the major protein within the HDL particle) in the presence of MPO can easily be monitored by measuring the

Correspondence to R. J. Schaur, Institute of Molecular Biology, Biochemistry and Microbiology, University of Graz, Schubertstrasse 1, A-8010 Graz, Austria. Fax: + 43 316 380 9857, Tel.: + 43 316 380 5488, E-mail: rudolf.schaur@kfunigraz.ac.at
Abbreviations: apo B-100, apolipoprotein B 100; apo A-I, apolipoprotein A-I; HDL, high-density lipoprotein; LDL, low-density lipoprotein; MPO, myeloperoxidase.

Enzyme: myeloperoxidase (MPO; EC 1.11.1.7).
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decrease in tryptophan fluorescence. Although it has been shown previously [11] that tryptophan oxidation is a very early and rapid event in lipoprotein modification by HOCl, we do not claim that tryptophan oxidation itself is the crucial event for rendering LDL proatherogenic. In our view, this is a useful and sensitive monitoring method for studying the kinetics of lipoprotein oxidation under near-*in vivo* conditions comparable with the conjugated diene assay for the resistance of lipoproteins to lipid peroxidation induced by copper [25]. The rationale for the experimental conditions to mimic the *in vivo* situation was to choose pH 6.0 and to reduce the concentration of all the reactants as far as possible, as the *in vivo* conditions for both the formation of HOCl and the reaction of HOCl with lipoproteins are not known. Thus, in the standard LDL tryptophan oxidation assay, the concentration of LDL was reduced by a factor of 20 ($0.0125 \text{ mg}\cdot\text{mL}^{-1}$ vs. $0.25 \text{ mg}\cdot\text{mL}^{-1}$) compared with the widely used conjugated diene assay.

A comparison with other methods of lipoprotein oxidation has been made previously [11]. The 37 tryptophan residues of apo B-100 were found to be virtually completely destroyed if LDL was exposed to HOCl [26] or MPO [11,27]. There is preliminary evidence that not only LDL but also HDL can be modified by the MPO/H₂O₂/Cl⁻ system, as treatment of HDL resulted in significantly enhanced turnover rates by macrophages [28]. This reaction of the MPO/H₂O₂/Cl⁻ system suggests that HDL changes from an antiatherogenic lipoprotein, which plays a key role in reverse cholesterol transport from the periphery to the liver, into a proatherogenic particle thought to initiate foam cell formation *in vivo* [28].

MATERIALS AND METHODS

All concentrations refer to final concentrations if not otherwise stated. Human MPO (EC 1.11.1.7) from Calbiochem (Bad Soden, Germany) was supplied by Bio-Trade (Vienna, Austria). H₂O₂ supplied by Merck (Darmstadt, Germany) was diluted daily from a stock solution.

Plasma preparation and preparation of LDL, HDL, and their subclasses

Plasma was prepared as described elsewhere [29]. LDL was obtained by ultracentrifugation using a single-step discontinuous gradient in a Beckman NVT 65 rotor at $342\,000 \text{ g}$ for 2 h at 10°C [30]. HDL was prepared as described for LDL [30] but with centrifugation at $543\,000 \text{ g}$ with a Beckman TLA 100.4 rotor for 4 h at 10°C . Discrete subclasses of LDL were isolated from plasma of normolipidemic healthy volunteers and fractionated by isopycnic density-gradient ultracentrifugation as described previously [31]. HDL₂ and HDL₃ were isolated from plasma of normolipidemic healthy volunteers and fractionated by sequential flotation ultracentrifugation in a fixed-angle rotor as described previously [32].

The apoprotein concentrations were calculated from the respective total cholesterol values (CHOD-PAD kit, Boehringer-Mannheim, Germany) and the known percentage values of total cholesterol and protein from the literature [31]. For some experiments, LDL subclass concentration was standardized to equal LDL protein concentration, which was determined with the Micro BCA Protein Assay kit (Pierce, Rockford, IL, USA).

Tryptophan fluorescence

The decrease in lipoprotein-bound tryptophan fluorescence in the presence of MPO was measured on a Perkin-Elmer LS 50

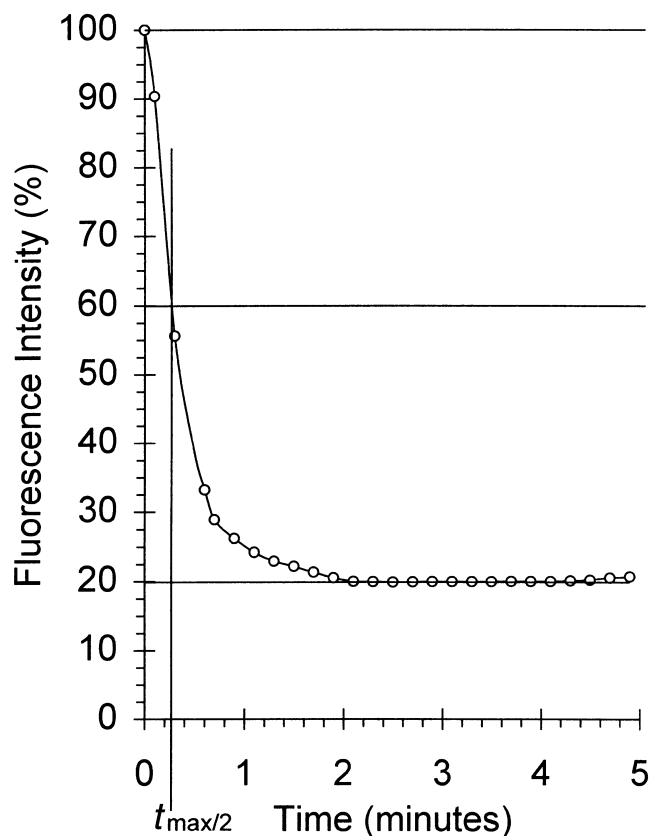


Fig. 1. Typical course of tryptophan fluorescence changes and calculation of $t_{\max/2}$. A $12.5 \mu\text{g}\cdot\text{mL}^{-1}$ sample of LDL ($2.79 \mu\text{g protein}\cdot\text{mL}^{-1}$) was incubated in NaCl/P_i, pH 6.0, at 37°C with $0.1 \mu\text{g}\cdot\text{mL}^{-1}$ MPO, and the reaction was started by addition of $0.1 \text{ mmol}\cdot\text{L}^{-1}$ H₂O₂. $t_{\max/2}$, a unit for the velocity of tryptophan oxidation in MPO-induced LDL modification, is the time needed to observe a reduction in fluorescence of 50% of the difference between initial and residual fluorescence intensity.

B luminescence spectrometer using the time-drive method at an emission wavelength of 331 nm, with excitation set at 282 nm. The emission and excitation slits were set in order to obtain optimal fluorescence output. LDL α -tocopherol fluorescence (Ex/Em = 290/323 nm) does not interfere with the tryptophan fluorescence even at high concentrations [33], and the LDL oxidation product dityrosine is not likely to interfere, because it emits fluorescent light at 410 nm.

All kinetic experiments were performed as follows (if not otherwise stated). In a cuvette, LDL or HDL was diluted with NaCl/P_i (pH 6.0, $150 \text{ mmol}\cdot\text{L}^{-1}$ NaCl, $10 \text{ mmol}\cdot\text{L}^{-1}$ NaH₂PO_{4}\cdot\text{H}_2\text{O}) to the concentrations indicated in the figure legends. Then $0.1 \mu\text{g}\cdot\text{mL}^{-1}$ MPO was added and the initial fluorescence intensity value was recorded (= 100%, Fig. 1). Subsequently the reaction was started by addition of $0.1 \text{ mmol}\cdot\text{L}^{-1}$ H₂O₂ (zero time). After mixing for 0.1 min, the next data point was collected and then the kinetics of modification were measured every 0.2 min. A continuous curve was obtained from discrete time points with the standard curve-smoothing function of the Microsoft EXCEL 5.0 software package.}

RESULTS

Evaluation of the kinetic curves

Common to all experiments with the complete MPO/H₂O₂/Cl⁻ system was a highly reproducible kinetic pattern consisting of a

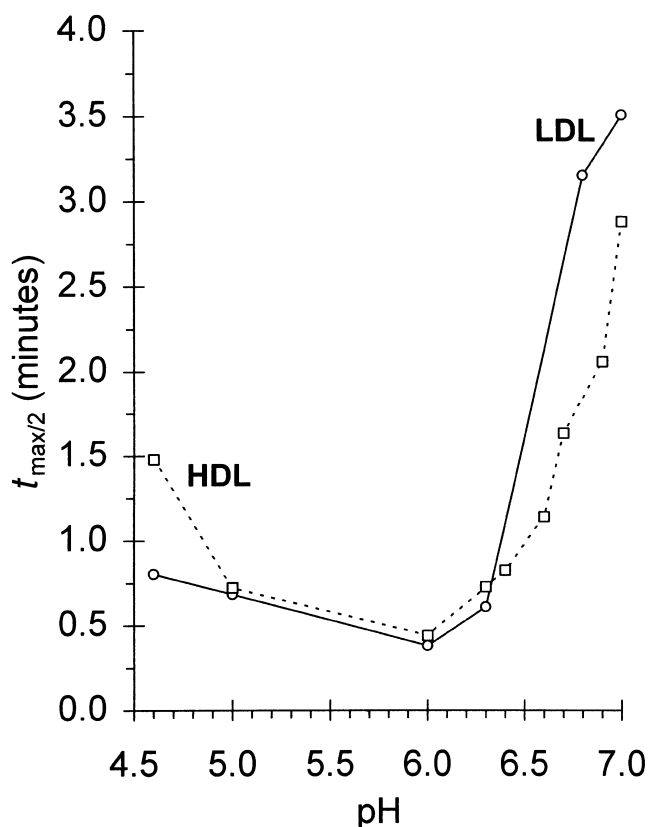


Fig. 2. Comparison of the kinetics of LDL and HDL with respect to dependence on pH. A $12.5 \mu\text{g}\cdot\text{mL}^{-1}$ sample of LDL total mass or $19.4 \mu\text{g}\cdot\text{mL}^{-1}$ HDL total mass was incubated in NaCl/ P_i at 37°C with $0.1 \mu\text{g}\cdot\text{mL}^{-1}$ MPO. Reactions were started by the addition of $0.1 \text{mmol}\cdot\text{L}^{-1}$ H_2O_2 , and the $t_{max/2}$ of lipoprotein modification at different pH values was determined. Data are represented as means of two (LDL) or three (HDL) experiments. The deviation of single values from the mean was less than 10% for LDL and less than 20% for HDL.

rapid decrease which leveled off (Fig. 1). Only the complete MPO/ $\text{H}_2\text{O}_2/\text{Cl}^-$ system with Cl^- and H_2O_2 was able to induce significant LDL modification, indicating that HOCl was the modifying agent. Control experiments without lipoproteins showed that the residual fluorescence is contributed by impurities contained in the buffer and the other components of the reaction mixture. Thus virtually all tryptophan residues are destroyed under these conditions. The kinetics of tryptophan oxidation in apoproteins of LDL and HDL is not thought to follow the first-order law and appeared to be a fast process, largely completed in less than 3 min, under optimal conditions.

Initial rates (v_0) were determined by evaluating the decrease in fluorescence within the first 18 s of the reaction, but this parameter was of low reproducibility in cases of slow kinetics (data not shown). Therefore the parameter half time ($t_{max/2}$) was introduced to characterize the fluorescence changes in quantitative terms for practical purposes. It is defined as the time required to observe a reduction in fluorescence of 50% of the difference between initial and residual fluorescence intensity. It may be considered as a parameter for the velocity of tryptophan oxidation in MPO-induced LDL and HDL modification similar to the parameter lag time for the quantification of the resistance of lipoproteins to lipid peroxidation determined by the widely used conjugated diene assay [25]. Evaluation of $t_{max/2}$ is shown in Fig. 1.

Effect of different reaction temperatures and pH

Experiments performed under otherwise identical conditions showed an accelerated kinetic course at 37°C when compared with 25°C , but the general kinetic patterns were comparable (data not shown).

Optimal modification of both LDL and HDL by the MPO/ $\text{H}_2\text{O}_2/\text{Cl}^-$ system was observed at pH 6.0 (Fig. 2). Hardly any LDL and HDL modification was observed at pH 7.4: $t_{max/2}$ values for LDL and HDL were far above 25 min (data not shown).

Effect of MPO

Increasing the MPO concentration resulted in a non-linear increase in the rate of LDL and HDL modification in terms of tryptophan oxidation (Fig. 3). Varying MPO concentration at $12.5 \mu\text{g}\cdot\text{mL}^{-1}$ LDL total mass (= $2.79 \mu\text{g}\cdot\text{mL}^{-1}$ LDL protein, $0.4 \mu\text{g}\cdot\text{mL}^{-1}$ tryptophan) and $19.4 \mu\text{g}\cdot\text{mL}^{-1}$ HDL total mass (= $9.7 \mu\text{g}\cdot\text{mL}^{-1}$ HDL protein, $4 \mu\text{g}\cdot\text{mL}^{-1}$ tryptophan) showed a large increase in the rate of LDL and HDL modification at between $0.025 \mu\text{g}\cdot\text{mL}^{-1}$ and $0.1 \mu\text{g}\cdot\text{mL}^{-1}$ MPO. At higher MPO concentrations (0.2 – $0.5 \mu\text{g}\cdot\text{mL}^{-1}$), $t_{max/2}$ values leveled off.

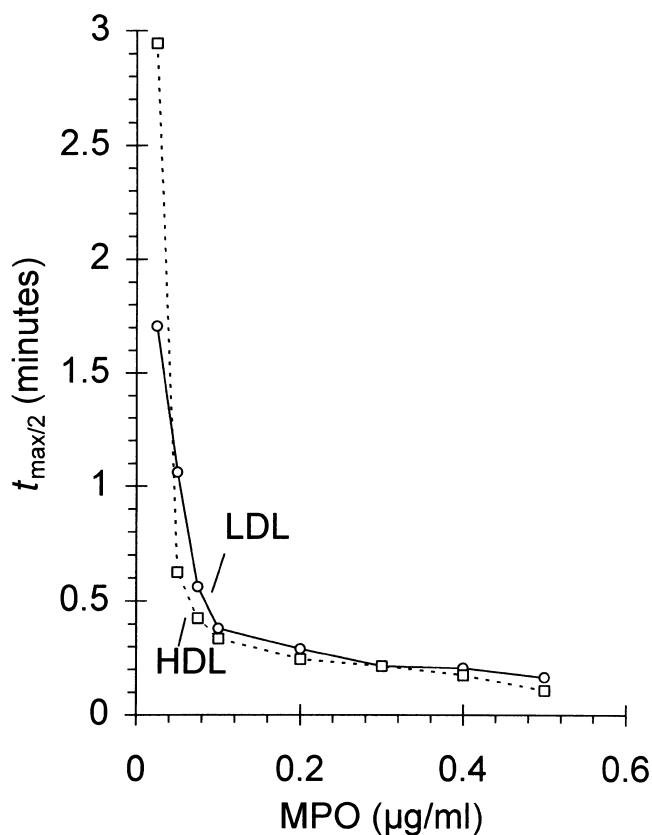


Fig. 3. Comparison of the kinetics of LDL and HDL with respect to dependence on the enzyme concentration. A $12.5 \mu\text{g}\cdot\text{mL}^{-1}$ sample of LDL total mass or $19.4 \mu\text{g}\cdot\text{mL}^{-1}$ HDL total mass was incubated in NaCl/ P_i , pH 6.0, at 37°C . Reactions were started by addition of $0.1 \text{mmol}\cdot\text{L}^{-1}$ H_2O_2 , and the $t_{max/2}$ of lipoprotein modification at various concentrations of MPO was determined. Data are represented as means of two experiments. The deviation of single values from the mean was less than 10% for LDL and less than 15% for HDL.

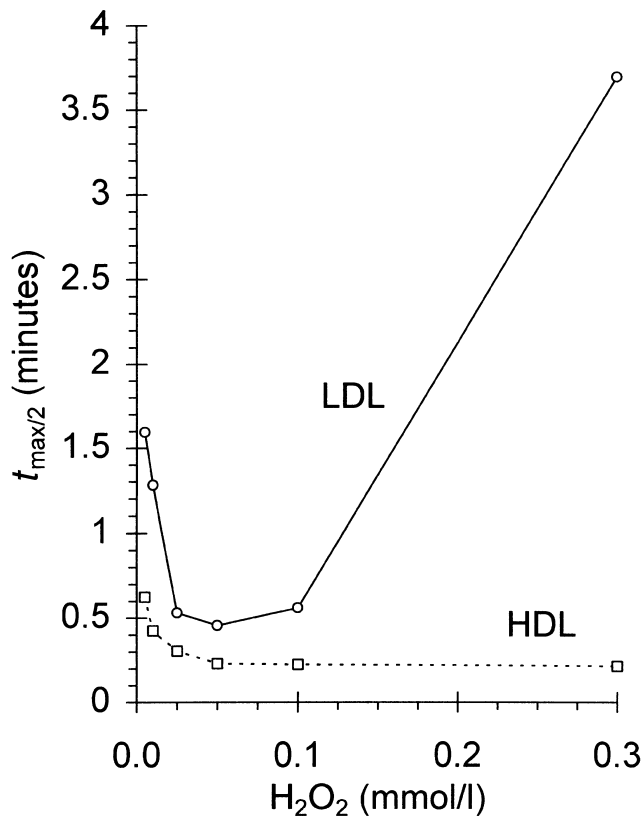


Fig. 4. Comparison of the kinetics of LDL and HDL with respect to dependence on the H₂O₂ concentration. A 12.5 µg·mL⁻¹ sample of LDL (2.79 µg protein·mL⁻¹ LDL) or 5.6 µg·mL⁻¹ HDL total mass (2.79 µg protein·mL⁻¹ HDL) was incubated in NaCl/P_i, pH 6.0, at 37 °C with 0.1 µg·mL⁻¹ MPO. Reactions were started by the addition of various concentrations of H₂O₂, and the *t*_{max/2} of lipoprotein modification was determined. Data are represented as means of two experiments. The deviation of single values from the mean was less than 25% for LDL and less than 15% for HDL. Thus there was no overlap between the two curves.

Effect of H₂O₂

Tryptophan oxidation of apo B-100 of LDL (2.79 µg·mL⁻¹ LDL protein = 12.5 µg·mL⁻¹ LDL total mass = 0.4 µg·mL⁻¹ tryptophan) appeared to be significantly slower than tryptophan oxidation of apo A-I of HDL (2.79 µg·mL⁻¹ HDL protein = 5.6 µg·mL⁻¹ HDL total mass = 1.2 µg·mL⁻¹ tryptophan, Fig. 4). LDL modification in terms of *t*_{max/2} is decreased by a factor of 0.33 at 0.01 mmol·L⁻¹ H₂O₂ and by a factor of 0.4 at 0.1 mmol·L⁻¹ H₂O₂ compared with HDL. A biphasic correlation between the H₂O₂ concentration and *t*_{max/2} was observed for both lipoproteins. High concentrations of H₂O₂ in the range from 0.2 mmol·L⁻¹ to 0.5 mmol·L⁻¹ were found to inhibit LDL modification in terms of tryptophan oxidation. Inhibition of HDL modification was found at concentrations above 0.5 mmol·L⁻¹ H₂O₂ (data not shown).

Effect of lipoprotein concentration

Varying the LDL concentration in the range 0.56–3.9 µg protein·mL⁻¹ and the HDL concentration in the range 0.25–3.88 µg protein·mL⁻¹ at 0.1 µg·mL⁻¹ MPO and 0.1 mmol·L⁻¹ H₂O₂ showed a biphasic dependence of the modification rate, with a maximum at 1.12 µg protein·mL⁻¹

LDL and 1.0 µg protein·mL⁻¹ HDL (Fig. 5A). The rate of HDL modification decreased above 1.0 µg protein·mL⁻¹ and also at lower HDL protein concentrations (0.25–0.5 µg protein·mL⁻¹), as was also observed for LDL. Modification of LDL (apo B-100) again appeared to be slower than modification of HDL (apo A-I).

Comparison of LDL and HDL modification

A quantitative comparison of the rate of LDL and HDL modification in terms of tryptophan oxidation showed that modification of apo B-100 of LDL is indeed a slower process than modification of apo A-I of HDL (Fig. 5A). At an identical protein concentration of 2.79 µg·mL⁻¹ (which corresponds to 12.5 µg·mL⁻¹ LDL total mass and 5.6 µg·mL⁻¹ HDL total mass), the kinetics of HDL modification in terms of *t*_{max/2} was reduced by a factor of 0.63 at optimal conditions (0.1 mmol·L⁻¹ H₂O₂, 0.1 µg·mL⁻¹ MPO) when compared with LDL. At an identical lipoprotein mass concentration, the tryptophan concentration in the apoprotein moiety of HDL is ≈ six times higher than in apo B-100. At identical protein mass concentration, the tryptophan concentration in the apoprotein moiety of HDL is ≈ 2.8 times higher than in apo B-100. When standardized to equal tryptophan concentration, the difference in *t*_{max/2} in the higher concentration range was slightly increased between the two lipoproteins compared with equal lipoprotein mass concentration (Fig. 5B).

Kinetics of tryptophan oxidation in LDL subclasses

No significant differences in the rate of MPO-induced LDL subclass modification were observed, when the assays were standardized to equal LDL protein concentrations (2.79 µg·mL⁻¹ LDL protein; Fig. 6A). However, a biphasic dependence of the modification rate with a maximum at subclass 8 was found when the assays were standardized to equal LDL total mass concentrations (Fig. 6B).

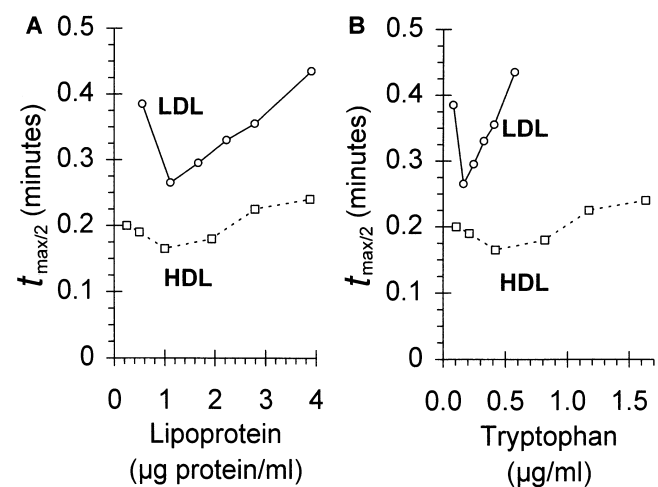


Fig. 5. Comparison of the kinetics of LDL and HDL with respect to dependence on the lipoprotein (A) and tryptophan (B) concentration. Lipoproteins were incubated in NaCl/P_i, pH 6.0, at 37 °C with 0.1 µg·mL⁻¹ MPO. Reactions were started by addition of 0.1 mmol·L⁻¹ H₂O₂, and the *t*_{max/2} of lipoprotein modification was determined. Data are represented as means of two experiments. The deviation of single values from the mean was less than 10% for LDL and less than 15% for HDL. Thus there was no overlap between the two curves.

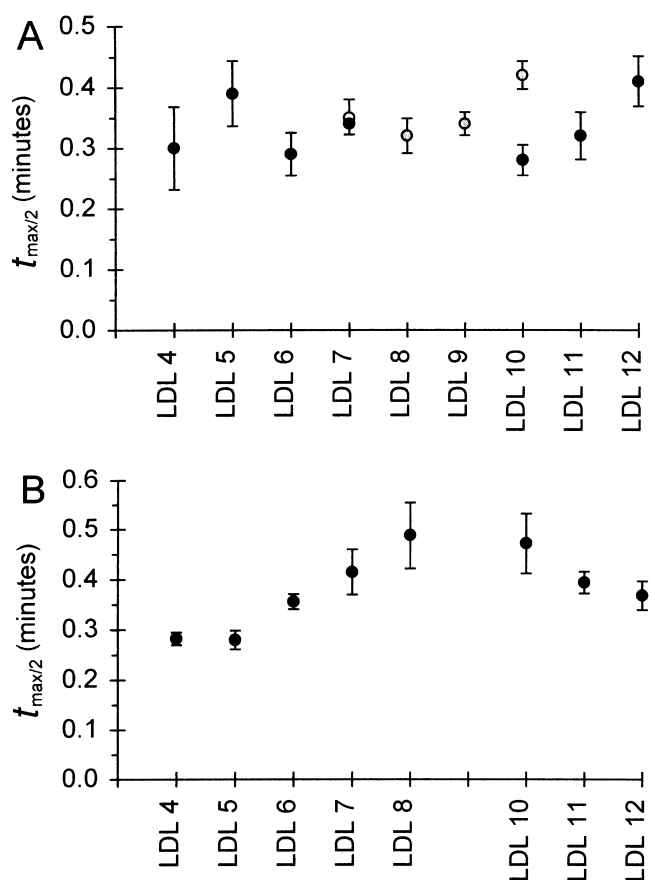


Fig. 6. Kinetics of tryptophan oxidation in LDL subclasses. LDL subclasses were incubated in NaCl/P_i, pH 6.0, at 37 °C with 0.1 µg·mL⁻¹ MPO. Reactions were started by addition of 0.1 mmol·L⁻¹ H₂O₂, and the $t_{max/2}$ of lipoprotein modification was determined. (A) LDL subclasses were standardized on an equal protein concentration of 2.79 µg·mL⁻¹. Subclasses were obtained from three different donors: full symbol, donor 1; open symbol, donor 2; gray symbol, donor 3. Data are represented as means of five experiments (except for LDL 4 where $n = 4$). (B) LDL subclasses were standardized on an equal total mass concentration of 12.5 µg·mL⁻¹. All subclasses were obtained from a single donor (subclass 9 was not available for measurements). Data are represented as means of five experiments (except for LDL 4 and LDL 7 where $n = 4$).

Kinetics of tryptophan oxidation in HDL, HDL₂ and HDL₃

The kinetics of HDL (5.6 µg·mL⁻¹ total mass), HDL₂ (6.6 µg·mL⁻¹ total mass) and HDL₃ (5.0 µg·mL⁻¹ total mass) modification with respect to dependence on H₂O₂ concentration (0.01 mmol·L⁻¹ to 0.5 mmol·L⁻¹) at equal HDL protein concentrations (2.79 µg protein·mL⁻¹; data not shown) showed a biphasic correlation between H₂O₂ concentration and $t_{max/2}$: high concentrations of H₂O₂ were found to inhibit HDL modification in terms of tryptophan oxidation. The differences in HDL, HDL₂ and HDL₃ modification were very small and not significant. The modification rate of HDL yields the middle values between HDL₂ and HDL₃ (data not shown). At 0.1 mmol·L⁻¹ H₂O₂ the following mean $t_{max/2}$ values were found: HDL, 0.22 min; HDL₂, 0.19 min; HDL₃, 0.23 min.

Similarly, varying the HDL, HDL₂ and HDL₃ concentration in the range 0.25–10 µg·mL⁻¹ at 0.1 µg·mL⁻¹ MPO and 0.1 mmol·L⁻¹ H₂O₂ showed a biphasic correlation with very small differences in $t_{max/2}$ that were not significant (data not shown).

DISCUSSION

The role of MPO in the pathogenesis of atherosclerosis is poorly understood, but a possible function could be both the protein and lipid modification of LDL, in terms of several oxidation and chlorination reactions [19,27,34], leading to preferential uptake by macrophages. There is preliminary evidence that not only LDL is modified by the MPO/H₂O₂/Cl⁻ system, as treatment of HDL also resulted in significantly enhanced rates of turnover by macrophages [28]. This reaction of the MPO/H₂O₂/Cl⁻ system may change HDL from an antiatherogenic lipoprotein into a proatherogenic particle thought to initiate foam cell formation *in vivo* [28].

Tryptophan is one of the most susceptible amino acids with respect to oxidation by HOCl. Therefore fluorescence of tryptophan was used to establish a sensitive method for measuring the overall protein oxidation of lipoproteins, comparable with the conjugated diene assay for the resistance of lipoproteins to lipid peroxidation induced by copper [25]. Jerlich *et al.* [11] suggested that HOCl, the major product of MPO, is responsible for lipoprotein modification, as in the absence of H₂O₂, chloride or the enzyme, itself no modification was observed.

Effect of pH

MPO is generally regarded as having a pH optimum of about 5–6, which may occur in an inflammatory focus [35]. The extracellular pH of atherosclerotic lesions is not known, but there is circumstantial evidence to suggest that it may be acidic [36]. The pH of phagolysosomes of neutrophils is not constant: 15–45 min after activation, it is slightly alkaline; thereafter the phagolysosomes become acidic (pH about 6) [36,37]. Sepe & Clark [38] found that the disruption of liposomes by the MPO/H₂O₂/Cl⁻ system is pH-dependent, increasing dramatically when the pH was decreased from neutrality to 6. This is in agreement with results of this study showing that a pH of 6.4 was necessary to observe at least a moderate rate of modification and hardly any lipoprotein modification could be observed at a pH above 7.0. Hazen *et al.* [39] have already shown that MPO at acidic pH is more active towards LDL with regard to cholesterol chlorohydrin formation. This optimum at pH 6.0 could be a natural release mechanism, which renders MPO active only in the case of inflammation or invasion of various micro-organisms.

Effect of the concentration of H₂O₂ and lipoproteins

Higher concentrations of H₂O₂ showed an inhibitory effect on both lipoproteins, which can be explained by the fact that, at high H₂O₂ concentrations, MPO breaks down H₂O₂ without forming HOCl, functioning more as a catalase than a peroxidase [40]. Alternatively, excessive production of HOCl may lead to rapid inactivation of MPO itself, or H₂O₂ might react with HOCl to give singlet oxygen.

The biphasic effect of increasing lipoprotein concentrations on its degradation is striking. According to the law of mass action, one would expect that an increase in tryptophan concentration augments $t_{max/2}$, because more tryptophan has to be degraded, which was found only in the higher concentration range of LDL and HDL. The decrease in $t_{max/2}$ at low lipoprotein concentrations is difficult to interpret at the moment, as it would require knowledge of the rate-determining step(s) of the reaction.

Differences between LDL and HDL

MPO-induced tryptophan oxidation of HDL was found to be more rapid than that of LDL when standardized to equal protein or tryptophan concentrations. This can be explained by the fact that LDL and HDL have different relative contents of protein and lipids per particle. Comparing total lipoprotein mass, the percentage of lipids in LDL is 1.5 times higher than in HDL, which may result in less accessibility of HOCl to protein components of LDL. An alternative explanation is the difference in size of the LDL and the HDL particle. The diameter of LDL particles (19–21 nm) is twice that of HDL particles (8–10 nm) [41], which may also affect the accessibility of HOCl to attack protein components.

Differences between LDL subclasses

No significant differences in the rate of modification of LDL subclasses were observed when the assays were standardized to equal protein concentrations (Fig. 6A). The small variations in LDL-modification rates observed were probably due to the restricted reproducibility of the protein determination and the very low concentrations of all components used for fluorimetry.

In contrast, a biphasic dependence of the rate of modification of LDL subclasses with a maximum at subclass 8 was observed when the assays were standardized to equal LDL total mass concentrations. This could be explained by the fact that differences in protein conformation, as reflected in the lysine microenvironments, exist in the different LDL subspecies [42]. Similarly, Chen *et al.* [43] observed significant differences in local conformation of apo B-100 when studying the proteolytic accessibility in various LDL subspecies. Possibly the accessibility of tryptophan residues also varies in different LDL subspecies.

Moreover intermediate subclasses contain the highest percentage of apo B-100 among the apoproteins of LDL. Apo B-100 has the highest content of tryptophan, namely 37 residues per protein molecule, while the other apoproteins (AI, AII, AIV, CI, CII, CIII, E) contain no more than eight tryptophan residues per particle [33] (Fig. 6B).

Relevance to the situation *in vivo*

For the experiments in this study, conditions were used that resemble the physiological situation in the phagosome of neutrophils, especially a slightly acidic pH of 6.0. The sensitivity of the method allowed us to use very low concentrations of H₂O₂ and the enzyme, approaching the conditions of the *in vivo* situation (optimal concentration used: MPO, 0.1 µg·mL⁻¹, H₂O₂, 0.1 mmol·L⁻¹). During phagocytosis, the concentration of H₂O₂ in the extracellular medium may reach a value of 0.6 mmol·L⁻¹ [44]. In mature neutrophils, MPO is present at exceptionally high concentration; estimates have varied from 1–2% to more than 5% of dry weight of the cells [21]. For the *in vivo* relevance, it is also important that MPO appears to be active during all stages of the pathogenesis of atherosclerosis [9]. Therefore it can be concluded that MPO-generated HOCl is able to contribute to the modification of LDL and HDL to a form recognizable for uncontrolled uptake by macrophages and initiation of foam cell formation *in vivo*, as observed by O'Connell *et al.* [45] for HOCl-modified LDL and by Panzenboeck *et al.* [28] for HOCl-modified HDL₃.

Our data indicate that HOCl is able to modify HDL more rapidly than LDL in terms of tryptophan destruction. Thus,

preferred oxidation of HDL may destroy the important physiological key role of HDL in atherosclerosis protection, i.e. reverse cholesterol transport from periphery to the liver, and possibly may have a retarding effect on MPO-induced oxidation of LDL, which obviously would be hard to demonstrate in an *in vitro* assay.

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