Modification of HDL3 by mild oxidative stress increases ATP-binding cassette transporter 1-mediated cholesterol efflux

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Abstract

Objective: Elevated levels of high-density lipoprotein (HDL) cholesterol are inversely related to the risk of cardiovascular disease. The anti-atherosclerotic function of HDL is mainly ascribed to its role in reverse cholesterol transport, and requires the integrity of HDL structure. Experimental evidence suggests that the ability of HDL to promote removal of excess cholesterol from peripheral cells is impaired upon oxidation. On the other hand, tyrosylation of HDL enhances its protective function, suggesting that not all forms of modified lipoprotein may be atherogenic. In the present study we investigated the effect of a mild oxidation of HDL3 on its function as cholesterol acceptor.

Methods and results: A mild oxidative stress (induced by 15 min exposure of HDL3 to 1 μM Cu++ or to 15-lipoxygenase) caused the formation of pre-β-migrating particles. Compared to native lipoprotein, mildly modified HDL3 induced a significant ATP-binding cassette transporter 1 (ABCA1)-mediated increase of cholesterol and phospholipids efflux from J774 macrophages. This effect was abolished by an inhibitor of ABCA1-mediated lipid efflux (glyburide) and was absent in Tangier fibroblasts.

Conclusions: A mild oxidative modification of HDL3 may improve its function as cholesterol acceptor, increasing ABCA1-mediated lipid efflux from macrophages, a process that may reduce foam cell formation.

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Keywords: High-density lipoprotein; Reverse cholesterol transport; Oxidative stress; 15-lipoxygenase; pre-β-HDL; ATP-binding cassette transporter 1

1. Introduction

A number of studies have established that elevated concentrations of plasma high-density lipoprotein (HDL) cholesterol are inversely related to the risk of cardiovascular disease (CHD) [1–3]. The atheroprotective role of HDL is believed to depend mainly upon its ability to remove cholesterol from peripheral cells, a process known as reverse cholesterol transport [4,5].

Human HDL consists of distinct subfractions that, besides differences in shape, density, size, and charge, differ in the mechanisms by which they promote the removal of cholesterol from cells [6,7]. Among the HDL subfractions, pre-β1-HDL, which contains apoA-I as only apoprotein and exhibits pre-β electronephoretic mobility, appears to be the most efficient acceptor of cellular cholesterol [7–9], via the interaction with ATP-binding cassette transporter 1 (ABCA1) [10–12]. The physiological mechanism by which pre-β1-HDL is generated in plasma is not fully understood, but there are at least two potential sources: i) it may be derived from the interaction of lipid poor apoA-I secreted from liver or intestine with cell membrane lipids [13,14]; ii) it may be formed during the conversion of mature, spherical HDL (α-HDL) [15]. In vitro, pre-β1-HDL can be formed following the interaction of free apoA-I with cholesterol-loaded macrophages [16] or fibroblasts [17]. Furthermore, the concentration of pre-β1-HDL is increased under pathophysiological conditions, such as hypercholesterolemia [18], showing a positive correlation...
with LDL concentration [19]; increased amounts of pre-β-HDL are also present in cholesterol-fed rabbits and mice [20].

The structural integrity of HDL is essential for their function in the reverse cholesterol transport. In fact, severe oxidation may impact the biological role of HDL by impairing their ability to promote cholesterol efflux in vitro [21–23]. There is growing pathophysiological evidence that increased generation of reactive oxygen species and oxidative stress participate in vivo to pro-atherogenic mechanisms [24–26]. 15-lipoxygenase [27,28], an enzyme that has been implicated in the conversion of native LDL to an atherogenic form [28,29], may contribute to such a mechanism. In a recent paper, we have shown that 15-lipoxygenase-mediated modification of HDL3 significantly reduces its role as efficient cholesterol acceptor [30].

Data obtained with transgenic rabbits overexpressing 15-lipoxygenase [31,32], however, suggest also antiatherogenic effects for this enzyme [33]. In this work we studied the functional effect of HDL exposure to a mild oxidative stress, induced by low concentration of copper ions or by 15-lipoxygenase for short periods. These mild modifications caused a transient increase of pre-β-migrating particles from α-HDL3, generating modified lipoprotein species which exhibited a significantly improved capacity to stimulate reverse cholesterol transport from macrophages.

2. Methods

2.1. Materials

MEM, DMEM, fetal bovine serum (FBS), bovine serum albumin (BSA), 22R-OH cholesterol (22-OH), 9-cis-retinoic acid (9cRA), glyburide and Oil red O were from Sigma (St. Louis, MO, USA). [3H]-cholesterol, methyl-[3H]-choline chloride, PD10 columns and ECL were from Amersham Biosciences (Uppsala, Sweden). Antiserum anti-human apoA-I was from Dade Behring (Marburg, Germany). SR-BI blocking antibody was from Abcam (Cambridge, UK). Native reticuloocyte-type 15-LO was prepared as described [34]. Cholesterol kit was from Clonital, Italy.

2.2. Cell culture

J774 macrophages were cultured in MEM containing 10% FBS; normal human skin fibroblast and Tangier fibroblasts were cultured in DMEM supplemented with 10% FBS.

2.3. Isolation of plasma lipoproteins

The use of human material in this study conforms to the principles outlined in the Declaration of Helsinki. LDL (d=1.019–1.063 g/ml) and HDL3 (d=1.125–1.21 g/ml) were isolated from fresh plasma of normolipidemic healthy volunteers by sequential ultracentrifugation [35]. Protein content was determined by the method of Lowry using BSA as standard [36]. LDL were acetylated by repeated additions of acetic anhydride [37].

2.4. Modification of HDL3

HDL3 (1 mg/ml) were modified by 15-lipoxygenase (2 µl/ml) [30] or by CuSO4 1 µM for 15 min up to 24 h at 37 °C. The oxidation was blocked by the addition of 10 µM BHT and by lowering the temperature to 4 °C. The TBARS content and the apoproteins cross-linking in mildly modified HDL3 were evaluated as previously described [30].

2.5. Agarose gel electrophoresis and western blotting

Aliquots of native and modified HDL3 were electrophoresed on a 0.8% agarose gel, then transferred onto a nitrocellulose membrane. After blocking with 5% nonfat milk in PBS-T (PBS-0.1% Tween) for 1 h at room temperature, apoA-I was identified using an antiserum anti-human apoA-I from rabbit (1:20000) followed by a goat anti-rabbit IgG peroxidase-conjugated (1:20000). Immuno-complexes were visualized using an ECL western blotting detection system followed by autoradiography.

Fig. 1. Mild modified HDL3 increased cholesterol efflux. J774 were labeled with [3H]-cholesterol as described in Methods, then incubated for 6 h with 10 µg/ml of native HDL3 or HDL3 modified with Cu++ 1 µM or 15-LO for 15 min. Values are mean±SD of 6 independent experiments. *p<0.0005 vs native HDL3.

Fig. 2. Mild oxidative modification of HDL3 generated pre-β-migrating particles. The distribution of apoA-I in HDL3 subclasses of native, Cu++ 1 µM or 15-LO-modified HDL3 was analyzed by 0.8% agarose gel electrophoresis, followed by western blotting using an anti-apoA-I antibody.
2.6. Cholesterol efflux experiments

J774 were labeled with \( ^3 \text{H}-\text{cholesterol} \) (1 \( \mu \text{Ci/ml} \)) in MEM containing 0.2% BSA and 50 \( \mu \text{g/ml} \) AcLDL; after 24 h, cells were washed once with PBS and incubated for 24 h in MEM+0.2% BSA in the presence of 22-OH/9cRA (10 \( \mu \text{M} \)). Native HDL3 or HDL3 modified with Cu\(^{2+} \) 1 \( \mu \text{M} \) or 15-LO for 15 min (10 \( \mu \text{g/ml} \)) were added to cells for 4 h. At the end of the experiment, media were collected, centrifuged to remove cellular debris and lipids were extracted with hexane:2-propanol (3:2). Radioactivity in the lipid extracts was quantified by liquid scintillation. Cell monolayers were lysed with 0.1N NaOH, lipids were extracted with hexane:2-propanol and aliquots were used to determine the intracellular radioactivity content.

In some experiments, glyburide (1 mM) was added together with the lipoproteins to the medium; alternatively, cells were pre-incubated with a SR-BI blocking antibody (1:1000 dilution) before the incubation with lipoproteins [30].

2.7. Phospholipid efflux experiments

J774 were incubated in MEM containing 0.2% BSA, 50 \( \mu \text{g/ml} \) AcLDL and methyl-\( ^3 \text{H}-\text{choline chloride} \) (2 \( \mu \text{Ci/ml} \)). After 24 h, cells were washed with PBS and incubated for 24 h in MEM+0.2% BSA in the presence of 22-OH/9cRA (10 \( \mu \text{M} \)). Native HDL3 or HDL3 modified with Cu\(^{2+} \) 1 \( \mu \text{M} \) or 15-LO for 15 min (10 \( \mu \text{g/ml} \)) were added to cells for 4 h. At the end of the experiment, media were collected, centrifuged to remove cellular debris and lipids were extracted with hexane:2-propanol (3:2). Radioactivity in the lipid extracts was quantified by liquid scintillation. Cell monolayers were lysed with 0.1N NaOH, lipids were extracted with hexane:2-propanol and aliquots were used to determine the intracellular radioactivity content.

2.8. Purification and modification of \( \alpha \)-HDL3 particles

A discontinuous salt density gradient was created in an ultracentrifuge tube. A solution containing 2 mg of HDL3 and 20 mg of BSA was adjusted to \( d=1.25 \) g/ml by the addition of KBr. Two ml of the obtained solution was placed in a Beckman ultracentrifuge tube and layered with: 2 ml of \( d=1.21 \) g/ml, 2 ml of \( d=1.18 \) g/ml, 2 ml of \( d=1.16 \) g/ml, 1 ml of \( d=1.14 \) g/ml, 1 ml of \( d=1.12 \) g/ml and 2 ml of

Fig. 4. Purification (A) and modification (B) of \( \alpha \)-migrating particles. (A) \( \alpha \)-migrating particles were isolated from HDL3 by a discontinuous salt density gradient (1.11–1.25 g/ml) and ultracentrifugation at 40,000 rpm at 5 °C for 18 h. The distribution of apoA-I in each fraction was determined by 0.8% agarose gel electrophoresis and western blotting using an anti-human apoA-I antiserum. (B) Fractions consisting of purified \( \alpha \)-particles were modified with Cu\(^{2+} \) 1 \( \mu \text{M} \) or 15-LO for the indicated times. After modification, the distribution of apoA-I-containing particles was analysed by 0.8% agarose gel electrophoresis and western blotting.

Fig. 3. Mild modified HDL3 increased ABCA1-mediated cholesterol (A) and phospholipid (B) efflux and decreased total cholesterol content (C). J774 labeled with \( ^3 \text{H}-\text{cholesterol} \) (A) or methyl-\( ^3 \text{H}-\text{choline chloride} \) (B) were pre-incubated with 22-OH/9cRA (10 \( \mu \text{M} \)) and then incubated for 6 h (A) or 4 h (B) with 10 \( \mu \text{g/ml} \) of native HDL3 or HDL3 modified with Cu\(^{2+} \) 1 \( \mu \text{M} \) or 15-LO for 15 min. Cell monolayers were lysed in 0.1 N NaOH and aliquots were used to determine the intracellular radioactivity content. The \( ^3 \text{H}-\text{cholesterol} \) release was calculated as the ratio of radioactivity released in the medium to the medium plus cell (total) radioactivity.

Values are mean±SD from 6 (A) and 3 (B, C) independent experiments. (A) *p<0.00005 vs native HDL3; (B) #p<0.05 vs native HDL3.
d = 1.11 g/ml. Samples were centrifuged at 40,000 rpm at 5 °C for 18 h in a Beckman SW41 rotor. Twelve fractions (1 ml each) were collected from the top of the tube. To study the distribution of apoA-I in HDL3 particles, aliquots of fractions obtained from the density gradient were subjected to agarose gel electrophoresis and western blotting as described above. Fractions consisting of purified α-particles were retrieved, concentrated in a Centricon filter unit fitted with a YM10 membrane and desalted in PBS using a Sephadex G25 (PD10) column. The protein content was evaluated by the Lowry method.

α-HDL3 were modified under the same experimental conditions described for HDL3. After modification, the distribution of apoA-I-containing particles was analyzed by agarose gel electrophoresis and western blotting, as described.

2.9. Oil red O staining

Foam cell formation was induced by cholesterol-loading with acetylated LDL (50 μg/ml) for 24 h; after this time, cells were washed twice with PBS and incubated for 24 h with 100 μg/ml of HDL3 native or modified for 15 min with 15-LO. At the end of the incubation, cells were fixed with 5% paraformaldehyde for 1 h, then neutral lipids were stained with Oil red O (0.2% in isopropanol) for 30 min. Cells were rinsed thrice with water and the stained lipid droplets were visualized by light microscopy. For quantification, the dye was extracted in isopropanol and the absorbance was measured at 515 nm. Cell protein concentration was determined by the method of Lowry and absorbance values were normalized to protein.

2.10. Total cholesterol determination.

After cholesterol-loading with AcLDL (50 μg/ml), cells were incubated for 24 h with 22-OH/9cRA (10 μM/1 μM) to induce ABCA1 expression, then incubated for 6 h with 10 μg/ml HDL3 native or modified for 15 min with 15-LO. Cellular lipids were extracted in hexane/isopropanol (3:2) and cholesterol content was evaluated by a colorimetric assay according to the instructions of the manufacturer and
normalized by protein concentration, determined by the method of Lowry.

3. Results

In agreement with our previous results [30], the incubation of HDL₃ with low concentrations of Cu⁺⁺ (1 μM) or with 15-LO for 15 min neither increased the lipoprotein TBARS content, nor induced an appreciable apolipoprotein cross-linking (data not shown). However, compared to native lipoprotein, HDL₃ modified for 15 min with Cu⁺⁺ 1 μM or 15-LO induced a higher cholesterol efflux from J774 macrophages (Fig. 1). The increase was independent of the modification type (chemical or enzymatic) and, as expected from previous data [30], disappeared at 24 h. Analysis of HDL₃ particles showed that incubation with Cu⁺⁺ 1 μM or 15-LO for 15 min induced the appearance of particles with pre-β electrophoretic mobility up to 2 h modification (Fig. 2), undetectable at 24 h (Fig. 2).

We therefore addressed the question as to whether the observed higher cholesterol efflux induced by mildly modified HDL₃ might be related to the increase of pre-β-migrating particles, possibly through ABCA1 activity. To this end, J774 cells were pre-incubated with 22-OH/9cRA to increase ABCA1 expression [30,38]. Accordingly, these cells exhibited a significantly higher cholesterol efflux when incubated with mildly modified HDL₃, compared with cells incubated with native HDL₃ (Fig. 3A). In agreement with this finding, total cholesterol levels were lower in the presence of mildly modified HDL₃ (Fig. 3B).

ABCA1 is involved in the reverse cholesterol transport, but also triggers the efflux of phospholipids to lipid poor apoA-I [39]; for this reason, we studied the impact of mildly modified HDL₃ on this process: in cells overexpressing...
ABCA1 the incubation with HDL3 modified with Cu++ 1 μM or 15-LO for 15 min induced a significant increase in phospholipid efflux compared to native HDL3 (Fig. 3C).

As native HDL3 did already contain a minor fraction pre-β migrating particles (Fig. 2) whose amount was increased after a mild oxidative modification, we aimed at clarifying whether those particles were generated ex-novo from α-particles or derived from a conformational rearrangement of pre-β particles already present in native lipoprotein. To this end, α-migrating particles were purified from native HDL3 by density gradient ultracentrifugation (Fig. 4A) and modified under the same experimental conditions described for HDL3. In keeping with the data with whole HDL3, short-term modification of α-HDL3 with Cu++ 1 μM or 15-LO generated pre-β-migrating particles up to 2 h, which disappeared at longer incubation periods (Fig. 4B), indicating an ex-novo formation of the pre-β particles after mild oxidative modification. A higher concentration of Cu++ (20 μM) failed to induce the formation of pre-β particles from α-HDL3 (Fig. 4B). α-HDL3 modified with Cu++ 1 μM or 15-LO for 15 min induced, when compared to native α-HDL3, a higher cholesterol efflux from J774 cells under basal conditions (Fig. 5A). In cells overexpressing ABCA1, mildly modified α-HDL3 further stimulated the cholesterol efflux process (Fig. 5A), while no significant effect was found with native α-HDL3. These data suggested that the newly formed pre-β-migrating particles efficiently promoted cholesterol efflux via an ABCA1-mediated mechanism; this finding was further confirmed using as cholesterol acceptor isolated pre-β-particles purified by density gradient ultracentrifugation (data not shown).

On the other hand, the removal of pre-β-migrating particles from α-HDL3 modified with 15-LO for 15 min abolished the increased cholesterol efflux obtained with mildly modified α-HDL3 (Fig. 5B), further indicating that pre-β particles were responsible for the observed effect.

To show that ABCA1 activity was actually involved in the increased cholesterol efflux observed with mildly modified HDL3, J774 overexpressing ABCA1 were pre-incubated with glyburide, an inhibitor of ABCA1-mediated lipid efflux to apo-A-I [40]. Glyburide reduced the cholesterol efflux to native HDL3 by 36%, and to mild modified HDL3 by ~70% (Fig. 6), indicating an essential contribution of ABCA1 activity in the mildly modified HDL3-induced cholesterol efflux. The presence of a SR-BI blocking antibody, which reduces SR-BI-mediated cholesterol efflux to α-HDL3 [30], could not inhibit cholesterol efflux to mildly modified HDL3 (Fig. 6).

The most direct evidence that ABCA1 transporter is involved in the increased cholesterol efflux induced by pre-β HDL particles generated in mildly modified HDL3 was obtained using Tangier fibroblasts as cellular model. These cells do not express a functional ABCA1 transporter and thus, the pre-β particles-induced cholesterol efflux is predicted to be absent [41]. As expected, modification of α-HDL3 with Cu++ 1 μM or 15-LO for 15 min caused an increase in cholesterol efflux from normal fibroblasts pre-treated with 22-OH/9cRA to overexpress ABCA1 (Fig. 7). In contrast, under the same experimental conditions, Tangier fibroblasts did not show a stimulatory effect on cholesterol release in the presence of mildly modified HDL3 (Fig. 7).

Finally, mildly modified HDL3 were tested for the ability to reduce foam cell formation. After loading with AcLDL, J774 exhibited extensive Oil red O staining (Fig. 8B, E) compared to unloaded cells (Fig 8A). As expected, native HDL3 significantly reduced neutral lipid content (Fig. 8C, E); HDL3 modified for 15 min with 15-LO further decreased cell lipid content (Fig 8D, E).

4. Discussion

Lipoproteins modification is usually regarded as a deleterious process, which affects their physiological properties, triggering events that can promote atherogenesis. HDL modification impairs its ability to promote cholesterol efflux from cultured cells, suggesting that changes in the protein and/or lipid moiety alter the athero-protective role of HDL [21–23,30]. Not all modification of HDL, however, shift HDL towards a pro-atherogenic particle. Tyrosylation, for instance, enhances the removal of cholesterol from cultured fibroblasts and macrophages [42] and the administration of tyrosylated HDL to apo-E-deficient mice induces a significant decrease of atherosclerotic lesions [43]. The mechanism by which tyrosylated HDL exerts its protective role seems to be independent of passive cholesterol desorption from the cell membrane, suggesting a possible involvement of ABCA1 activity [44]. These observations suggest that lipoprotein modification does not always confer pro-atherogenic properties. In the present work we show that mild oxidative modification (enzymatic or non-enzymatic) of HDL3 enhances its ability to induce cholesterol efflux from cells and provides evidence that newly formed pre-β-HDL3 are responsible for this effect through the interaction with ABCA1.

Experimental evidence suggests that oxidative stress represents a key factor in the initiation of vascular dysfunctions associated with atherosclerosis. Both systemic factors, such as hypercholesterolemia [45] and local factors, such as activation of macrophages and T-cells, may contribute to oxidative stress [26]; diet-induced atherosclerosis in different animal models is related, in fact, to an enhanced xanthine oxidase activity and ROS production [45,46]. The tight regulation of both production and removal of reactive oxygen species induces transient fluctuations in oxidant levels modulating gene expression and thus, metabolic switches [47]. Extracellular stimuli like angiotensin II or TNFα [48,49], or pathophysiological conditions such as hypercholesterolemia can shift the balance to a pro-oxidant state, resulting in a decreased activity of antioxidant enzymes (SOD, catalase, GPx) and in lipoprotein modification. Copper is normally tightly sequestered in biological...
The interaction between pre-in a reduced cellular cholesterol content. A crucial role for HDL-mediated reverse cholesterol transport, thus resulting in an anti-atherogenic role, with an improvement of mild modified HDL 3 and ABCA1 can be assumed, as proposed by experiments using an ABCA1 inhibitor or Tangier fibroblasts expressing a non-functional form of ABCA1 [41]. At later time points, when HDL particles are converted into more extensively modified species, the cholesterol accepting properties are lost [30] and reverse cholesterol transport is impaired. This effect may contribute in vivo to the formation of lipid-laden foam cells.

The data obtained in the present study suggest also that pre-β-migrating particles detected in mildly oxidized HDL 3 were generated ex-novo from α-particles and did not derive from a conformational change of apoA-I in the pre-β-HDL already present in the lipoprotein preparation. This finding is supported by the observation that oxidation of lipid-bound apoA-I significantly decreases its stability [54]; destabilized apoA-I can be readily released from HDL, providing a pool of lipid-free/lipid-poor apoA-I that exhibits a pre-β electrophoretic mobility. When oxidation proceeds, apoA-I becomes further oxidized, thus increasing the negative particle charge, and this could explain the loss of pre-β-migrating particles observed by agarose gel electrophoresis. Moreover, an extensive HDL 3 oxidation leads to an increased particles size (not shown) with a reduced ABCA1-mediated cholesterol efflux.

In hypercholesterolemic subjects plasma level of pre-β1-HDL is increased [18,55]. Moreover, sera from patients with low HDL cholesterol, such as in hypertriglyceridemic, trigger a significant increase in cholesterol efflux from J774 cells overexpressing ABCA1, when compared to normolipidemic controls [55]. This effect was attributed to the increased levels of pre-β-HDL particles in hypertriglyceridemic serum. Since hypercholesterolemia is associated with an increased oxidative potential [45,56], the processes investigated here might be of pathophysiological relevance in vivo, as our data indicate that pre-β-migrating particles can be formed by mild oxidative modification of HDL 3. Whether pre-β-particles generated under our experimental conditions are structurally related to those detected in hypercholesterolemic plasma remains to be addressed. We can only suggest a common ABCA1-mediated mechanism of cholesterol efflux.

Taken together these data are consistent with the hypothesis that mild oxidative modification of HDL, which leads to the formation of pre-β-HDL migrating particles, might be considered an anti-atherogenic process enhancing reverse cholesterol transport and thus, impairing intracellular lipid deposition in peripheral cells. However, when oxidation proceeds further, HDL lose their cholesterol effluxing properties [30]. These results provide additional evidence for the previous suggestion [57] that a low degree of oxidation is needed for a balanced steady state of lipid metabolism. Deviations in either direction might induce cascades leading to intracellular lipid deposition. Moreover, these results may also contribute to explain the clinical failure of antioxidant therapy in cardiovascular disease [58–60].

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