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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–H_2O_2–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.

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–H_2O_2–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 fibroatheromatic 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–H_2O_2–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)

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[1] *Abbreviations used:* HDL, high-density lipoprotein; MPO, myeloperoxidase; HOCl, hypochlorous acid.

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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-Salpétrière (Paris, France) and stored at −80 °C. Sucrose (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 M^{-1} 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 $NaHCO_3$ (4%, w/v; pH 8.4) and 50 μl trinitrobenzene sulfonic acid in H_2O (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