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Distinct HDL subclasses present similar intrinsic susceptibility to oxidation by HOCl

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Abstract

The heme protein myeloperoxidase (MPO) functions as a catalyst for lipoprotein oxidation. Hypochlorous acid (HOCl), a potent two-electron oxidant formed by the MPO–H₂O₂–chloride system of activated phagocytes, modifies antiatherogenic high-density lipoprotein (HDL). The structural heterogeneity and oxidative susceptibility of HDL particle subfractions were probed with HOCl. All distinct five HDL subfraction were modified by HOCl as demonstrated by the consumption of tryptophan residues and free amino groups, cross-linking of apolipoprotein AI, formation of HOCl-modified epitopes, increased electrophoretic mobility and altered content of unsaturated fatty acids in HDL subclasses. Small, dense HDL3 were less susceptible to oxidative modification than large, light HDL2 on a total mass basis at a fixed HOCl:HDL mass ratio of 1:32, but in contrast not on a particle number basis at a fixed HOCl:HDL molar ratio of 97:1. We conclude that structural and physicochemical differences between HDL subclasses do not influence their intrinsic susceptibility to oxidative attack by HOCl.

Keywords

Antiatherogenic lipoprotein; Hypochlorite; Myeloperoxidase-hydrogen peroxide-halide system; Tryptophan residues; Lysine modification; Apolipoprotein AI

Introduction

Previous studies have demonstrated an inverse relationship between plasma levels of high-density lipoprotein (HDL)¹-cholesterol and atherosclerotic cardiovascular disease [1]. Gordon and colleagues observed that a decrease of 1 mg/dl in HDL-cholesterol concentrations is associated with an increase of 2–3% in cardiovascular risk [2]. Moreover, the Framingham Heart Study revealed that circulating levels of HDL-cholesterol represent the strongest independent risk factor of cardiovascular disease among other risk factors, including elevated plasma levels of low-density lipoprotein-cholesterol [3]. Such

relationship led to the widely accepted hypothesis that HDL, and in particular its major apolipoprotein, apoAI, is atheroprotective. Besides its capacity to remove cholesterol from artery wall macrophages, an array of anti-inflammatory, anti-thrombotic, and anti-oxidant properties have been reported for HDL (see [4-6] for review).

However, modification of HDL may considerably impair its anti-atherosclerotic capacity and render native HDL into a pro-atherogenic and pro-inflammatory lipoprotein particle [6]. Among different routes of *in vivo* modification, myeloperoxidase (MPO) has turned out as a prominent catalyst for lipoprotein oxidation [7]. The potent oxidant hypochlorous acid (HOCl), formed by the MPO-H₂O₂-halide system of activated phagocytes, contributes to their microbicidal activity *in vitro* and *in vivo* [8]. However, evidence has emerged that chronic and prolonged production of HOCl contributes to tissue damage and the initiation and propagation of acute and chronic vascular disease [8,9]. Pronounced staining for HOCl-modified epitopes and MPO has been found in acute and chronic vascular inflammatory diseases, e.g. in glomerulosclerosis and glomerulonephritis [10] as well as in complicated and fibro-atheromatic human lesions [11,12]. Most importantly, immunohistochemical staining revealed pronounced *in situ* colocalization of HOCl-modified epitopes and apoAI in human lesion material [13]. HDL modified *in vitro* by HOCl, added as a reagent or generated by the MPO-H₂O₂-halide system, was more susceptible to uptake and degradation by macrophages [14], thus turning HDL from a lipid-removing to a lipid-loading lipoprotein [15]. ApoAI has been identified as a selective target for oxidation [16,17] that in further consequence leads to impaired capacity of HDL to efflux cholesterol via ATP-binding cassette transporter A1 [18-20]. Identification of a specific interaction site between the apoAI moiety of HDL and MPO as well as the modification of specific amino acids on apoAI relevant for functional activity of HDL, primarily cellular cholesterol efflux, supports the notion that oxidation of HDL/apoAI may be directly mediated by MPO [17].

Functional plasma HDL consists of spherical or discoidal particles of high hydrated density; HDL fractionation by ultracentrifugation reveals two major subclasses, HDL2 (1.063–1.125 g/ml) and HDL3 (1.125–1.21 g/ml) [6]. Such heterogeneity results from differences in relative contents of apolipoproteins and lipids and is intimately related to differences in intravascular metabolism and biological function [21]. In particular, small, dense HDL3 display superior capacity to protect low-density lipoprotein from lipid peroxidation and is more resistant to oxidation than large, light HDL2. We therefore hypothesized that small, dense HDL3 might be distinct in their resistance to oxidative attack by HOCl as compared to large, light HDL2. Our present findings, however, reveal that the susceptibility of HDL subclasses to oxidation by HOCl critically depends on the concentration basis (particle number or mass) employed for their comparison, and that HDL subclasses do not differ in their intrinsic susceptibility to oxidative modification by HOCl on a particle number basis.

Materials and methods

Fractionation of HDL

Pooled plasma was obtained from normolipidemic subjects from the Blood Transfusion Centre of the Hospital La Pitié-Salpêtrière (Paris, France) and stored at –80 °C. Sucrose

¹Abbreviations used:

HDL high-density lipoprotein

MPO myeloperoxidase

HOCl hypochlorous acid

(SigmaUltra, purity > 99.5%; final concentration, 0.6%) was added as a cryoprotectant for lipoproteins [22]. To isolate total HDL, EDTA (final concentration, 0.5 M) and gentamycin (final concentration, 0.1 mg/ml) were added to freshly thawed plasma at 4 °C followed by ultracentrifugation for 16 h (36,000 rpm, 15 °C) to remove chylomicrons ($d < 1.000$ g/ml) and very low-density lipoproteins (< 1.006 g/ml). The bottom fraction was subsequently centrifuged for 48 h (36,000 rpm, 15 °C) at a density of 1.063 g/ml to remove low-density lipoprotein. Finally, total HDL was isolated in the density range of 1.063–1.20 g/ml by ultracentrifugation for 24 h (36,000 rpm, 15 °C).

For isolation of HDL subclasses, total HDL was preparatively fractionated by isopycnic density gradient ultracentrifugation exactly as described [23–25]. Five major HDL subclasses were isolated, i.e., large, light HDL2b (d 1.063–1.087 g/ml) and HDL2a (d 1.088–1.110 g/ml), and small, dense HDL3a (d 1.110–1.129 g/ml), HDL3b (d 1.129–1.154 g/ml) and HDL3c (d 1.154–1.170 g/ml). All HDL subclasses isolated by this procedure are essentially albumin-free (<1% of total protein, i.e., <0.05 mg/dl). KBr and EDTA were removed by exhaustive dialysis against PBS at 4 °C. Isolated lipoproteins were stored at 4 °C and used within 8 days.

Chemical analysis of HDL subclasses

Total and free cholesterol, phospholipid and triglyceride content of isolated lipoprotein subclasses were determined using commercially available enzymatic assays from Wako Chemicals/Diasys (Bouffemont, France) and Biomerieux (Craponne, France) [26]. The cholesteryl ester content was calculated by multiplying the difference between total and free cholesterol by 1.67 [23]. Total protein content was estimated by BCA assay from PerBio Science (Bezons, France). Total lipoprotein mass was calculated as the sum of the mass of cholesteryl ester, free cholesterol, phospholipid, triglyceride and protein components. To calculate molar concentrations of HDL subclasses, molecular masses of 424 (HDL2b), 380 (HDL2a), 350 (HDL3a), 194 (HDL3b) and 161 kDa (HDL3c) were used [25].

Oxidation of HDL subclasses

Modification of individual HDL subclasses by HOCl was performed as described previously [14]. Briefly, the pH of reagent NaOCl (0.1 M) was adjusted to 7.4 using 0.1 M HCl. The concentration of NaOCl was calculated using a molar absorption coefficient of $350 \text{ M}^{-1} \text{ cm}^{-1}$ at 292 nm. Subsequently, HDL subclasses were incubated at 37 °C for 30 min in the presence of the HOCl solution.

The susceptibility of HDL subclasses to HOCl-mediated oxidation was compared on a total mass and on a particle number basis. To compare the oxidative susceptibility of HDL subclasses on a total mass basis, the lipoprotein particles were incubated at a concentration of 160 mg total HDL mass/dl, corresponding to 55–105 mg protein/dl or to 4–10 μM , in the presence of 960 μM HOCl; this resulted in a fixed oxidant:lipoprotein mass ratio of 1:32. In these experiments, the oxidant: lipoprotein molar ratio varied from 97:1 (HDL3c) to 254:1 (HDL2b). To compare the oxidative susceptibility of HDL subclasses on a particle number basis, lipoprotein particles (9.9 μM) were incubated with HOCl (960 μM), resulting in the oxidant:lipoprotein molar ratio of 97:1. The HOCl concentration of 960 μM was chosen to model the physiopathologically relevant HOCl/HDL molar ratio of approximately 100:1, as HOCl concentrations *in vivo* intima may reach several hundreds of μM [15,27,28], whereas circulating concentrations of each of five HDL subpopulations studied by us are about 1–2 μM [25].

Characterisation of native and oxidized HDL subclasses

Measurement of reactive amino groups—Reactive apolipoprotein amino groups were quantitated with trinitrobenzene sulfonic acid [29]. Briefly, 50 µg protein of native or modified HDL subclasses were mixed with 1 ml of NaHCO₃ (4%, w/v; pH 8.4) and 50 µl trinitrobenzene sulfonic acid in H₂O (0.1%, v/v). After incubation for 1 h (37 °C), 100 µl of HCl (1 N) and 100 µl of SDS (10%) were added. Absorbance was measured at 340 nm. The standard curve (produced using valine) was linear in the range 5–50 nmol of reactive amino groups.

Relative electrophoretic mobility (REM)—Electrophoretic mobility of HDL subclasses was determined using 0.8% agarose gels containing bovine serum albumin. Native and HOCl-modified HDL subclasses were subjected to electrophoresis in 0.05 M barbital buffer (pH 8.6) at 400 V for 6 min. The increase in the electrophoretic mobility of each oxidized HDL subclass was calculated relative to that of corresponding native HDL subclass.

Measurement of tryptophan residues—Content of tryptophan residues was evaluated in 100 µl of each HDL subclasses as fluorescence at 335 nm with excitation at 280 nm [30]. Fluorescence intensity was normalized to mg HDL protein. Modification of tryptophan residues was expressed relative to initial tryptophan levels in native (non-oxidized) HDL subclasses.

SDS–PAGE and Western blotting—SDS–PAGE of HDL-associated apolipoproteins was performed using 5–15% polyacrylamide gradient gels at 150 V for 90 min in a Bio-Rad mini protein chamber (Bio-Rad, Austria) [14]. For Western blotting experiments proteins were electrophoretically transferred to nitrocellulose membranes (150 mA, 4 °C, 90 min). Immunochemical detection of HOCl-modified apolipoproteins was performed with a monoclonal antibody clone 2D10G9 ([31], dilution 1:50) followed by horseradish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad, 1:5000). Immunochemical detection of apoAI in the same lipoprotein samples was performed with rabbit polyclonal anti-human apoAI (Behring, Germany) as a primary antibody, followed by horseradish peroxidase-conjugated goat anti-rabbit IgGs as secondary antibodies. Detection of immunoreactive bands was performed using ECL® (enhanced chemiluminescence; Amersham); films were scanned and densitometric evaluation performed using a Kodak Image Station 440CF (Perkin-Elmer, France).

Measurement of fatty acids (FAs)—FA analysis of HDL subclasses was performed as described [32]. Briefly, HDL-associated lipids were converted to the corresponding FA methyl esters in toluene (0.5 ml) and methanol/BF₃ complex (Sigma; 20%, v/v; 1 ml) with heptadecanoic acid as internal standard (100 µg). Separation of FA methyl esters (2 µl samples) was performed on a WCOT fused silica 25 m FFAP-CB column (0.32 mm ID; Chrompack) using a HP 5890 gas chromatograph equipped with a flame ionization detector and a split/splitless injector (Hewlett–Packard Co.). After an initial isothermal period of 5 min at 150 °C the temperature was programmed to 190 °C at 43 °C/min and then to 215 °C at 23 °C/min, with a hold at 215 °C for 15 min. Concentrations of individual FAs (myristic (C14:0), palmitic (C16:0), palmitoleic (C16:1), stearic (C18:0), oleic (C18:1), linoleic (C18:2), linolenic (C18:3), arachidonic (C20:4) and eicosapentaenoic (C20:5) acids) were calculated by peak area comparison with the internal standard.

Statistical analysis

All data are means ± SD unless otherwise indicated. Differences between HDL subfractions were analyzed by Wilcoxon non-parametrical test for dependent samples or by Student's *t*-

test when applicable. Pearson's moment-product correlation coefficients were calculated to evaluate relationships between variables.

Results and discussion

Treatment of human HDL3 with HOCl, generated by the MPO-H₂O₂-halide system or added as a reagent, results in the modification of the protein moiety of the lipoprotein particle; cysteine, methionine, tyrosine, tryptophan and primarily lysine-derived amino groups are highly susceptible to HOCl-mediated attack [13,14]. Consistent with data reported for total HDL3 [14], treatment of all five distinct HDL subclasses with HOCl led to the consumption of free amino groups (Fig. 1). Amino groups of small, dense HDL were significantly less susceptible to oxidation by HOCl (39% and 29% oxidized in HDL3b and HDL3c, respectively) than those of large, light HDL2b (62% oxidized, $p < 0.05$ vs. HDL3b and HDL3c) when compared on a total mass basis (Fig. 1A). However, when HDL subclasses were compared on a particle number basis, no significant difference in the degree of amino group consumption was detected between HDL subclasses (Fig. 1B).

As modification of free amino groups by HOCl is paralleled by changes in the net charge of the lipoprotein particle, the REM of modified HDL subclasses was followed in agarose gels [33]. When HDL subclasses were compared on a total mass basis, REM of both large, light HDL2 (1.17-fold in HDL2b and 1.15-fold in HDL2a) and of small, dense HDL3a (1.15-fold) subfractions were increased after HOCl treatment. However, REM of small, dense HDL3b and HDL3c remained unchanged (Fig. 2A). Such increases in REM of HDL2b, 2a and 3a were significantly different from changes in REM of HDL3c ($p < 0.05$). By contrast, when HDL subclasses were compared on a particle basis, REM was similar in all HDL subclasses and close to that observed for the corresponding native HDL subclasses (Fig. 2B), resembling the patterns observed with amino group consumption (Fig. 1B). Indeed, when data obtained using all HDL subclasses under two different conditions of oxidation were pooled, increase in HDL REM revealed positive correlations with the consumption of free amino groups ($r = 0.41$, $p < 0.05$).

Treatment of HDL with HOCl leads to the formation of intra- and intermolecular cross-links of apoAI [14-16]. Consistent with this finding, both monomeric and aggregated forms of apoAI were found in all five HDL subclasses following incubation with HOCl; in addition to monomeric apoAI (28 kDa), immunoreactive bands with apparent molecular masses of 46, 63 and 91 kDa became apparent, suggesting the formation of dimeric up to tetrameric apoAI products (Fig. 3); in parallel, apoAI/AII heterodimers are also likely to occur under these conditions [15]. Densitometric evaluation of immunoreactive bands revealed that signal intensity of aggregated apoAI was up to 5-fold higher as compared to monomeric apoAI. However, no apparent difference in the level of aggregated apoAI was observed between HDL subclasses independently of the concentration basis employed for such comparison (total mass or particle number; Fig. 3A and B).

Stripping membranes and incubation with monoclonal antibody 2D10G9 [31] as a primary antibody revealed the presence of HOCl-modified epitopes in all oxidized HDL subclasses but not in their native counterparts (Fig. 4C and D). Furthermore, immunoreactivity of HOCl-modified epitopes decreased with the density of HDL subclasses when the latter were compared on a total mass basis, as staining was largely associated with large, light HDL (Fig. 4C). Indeed, HDL2b, 2a and 3a together represented 73% of the total signal detected whereas HDL3b and HDL3c only contributed to 16% and 11% respectively. Moreover, in this experiment, the immunoreactive signal detected in HDL3c was significantly lower as compared to other HDL subclasses ($p < 0.01$ vs HDL2b; $p < 0.05$ vs HDL2a, 3a and 3b; Fig. 4A). By contrast, no significant difference in the contribution of each HDL subclasses to the

total signal of HOCl-modified epitopes was detected by Western blotting, when these subclasses were compared on a particle number basis (Fig. 4B and D). Signal patterns from HOCl-modified epitopes (Fig. 4A and B) were similar to those of REM (Fig. 2A and B); as a result, the appearance of HOCl-modified epitopes and HDL REM were significantly and positively correlated ($r = 0.40$, $p < 0.05$).

In addition to lysine residues (major carrier of free amino groups), tryptophan residues are equally highly sensitive to HOCl oxidation [34]. When HDL subclasses were compared on a total mass basis, tryptophan residues were almost completely consumed (by >80%, Fig. 5A). Tryptophan residues of HDL3c tended to be more resistant to oxidative modification by HOCl as compared to other HDL subclasses, particularly vs. HDL2a and HDL3b ($p < 0.05$; Fig. 5A). However, when HDL subclasses were compared on a particle number basis, tryptophan residues were less modified by reagent HOCl (Fig. 5B); this was especially true for large, light HDL, while small, dense HDL3b and HDL3c showed the highest susceptibility towards tryptophan modification ($p < 0.001$ vs. HDL2b, $p < 0.01$ vs. HDL3a and $p < 0.05$ vs. HDL2a). Moreover, consumption of tryptophan residues was positively correlated with the increase in HDL REM ($r = 0.40$, $p < 0.05$) and with accumulation of aggregated apoAI ($r = 0.76$, $p < 0.001$).

As HOCl may also attack double bonds of unsaturated FAs (leading to the formation of chlorohydrins), the loss of FAs was analyzed in HDL subclasses following HOCl treatment. When HDL subclasses were compared on a total mass basis, FAs were less rapidly consumed by HOCl than amino acids as demonstrated by the relative consumption of free amino group and tryptophan residues. Indeed, between 65% and 91% of the initial total FA content was present after oxidation (Fig. 6A) as compared to 15–18% of tryptophan residues (Fig. 5A) and 40–70% of free amino groups (Fig. 1A). Consumption of FAs decreased with HDL density and was significantly higher in HDL2a and HDL3a than in HDL3b and HDL3c ($p < 0.05$; Fig. 6A). Consistent with earlier data on FA content in total HDL3 modified by HOCl, added as reagent or generated by the MPO–H₂O₂–chloride system [14,32], unsaturated FAs were sensitive to HOCl modification in all five HDL subclasses (Table 1). However, when HDL subclasses were compared on a particle number basis, no difference was observed, with virtually no FAs consumed at a HOCl:lipoprotein molar ratio of 97:1 (Fig. 6B). Consumption of total unsaturated FAs was positively correlated with the consumption of tryptophan residues ($r = 0.48$, $p < 0.01$).

Taken together, our present results reveal that susceptibility of five different HDL subclasses to HOCl oxidation decreases with lipoprotein density when HDL subclasses are compared on a total mass basis. Indeed, small, dense HDL displayed diminished consumption of amino groups and FAs, reduced increment in REM and less pronounced formation of HOCl-modified epitopes in comparison to large, light HDL. However, when HDL subclasses were compared on a particle number basis, no difference between all five HDL subclasses became apparent. These data indicate that HDL subclasses do not differ per se in their intrinsic particle susceptibility to HOCl-induced oxidation, despite marked differences in physicochemical properties and chemical composition [6]. In the present study, the amount of protein which could be oxidized by HOCl was almost 2-fold superior in small, dense HDL3 than in large, light HDL2 when individual subclasses were compared on a total mass basis [26]. Consistent with this relationship, we observed approximately 2-fold lower consumption of amino groups and accumulation of HOCl-modified epitopes in HDL3c compared to HDL2b under these experimental conditions. These data suggest that the protein moieties of distinct HDL subclasses do not differ in their intrinsic susceptibility to modification by HOCl.

To directly assess the intrinsic susceptibility of HDL subclasses to oxidation by HOCl, their comparison was performed on a particle number basis. In this experiment, only slightly more protein (not higher than 1.4-fold) was present in large, light HDL as compared to small, dense HDL. Such similarity of protein concentrations can explain the absence of significant differences between biomarkers of protein oxidation in HDL subclasses observed under these conditions. Indeed, only a more rapid consumption of tryptophan residues was detected in small HDL as compared to large HDL in this experiment; moreover, we observed that tryptophan residues were consumed more rapidly by HOCl as compared to changes in other biomarkers of protein oxidation. Kinetic parameters of tryptophan oxidation in total HDL (a mixture of HDL2 and HDL3) are between those separately calculated for HDL2 and HDL3 [34]. The conformation of apoAI, which accounts for up to 75% of the total apolipoprotein content of HDL, strongly depends on HDL particle size [35] and can modulate the exposure of tryptophan residues to the aqueous environment. Importantly, tryptophan residues are more solvent-exposed in small, than in large, reconstituted HDL particles [36], the finding that may support the hypothesis that tryptophan residues are more accessible to oxidation in small than in large HDL [34].

Furthermore, when analyzing biomarkers of HOCl oxidation, the increase in REM was positively correlated with the consumption of amino groups and tryptophan residues, and with the presence of immunoreactive HOCl-epitopes. In addition, levels of these biomarkers of HDL oxidation showed largely similar profiles across HDL subclasses (Figs. 1, 4 and 5). Such correlational data suggest that these biomarkers of protein oxidation by HOCl reflect common oxidative pathways as proposed earlier [29,37].

In addition, consumption of tryptophan residues in HDL subclasses revealed a profile similar to the aggregation of apoAI when the susceptibility of HDL subclasses towards HOCl-oxidation was followed either on a total mass or on a particle number basis. Moreover, these two biomarkers of protein oxidation showed a strong positive correlation, suggesting that modification of tryptophan residues could be involved in apoAI aggregation. This hypothesis is supported by earlier data on the oxidation of apoAI by HOCl, added as reagent or generated by the MPO-H₂O₂-halide system [16]. Analysis of tryptic peptides of HOCl-modified, lipid-free and lipid-associated apoAI revealed that in addition to methionine residues, tryptophan residues also serve as an early biomarker of oxidation even at a 10-fold molar excess of the oxidant [16].

HOCl may directly react with unsaturated double bonds of FAs to form chlorohydrins [38]. Alternatively, lysine-derived chloramines can generate nitrogen-centered and subsequently carbon-centered radicals that, in addition to direct reaction of HOCl with unsaturated FAs, may initiate damage to lipids [39]. In the present study, we observed that unsaturated FAs of all five HDL subclasses represented preferential target for HOCl attack; other FAs demonstrated qualitatively similar consumption profiles across HDL subclasses (data not shown). When HDL subclasses were compared on a total mass basis, more FAs (2-fold) were present in large, light than in small, dense HDL, suggesting that oxidation may occur more rapidly in large, light particles. Indeed, we observed elevated consumption of FAs in large, light HDL as compared to small, dense HDL. However, when HDL subclasses were compared on a particle number basis at a fixed HOCl/HDL molar ratio (97:1), no significant difference between HDL subclasses was found. Consistent with these data, no remarkable loss of FAs was detected in HDL3 at an HOCl/HDL molar ratio of 100:1 [14,32]. Thus, HDL size and physicochemical properties do not appear to influence the susceptibility of HDL-associated FAs to exogenous HOCl.

Cross-immunoprecipitation studies revealed the presence of MPO in HDL-like particles isolated from human atherosclerotic lesions [20]. Observations that binding affinity of MPO

to HDL3 markedly increases as a function of increasing extent of modification by HOCl [40] is of further support for the notion that HDL, a physiological carrier of MPO, is highly susceptible to MPO-induced modification, as further revealed by the high content of MPO-produced oxidants in lesion-derived apoAI [17]. We found that all HDL subclasses could be modified by HOCl; therefore, we conclude that the concentration basis on which the comparison of HDL subclasses is performed can critically modify the conclusion regarding their relative susceptibility to HOCl-mediated oxidation. Indeed, different HDL subclasses play major roles in the conversion and remodeling of HDL particles, processes commonly mediated by different enzymes and/or proteins. Reactions mediated by plasma phospholipid transfer protein are impaired by HOCl-modification of HDL3 [41]; furthermore, modulation of lecithin-cholesterol acyl transferase activity by HOCl-mediated modification of apoAI has been reported [42]. Finally, as all HDL subclasses can be modified by HOCl, all major atheroprotective activities of HDL [6] may theoretically be affected by MPO-generated HOCl. Deficiency in HDL function as a result of MPO activity, such as that reported in Type 2 diabetes [43], may be involved in the development of cardiovascular disease in patients with metabolic disease. Clearly then, more studies of therapeutic interventions are needed to elucidate the relationship between MPO, HDL functionality and cardiovascular risk.

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Fig. 1.

Consumption of free amino groups following oxidation of HDL subclasses by HOCl. HDL subclasses were compared on a total mass (160 mg total HDL mass/dl incubated in the presence of 960 μ M HOCl for 30 min at 37 °C) (A; $n = 6$) and on a particle number (9.9 μ M HDL incubated with 960 μ M HOCl for 30 min at 37 °C) (B; $n = 3$) basis. Then reactive amino groups were quantitated with a method using trinitrobenzene sulfonic acid, and consumption was calculated for each HDL subclass relative to initial content detected in native HDL. The initial content of free amino groups in native HDL was 0.49, 0.34, 0.43, 0.31 and 0.35 mg/ μ g protein in HDL2b, 2a, 3a, 3b and 3c, respectively. Data are shown as means $\pm$ SEM; * $p < 0.05$ vs. HDL3c, § $p < 0.05$ vs HDL3b.

Fig. 2.

Relative electrophoretic mobility of HDL subclasses oxidized by HOCl. HDL subclasses were compared on a total mass (160 mg total HDL mass/dl incubated in the presence of 960 μ M HOCl for 30 min) (A; $n = 3$) and on a particle number (9.9 μ M HDL incubated with 960 μ M HOCl for 30 min) (B; $n = 3$) basis. Relative electrophoretic mobility of oxidized HDL subclasses was calculated relative to that of native HDL.; * $p < 0.05$ vs HDL3c.

Fig. 3.

Accumulation of aggregated apoAI in HDL subclasses oxidized by HOCl. HDL subclasses were compared on a total mass (160 mg total HDL mass/dl incubated in the presence of 960 μ M HOCl for 30 min) (A and C) and on a particle number (9.9 μ M HDL incubated with 960 μ M HOCl for 30 min) (B and D) basis. Monomeric and aggregated apoAI were analyzed by Western blotting with polyclonal antibody against apoAI. Blots were scanned and signals detected in each HDL subclass expressed as a ratio of the signal of aggregated apoAI to that of monomeric apoAI. Data are shown as means $\pm$ SEM of three experiments (A and B) or as representative gels (C and D).

Fig. 4.

Accumulation of HOCl-modified epitopes in HDL subclasses oxidized by HOCl. HOCl-modified epitopes were analyzed by Western blotting with a specific monoclonal antibody (clone 2D10G9). Blots were scanned and the signal detected in each HDL subclasses was expressed as a percentage of total signal calculated as the sum of signals from all HDL subfractions. HDL subfractions were compared on a particle number (A and C) and on a total mass (B and D) basis. Data are shown as means $\pm$ SEM of eight (A) or four (B) experiments or as representative gels (C and D); * $p < 0.05$, ** $p < 0.01$ vs HDL3c; § $p < 0.05$ vs HDL3b.

Fig. 5.

Consumption of tryptophan residues in HDL subclasses after oxidation by HOCl. HDL subclasses were compared on a total mass (160 mg total HDL mass/dl incubated in the presence of 960 μ M HOCl for 30 min) (A; $n = 3$) and on a particle number (9.9 μ M HDL incubated with 960 μ M HOCl for 30 min) (B; $n = 3$) basis. Content of tryptophan residues was evaluated in 100 μ l of each HDL subclass by fluorescence (em. 335 nm/exc. 280 nm) and normalized to mg HDL protein. Modification of tryptophan residues was expressed relative to initial tryptophan levels in native (non-oxidized) HDL subclasses; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs HDL3c; \$ $p < 0.05$, \$\$ $p < 0.01$, \$\$\$ $p < 0.001$ vs HDL3b.

Fig. 6.

Content of fatty acids in HDL subclasses after oxidation by HOCl. HDL subclasses were compared on a total mass (160 mg total HDL mass/dl incubated in the presence of 960 μ M HOCl for 30 min) (A; $n = 3$) and on a particle number (9.9 μ M HDL incubated with 960 μ M HOCl for 30 min) (B; $n = 3$) basis. FA analysis of HDL subclass was performed as described using a HP 5890 gas chromatograph equipped with a flame ionization detector. The initial content of total FA in HDL was 1.05, 0.89, 0.69, 0.46 and 0.36 mg/mg protein in HDL2b, 2a, 3a, 3b and 3c, respectively. Data are expressed relative to the initial FA content in native HDL and shown as means $\pm$ SEM; * $p < 0.05$, ** $p < 0.01$ vs HDL3c; § $p < 0.05$ vs HDL3b.

Table 1

HDL content of unsaturated FAs following HDL oxidation by HOCl

	C16:1	C18:1	C18:2	C20:4	C20:5
HDL2b	67.9 ± 13.4 (18.2)	66.9 ± 12.0 [§] (167.5)	64.8 ± 12.2 ^{Ê,§§} (327.2)	55.6 ± 10.1 ^{*Ê,§§} (110.0)	56.2 ± 10.1 ^{*Ê,§} (12.1)
HDL2a	62.4 ± 9.1 (15.2)	74.0 ± 3.5 (143.2)	71.4 ± 2.4 ^Ê (290.6)	64.1 ± 1.5 ^{ÊÊ,§} (102.3)	85.4 ± 3.4 ^{ÊÊ,§} (8.7)
HDL3a	76.0 ± 16.5 (11.0)	82.1 ± 3.6 ^{§§} (98.5)	79.6 ± 3.8 ^{Ê,§§} (204.2)	71.6 ± 4.1 ^{*ÊÊ,§§§} (70.4)	66.5 ± 3.7 ^{*ÊÊ,§§§} (8.5)
HDL3b	84.6 ± 11.7 (7.5)	89.0 ± 0.6 (72.7)	87.3 ± 3.1 ^{Ê,§} (152.2)	78.8 ± 3.7 ^{Ê,§} (52.4)	84.1 ± 3.8 (5.6)
HDL3c	94.0 ± 3.8 (5.1)	95.4 ± 1.9 (55.9)	89.2 ± 1.5 ^Ê (120.9)	81.6 ± 0.6 ^{*ÊÊ} (42.4)	74.0 ± 8.2 ^{*§} (5.2)

HDL subclasses were compared on a total mass basis (160 mg total HDL mass/dl incubated in the presence of 960 µM HOCl for 30 min). FA analysis of native and HOCl-modified HDL subclasses was performed as described [32] using a HP 5890 gas chromatograph equipped with a flame ionization detector. Data are expressed relative to the initial fatty acid content in native HDL ($n = 3$). Values are given as a percentage of FAs before oxidation. The initial content of each FA (expressed as µg/mg HDL protein in each HDL subclass) is shown in parentheses;

Abbreviations: C16:1, palmitoleic acid; C18:0, stearic acid; C18:1, oleic acid; C18:2, linoleic acid; C18:3, arachidonic acid; C20:4, arachidonic acid; C20:5, eicosapentaenoic acid.

* $p < 0.05$, vs. 14:0

Ê $p < 0.05$

ÊÊ $p < 0.01$ vs. 16:0

§ $p < 0.05$

§§ $p < 0.01$

§§§ $p < 0.001$ vs. 18:0.