Hypochlorite induces the formation of LDL^-, a potentially atherogenic low density lipoprotein subspecies

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Abstract Oxidation of low density lipoprotein (LDL) induced by hypochlorous acid (HOCl) leading to LDL^-, a minimally oxidized subspecies of LDL, was investigated. LDL^- is characterized by its greater electronegativity and oxidative status, and is found in plasma in vivo. Its concentration was found to be elevated under conditions that predispose humans to atherosclerosis. We found that HOCl also converts LDL rapidly to an even more oxidized state, identified as LDL^{2-}, which is more electronegative than LDL^-. After milder oxidation for short durations, formation of LDL^- takes place while less LDL^{2-} is formed. Under these conditions, addition of methionine not only suppressed further oxidation of LDL but also favored the formation of LDL^- over LDL^{2-}, possibly by removing chloramines at lysyl residues of LDL. The presence of lipoprotein-deficient plasma did not prevent HOCl-mediated conversion of LDL to more electronegative species. It is concluded that the HOCl-mediated conversion of LDL into more electronegative species might be physiologically relevant. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Low density lipoprotein; Hypochlorous acid; Minimally oxidized low density lipoprotein; Lipoprotein-deficient plasma

1. Introduction

Low density lipoprotein (LDL) is a heterogeneous collection of particles which vary in size [1], density [2], composition [3], and charge [4]. Based on differences in charge, plasma LDL is readily separated by anion exchange chromatography into three fractions. The major fraction, native LDL (nLDL), represents ~90–99% of total LDL. The other two fractions have been operatively termed LDL^- and LDL^{2-}, the terminology used refers to the increase in oxidized status and electronegativity [5]. LDL^{2-} constitutes 0.1–1% of total LDL and appears to be a highly oxidized subfraction of LDL. LDL^- represents 0.2–8% of LDL, and is predominantly found in the small, dense LDL fraction [6] which is strongly associated with an increased risk of atherosclerosis [7].

LDL^- is found in vivo in the circulation and displays subtle changes in protein composition. Agarose gel electrophoresis shows increased negative charge density compared to nLDL. LDL^- is enriched in oxidized lipids, e.g. lipid hydroperoxides and oxysterols [8]. It binds with diminished affinity to LDL receptors of human skin fibroblasts and endothelial cells [9] but is a poor ligand for macrophage scavenger receptor [10]. In contrast to nLDL, LDL^- is cytotoxic to cultured endothelial cells [11].

Inflammation is strongly associated with an increased risk of atherosclerosis [12]. Phagocytic white blood cells (monocytes, macrophages, and neutrophils) are the cellular hallmark of inflammation. One important pathway for the generation of reactive species by phagocytes involves the enzyme myeloperoxidase (MPO), a secreted heme protein [13]. Together with H_2O_2 MPO oxidizes chloride to hypochlorous acid (HOCl), a potent microbicidal agent which can oxidize many biological substances including LDL [14]. HOCl oxidizes thiols, reacts with unsaturated fatty acids in LDL to form chlorohydrins [15], and covalently modifies amino groups of proteins, disrupting the tertiary structure and function of target proteins (reviewed in [16]). Catalytically active MPO is present in human atherosclerotic lesions [17] suggesting that MPO promotes LDL oxidation in vivo.

To date, the origin of LDL^- remains unclear, although its role in atherogenesis is emerging. Three possible sources of LDL^- are discussed: (i) oxidation of LDL entrapped in the arterial wall [18], (ii) ingestion of oxidants or generation from postprandial lipoprotein remnants [19], and (iii) oxidation in plasma [20]. Our study reveals a potential role of HOCl in the generation of LDL^- as a further mechanism. Localized inflammation and MPO secretion may account for increases in plasma LDL^- levels. At high concentrations HOCl has been found to modify LDL predominantly to LDL^{2-}. By contrast, low concentrations of HOCl favor formation of LDL^-. Our findings indicate that LDL^- may be formed preferentially when readily oxidizable thioether groups, such as methionine, are present. By contrast, extensive oxidation yielding LDL^- is not suppressed by methionine suggesting that LDL^- formation by HOCl involves quantitative LDL oxidation via chloramines that subsequently decompose, reversibly oxidize or chlorinate other susceptible target molecules.

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Abbreviations: EDTA, ethylenediaminetetraacetic acid; MPO, myeloperoxidase; REM, relative electrophoretic mobility; LDL, low density lipoprotein; LDP, lipoprotein-deficient plasma

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2. Materials and methods

2.1. Materials

All reagents were of AR grade and obtained from either Merck (Darmstadt, Germany) or Sigma (St. Louis, MO, USA) if not otherwise stated.

2.2. Plasma preparation

Blood was drawn by venipuncture from one healthy normolipidemic volunteer after overnight fasting and plasma was immediately separated by low spin centrifugation at $1500 \times g$ for 10 min at 4°C. Sucrose solution (60% w/v) was added (final concentration 0.6%) and aliquots were stored at −80°C in the dark until LDL preparation (up to 2 months) [21].

2.3. Preparation of LDL and lipoprotein-deficient plasma (LDP)

LDL ($d = 1.019$–1.063 g/ml) and LDP (bottom fraction; $d = 1.41$ g/ml) were prepared from plasma by discontinuous density gradient ultracentrifugation [21] and stored under nitrogen at 4°C in the dark for up to 1 week after preparation. LDL (but not LDP) was gel filtrated with an Econo-Pac 10 DG column (Bio-Rad, Richmond, CA, USA) using phosphate-buffered saline (PBS, 150 mM NaCl, 10 mM $NaH_2PO_4 \cdot H_2O$, pH 7.4) containing 0.1% ethylenediaminetetraacetic acid (EDTA) (w/v) as an eluent. LDL cholesterol content was determined by the CHOD-PAD method using a commercial kit (Roche, Mannheim, Germany). Protein concentration was measured using a Protein Microtiter Plate assay (Bio-Rad, Richmond, CA, USA).

2.4. LDL oxidation

LDL (0.3 μM) was titrated with HOCl at various concentrations (0.03 μM up to 30 mM) in PBS pH 7.4 EDTA and incubated for either 1 or 60 min at 37°C under aerobic conditions. Diluted HOCl was prepared in double-distilled water always freshly prepared prior to oxidation. Immediately after initiation of oxidation by HOCl, aliquots (0.3 ml) were taken and the kinetics of the formation of conjugated dienes monitored for up to 2 h at 234 nm in a Beckman DU 640 spectrophotometer [22]. At the end of the respective reaction times, selected samples were incubated with L-methionine at a five-fold molar ratio compared to HOCl. The reaction mixtures were gel filtrated with an Econo-Pac 10 DG column thereby separating LDL from any unreacted HOCl and/or methionine, and eluted with 20 mM Tris–HCl pH 7.2. LDL was then subjected to HPLC analysis as described below. 3 μl of unmodified LDL, or of the reaction mixtures obtained immediately after addition of HOCl, or of modified LDL (after removal of HOCl by gel filtration) was loaded on Beckman Paragon Lipo-gels and the relative electrophoretic mobility (REM) determined according to the manufacturer's instructions.

2.5. Separation of LDL subspecies by HPLC

LDL in 20 mM Tris–HCl pH 7.2 was separated into subfractions according to the method described by Hodis et al. [11] with minor modifications [23].

2.6. Reisolation of LDL oxidized in the presence of LDP

Samples consisting of 0.3 μM LDL in medium containing undialyzed LDP (5–20%, v/v) were exposed to HOCl as described above. Sample density was adjusted to 1.25 g/cm^3 by adding 0.38 g solid NaBr/ml, and overlayered with 4 ml of 1.25, 2 ml of 1.05, and 3 ml of 1.0 g/cm^3 NaBr–EDTA (0.1%, pH 7.4). Ultracentrifugation using a Beckman SW41 rotor was carried out at 5°C for 22 h at 40 000 rpm in a Beckman L-70 ultracentrifuge. The reisolated LDL band was collected and immediately subjected to HPLC analysis as described above.

SDS–PAGE was performed [24] for the detection of possible covalent aggregates due to modifications of LDL by HOCl.

2.7. Statistics

Values are expressed as mean ± S.D. of at least three independent experiments. Statistical analysis (ANOVA) was performed using SigmaStat 2.03 software.

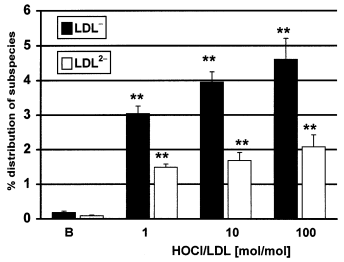

Fig. 1. Formation of LDL^- and LDL^{2-} by HOCl. B: Plasma baseline levels of LDL^- and LDL^{2-}. 1, 10, 100: LDL was titrated with increasing HOCl concentrations, and subspecies were separated by HPLC after 60 min of incubation at 37°C. Values represent mean ± S.D. ($n = 3$). ANOVA analysis of LDL^- and LDL^{2-} compared to B: $^{**}P < 0.001$.

3. Results

3.1. Formation of LDL subspecies by low concentrations of HOCl

HPLC separation of unmodified LDL revealed the following baseline pattern of LDL subfractions: 99.7 ± 0.05% nLDL, 0.18 ± 0.04% LDL^-, and 0.09 ± 0.02% LDL^{2-}. Subjecting LDL to oxidation by HOCl over 60 min at 37°C at a molar HOCl/LDL ratio as low as 10:1 led to a 17-fold increase in both LDL^- and LDL^{2-}. Raising the HOCl/LDL molar ratio to 100:1 further increased the levels of LDL^- and LDL^{2-} by 22-fold and 19-fold, respectively (Fig. 1).

3.2. Impact of incubation time on the generation of LDL^- and LDL^{2-} by HOCl

After an incubation time of only 1 min at a low molar HOCl to LDL ratio, LDL^- was the predominant species generated compared to LDL^{2-} levels (Fig. 2A). Above a HOCl/LDL ratio of 3000, LDL^{2-} levels (∼14%) exceeded those of LDL^- (∼8%). 60 min of incubation (Fig. 2B) did not markedly alter LDL^- levels compared to 1 min of incubation, but increased LDL^{2-} significantly, i.e. ∼24% of LDL^{2-} after 60 min compared to ∼14% after 1 min. Circular dichroism measurement of all 60 min samples revealed no increase in absorbance; SDS–PAGE showed aggregation only at the highest HOCl concentrations used (data not shown).

3.3. Methionine reduces the formation of LDL^{2-}

Post-addition of L-methionine suppressed LDL^{2-} formation by HOCl but did not alter LDL^- levels significantly regardless of incubation time (Fig. 3). Furthermore, L-methionine led to a significant decrease in REM indicating LDL^{2-} to be a major contributing factor of the increase in the REM of oxidized LDL (Fig. 4).

3.4. LDP does not prevent the formation of LDL^- and LDL^{2-} by HOCl

Oxidation of LDL in the presence of up to 20% LDP decreased the generation of LDL subspecies compared with HOCl alone. However, even 20% (v/v) LDP could not prevent the HOCl-induced five-fold increase of both LDL^- and LDL^{2-} concentrations (Fig. 5). The inhibitory effect was not dose-related for LDL^- formation but was for LDL^{2-}.

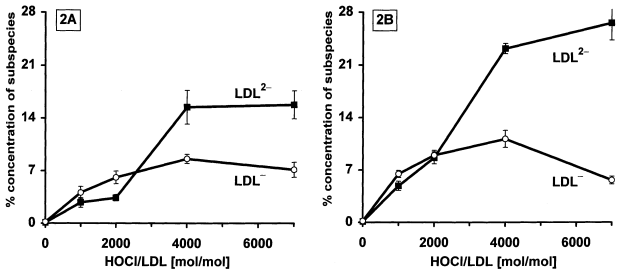

Fig. 2. Time-dependent formation of LDL^- and LDL^{2-} by HOCl. LDL was titrated with increasing HOCl concentrations, and subspecies were separated by HPLC after 1 min (A) or 60 min (B) of incubation at 37°C. Values represent mean ± S.D. ($n = 3$).

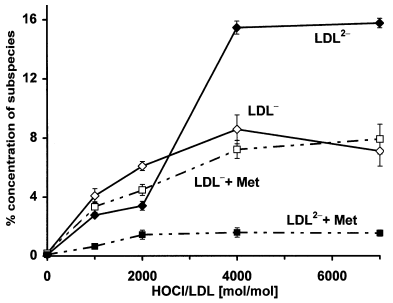

Fig. 3. Influence of L-methionine on HOCl-dependent generation of LDL^- and LDL^{2-} by HOCl. LDL was titrated with increasing HOCl concentrations, L-methionine was added after 1 min of incubation at 37°C, and LDL subspecies separated by HPLC. Values represent mean ± S.D. ($n = 3$).

4. Discussion

Our observations indicate that (i) HOCl generates LDL^- and LDL^{2-}; (ii) HOCl increases the negative charge of LDL in a concentration- rather than time-dependent manner; (iii) post-addition of methionine leads to the preferential formation of LDL^- over LDL^{2-}; and (iv) LDL^- formation by HOCl prevails in the presence of LDP.

Modification of LDL by HOCl lowers the number of sulfhydryl and amino groups and increases the negative charge density of the LDL surface [25] which is in line with the detected increase in REM in this study. One reason for the more negatively charged surface of HOCl-altered LDL is the possible formation of monochloramines. While amino groups have a positive charge at physiological pH values, monochloramines are uncharged [26]. Moreover, the side chains of cysteine, methionine, tryptophan and tyrosine were shown to be of higher reactivity towards HOCl compared with lysine residues of lipoproteins [27]. LDL^- formation at low concentrations of HOCl in the micromolar range can therefore be attributed to sulfhydryls, tryptophans and tyrosines as targets of an electrophilic substitution by HOCl in addition to oxidation/chlorination of lysyl residues.

LDL-bound chloramines might be formed at higher HOCl/LDL ratios. Indeed, distinctive peaks at 253 and 248 nm – indicative of chloramines (published absorption maxima range from 240 to 270 nm [28,29]) – were observed in HOCl-treated LDL even in the presence of L-methionine added to prevent further and extensive oxidation (data not shown). Subsequent addition of methionine led to preferential formation of LDL^- over LDL^{2-}. In principle, there are three possible pathways of methionine consumption by either HOCl or chloramines. First, free L-methionine might act as a HOCl scavenger by virtue of the thioether group scavenging up to three molecules of HOCl which leads to a sulfone residue via an intermediate sulfinylchloride and sulfoxide [30]. In our experiments, L-methionine was added 1 or 60 min after the start of the reaction between HOCl and LDL. Already after 1 min of incubation, at least in the case of low HOCl/LDL molar ratios, all molecules of HOCl will have reacted with the estimated 987 amino acid residues (i.e. sum of His, Cys, Met, Trp, Lys, Tyr and Phe) principally susceptible to chlorination/oxidation by HOCl [27]. It has also been shown that several residues are capable of consuming more than one molecule of HOCl [16]. It is therefore conceivable that under these conditions HOCl reacts quantitatively with LDL, and that addition of methionine after 1 or 60 min can lead to methionine–chloramine interactions rather than scavenging HOCl.

The second pathway of methionine reacting with HOCl and/or chloramines is based on the reversibility of chloramine formation by HOCl. A protein-bound amino group reacts with HOCl forming a chloramine [31]. The labile LDL-bound chloramine may liberate HOCl, and in the presence of targets susceptible to chlorination/oxidation, including free methionine, HOCl may either form another chloramine on the target molecule or otherwise modify LDL as described above.

Third, chloramines may decompose by a reaction that involves the generation of radical species. Homolytic cleavage of the N–Cl bond could form a protein-bound nitrogen-centered radical and a chlorine radical. These nitrogen-centered radicals have been shown to account for fragmentation, protein modification [32], and lipid peroxidation of LDL [33]. There are few indications for lipid peroxidation due to the observed lack of increase of absorption at 234 nm. This is in line with a recent study where neither epoxides nor peroxides were detectable, but solely chlorohydrins [15]. Decomposition of lysyl chloramines was shown to occur at millimolar concentrations

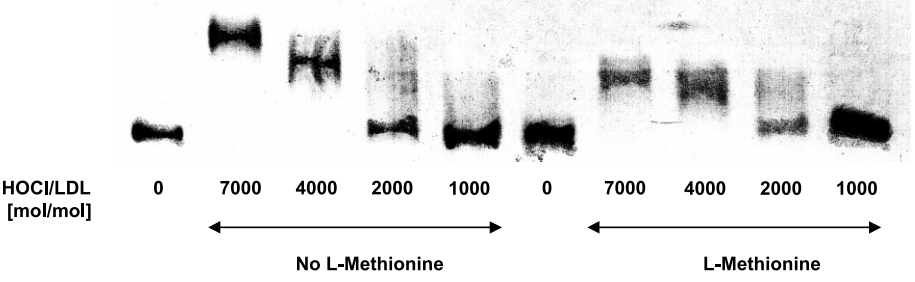

Fig. 4. Representative agarose gel showing the change in REM of LDL titrated with increasing HOCl concentrations, and the influence of L-methionine addition after 1 min of incubation at 37°C.

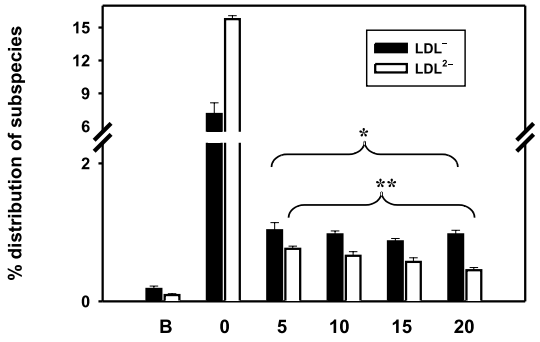

Fig. 5. LDL oxidation by HOCl in the presence of LDP. B: Plasma baseline levels of LDL^- and LDL^{2-}; 0: oxidation of isolated LDL by HOCl (7000:1 mol/mol) in the absence of LDP. 5–20: LDP (5–20% v/v) together with 0.3 μM LDL was oxidized by HOCl (7000:1 mol/mol) for 1 min at 37°C. Samples were ultracentrifuged to reisolate LDL, and LDL subspecies separated by HPLC. Values represent mean ± S.D. ($n = 3$). ANOVA showed highly significant differences for LDL^{2-} (**$P < 0.001$), and significant differences for LDL^- (*$P < 0.05$) compared to B.

of HOCl, hence it might only proceed at the highest molar HOCl/LDL ratio in our experiments.

By hydrolysis of an intermediate imine (Eq. 1), α-chloramines can break down to aldehydes (Eq. 2), concomitantly releasing ammonia [34]:

$$\text{R-CH}_2\text{-NHCl} \rightarrow \text{R-CH} = \text{NH} + \text{HCl} \quad (1)$$

$$\text{R-CH} = \text{NH} + \text{H}_2\text{O} \leftrightarrow \text{R-CH} = \text{O} + \text{NH}_3 \quad (2)$$

ε-Amino groups are likely to undergo similar reactions, therefore aldehydes formed on lysyl residues of LDL might interact, in a reversible reaction, with another ε-amino group leading to a Schiff base, a reaction which invokes inter- as well as intraparticle crosslinks causing aggregation as a consequence. SDS–PAGE revealed aggregation at high concentrations of HOCl, but hardly any increase in molecular weight at low molar HOCl/LDL ratios.

Most pathways which promote LDL oxidation in vitro are strongly inhibited by plasma. In contrast, HOCl converts LDL to LDL^- and LDL^{2-} in the presence of LDP. Chloramines formed by HOCl on LDP proteins may lead to oxidation/chlorination of LDL. This important observation suggests that the conversion of LDL to more electronegative species by HOCl secreted by activated, circulating phagocytes can indeed occur in the circulation and may be physiologically relevant.

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