Age-related impairment of HDL-mediated cholesterol efflux

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Running title: impairment of cholesterol efflux with aging

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Abstract

Our aim of this study was to investigate the effect of aging on the capacity of high-density lipoproteins (HDL) to promote reverse cholesterol transport (RCT). HDL were isolated from plasma of young (Y-HDL) and elderly (E-HDL) subjects. HDL-mediated cholesterol efflux was studied using THP1 and J774 macrophages. Our results show that E-HDL present a lower capacity to promote cholesterol efflux than Y-HDL (41.7 ± 1.4 % vs. 49.0 ± 2.2 % respectively, \( p=0.013 \)). Reduction in the HDL-mediated cholesterol efflux capacity with aging was more significant with HDL\(_3\) than HDL\(_2\) (Y-HDL\(_3\): 57.3 ± 1 % vs. E-HDL\(_3\): 50.9 ± 2 %, \( p=0.012 \)). Moreover, our results show that the ABCA-1-mediated cholesterol efflux is the more affected pathway in the cholesterol removing capacity. Interestingly, the composition and structure of HDL revealed a reduction in the phosphatidylincholine/sphingomyelin (PC/SPM) ratio (E-HDL: 32.7 ± 2.7 vs. Y-HDL: 40.0 ± 1.9 vs. \( p = 0.029 \)) and in the phospholipidic-layer membrane fluidity in E-HDL as compared to Y-HDL as well as an alteration in the apoA-I structure and charge. In conclusion, our results shown that elderly-derived HDL present a reduced capacity to promote cholesterol efflux, principally through the ABCA-1 pathway, and this may explain the increase of the incidence of cardiovascular diseases observed during aging.

**Keys word:** Aging, Reverse cholesterol transport, ABCA-1, apoA-I, Phospholipids.
Introduction

The inverse relationship between plasma levels of high density lipoproteins (HDL) and cardiovascular diseases (CVD) has been demonstrated in several epidemiological and interventional studies (1,2). The anti-atherogenic properties of HDL have been ascribed to their ability to inhibit LDL oxidation (3), and to prevent ox-LDL-induced cytotoxicity and monocyte transmigration (4,5). One of the long-standing mechanisms to explain the protective effect of HDL against CVD is their capacity to promote reverse cholesterol transport (RCT) (6).

The concept of RCT as suggested by Glomset, consists of a movement of cholesterol from the peripheral tissues to the liver, which starts by the efflux of free cholesterol (FC) and phospholipids from cells of peripheral tissues to pre-β migrating lipid-poor, or lipid-free apolipoprotein-Al (apoA-I), and to HDL₃ (7-9). The process of FC efflux occurs by 3 known pathways: 1) *Aqueous diffusion*: this process involves desorption of FC molecules from the donor lipid-water interface and diffusion of these molecules through the intervening aqueous phase until they collide with and are absorbed by an acceptor. 2) *Scavenger receptor type BI (SR-BI)-mediated FC flux*: the movement of FC via SR-BI is bi-directional, and like the aqueous diffusion mechanism, the net movement of FC via SR-BI depend on the direction of the cholesterol gradient (10). 3) *ATP-binding cassette (ABC)-mediated cholesterol efflux*: ABCA-1 and ABCG1/4 are members of a large family of ATP-dependant transporters that share common structural motifs for the active transport of a variety of substrates (11). In contrast to aqueous diffusion and SR-BI-mediated FC flux, the movement of FC by ABCA-1 and ABCG1/4 are unidirectional, and net efflux of cellular FC would always occur via this mechanism (12). The preferred cholesterol acceptors for ABCA-1 are lipid-poor apolipoproteins and all of the exchangeable apolipoproteins, such as apoA-I, apoA-II, apoA-IV, apoE and apoC (13). ABCA-1 has been shown to bind with apoA-I, indicating a very close association between the two proteins in mediating the cholesterol efflux process (14). Recently, it has been shown that another transporter, ABCG-1, promotes mass cholesterol efflux from cells to mature HDL particles (HDL₂ and HDL₃), but not to lipid-poor apoA-I (5, 6). Both ABCA-1 and ABCG-1 are abundant in macrophages, especially following cholesterol loading, suggesting their importance for cholesterol efflux process.

Cholesterol efflux was also correlated to the HDL lipid composition and structure (12). As an example, phosphatidylcholine (PC)-enriched HDL increases cholesterol efflux, whereas sphingomyelin (SPM)-enriched HDL decreases cholesterol influx to macrophages (12).
Moreover, several lipids that are common constituents of HDL are known to significantly affect the fluidity of lipid surfaces (e.g. PC and SPM) (15). Indeed, the phospholipid fatty acyl composition of lipoproteins is known to have subtle but measurable effects on the fluidity of lipoproteins phospholipidic layer (15,16). These changes may affect the ability of HDL particles to accommodate FC molecules that have desorbed from peripheral cells. Additionally, oxidative modifications of HDL affect its capacity to promote cholesterol efflux (17). Indeed, formation of peroxidation-derived lipid products is associated with changes of physico-chemical properties of HDL and especially a decrease in the fluidity of the HDL phospholipids bilayer (17).

In previous studies we have demonstrated that HDL from elderly subjects are more prone to lipid peroxidation (18), and present a significant reduction of their antioxidant property, along with a decrease in paraoxonase 1 activity (19). In this study, we investigated the capacity of HDL to promote cholesterol efflux during aging and to elucidate its biophysical and biochemical changes that influence this process. Hence, our results will contribute to a better understanding of the age-related increase in the incidence of CVD.
Materials and Methods

Chemicals

Acetic acid, sulfuric acid, sodium phosphate, thiobarbituric acid, n-butanol, methanol, ethanol, n-isopropanol, hexane, ammonium hydroxide, chloroform and methanol were purchased from Fisher (Montréal, Quebec). 1,1,3,3,- tetraethoxypropane, D-α-tocopherol, γ-tocopherol, butylated hydroxytoluene (BHT), cupric sulfate (CuSO₄), ethylenediaminetetraacetic acid (EDTA) and lithium perchlorate, DPH (1,6-diphenyl-1,3,5-hexatriene), phosphatidylcholine, sphingomyelin, 8-(4-chlorophenylthio) adenosine 3':5'-cyclic monophosphate (AMPC), and [³H]-cholesterol, were obtained from Sigma (St. Louis, MO). THP-1 and J774 cells were purchased from American type Culture Collection (ATCC) (Manassas, VA).

Subjects

Plasma was obtained from healthy volunteers (8 young, aged between 20–30 years and 9 elderly, aged between 65-70 years). Their demographic data are depicted in Table I. All subjects were considered all healthy since they were normolipidemic and have normal blood pressure. No subjects showed clinical signs of inflammation or diabetes. They were free of any medication including lipid-lowering medications and no oral antioxidants supplementation was used. They were all non-smokers and none of the women was currently taking oestrogen replacement therapy for menopause. The elderly subjects were living at home, functionally independent and cognitively intact (MMSE >28). Ethics Committee of the Sherbrooke Geriatric University Institute approved the study, and all subjects gave written informed consent.

Methods

Lipoprotein isolation

Human plasma was collected in citrate-tube, and HDL isolations were performed immediately according to the method of Sattler et al. (20). Isolated lipoproteins were dialysed overnight at 4 °C in 10⁻² M sodium phosphate buffer (pH 7.0) and then the protein concentrations were measured by commercial assay (Biorad, Mississauga, ON, Canada).

ApoA-I purification

ApoA-I was purified from HDL obtained from young and elderly subjects according to the method of Haidar et al. (21). Briefly, HDL were delipidated in acetone-ethanol solution and the protein fractions subjected to chromatographic separation using two sephacryl S-200 columns
ApoA-I pure fractions were pooled and dialysed in 100 mM NH₄HCO₃, then lyophilized and resuspended in phosphate buffer solution.

**Cell culture**

Human THP-1 monocytes and J774 macrophages were grown in RPMI 1640 and DMEM medium, respectively. Mediums were supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2-β-mercaptoethanol (only for THP-1, 50µM), L-glutamine (2 mM), glucose (1.5 mg/ml) and antibiotic (penicillin, 100 U/ml).

Differentiation of THP-1 monocytes into macrophages was induced by plating the cells at a density of $1.0 \times 10^5$ cells/cm² in the presence of 100 nM phorbol myristate acetate (PMA) for 96 h.

**Cholesterol efflux measurement**

THP-1 and J774 macrophages were incubated in fresh growth medium containing $[^3]$H]-cholesterol (0.2 µCi/ml) for 48 h. Labelled cells were washed, and incubated in serum-free medium containing 1% BSA for 24 h equilibration. The cells were then washed and subjected to different treatments. **1)** Control: fresh medium without HDL, **2)** fresh medium containing (50 µg/ml) of HDL and **3)** fresh medium containing HDL₃ or HDL₂ (50 µg/ml), obtained from young and elderly subjects.

In another series of experiments, J774-macrophages were labelled with $[^3]$H]-cholesterol (2 µCi/ml) for 24 h. The cells were then washed and incubated with 0.2% BSA in DMEM alone or with 0.3 mM AMPc for 12 h to yield ABCA-1-enriched cells (22). Then, the cells were washed and further incubated for 4 h at 37 °C in the presence of whole HDL, HDL sub-fractions (HDL₃ and HDL₂) or apo-AI obtained from young and elderly subjects.

Cholesterol efflux was determined by liquid scintillation counting and the percentage of radiolabeled cholesterol released (% cholesterol efflux) was calculated as (cpm in medium/[cpm in the cell + medium]) x 100.

Net cholesterol efflux was also assayed by measuring cell cholesterol mass content. Briefly, cellular lipids were extracted from (2x10⁶) J774 cells with hexane:isopropanol (3:2, v/v), and the hexane phase was dried under nitrogen. Total cholesterol concentrations in cells as well as in culture medium was measured by HPLC according the method of Katsanidis and Addis (2).
Western blotting and PCR analysis

Twenty five µg of cell lysate proteins were loaded per line and separated on 7.5% SDS-PAGE. After transfer, membranes were incubated with specific antibodies against ABCA-1 (Santa Cruz Biotechnology, CA, USA). Antigoat IgG-HRP antibody (Santa Cruz) was directed against the primary antibody and protein was detected using a chemiluminescent reagent (ECL).

RNA was extracted from J774 cells using the RNeasy mini Kit (QIAGEN) and reverse transcribed employing the GeneAmp Kit (Applied Biosystems). Reverse-transcribed (RT) RNA was used for PCR analysis with the following primer sequences (F: forward; R: reverse): β-actin (F: 5’- CACCCTgTgCTgCTACgAggCC-3’; R: 5’-ACCgCTgTTgCCAATAgTgATgA-3’), ABCA-1 (F: 5’-gCCCTACTgTCggTTgAC-3’; R: 5’-TgggCAACACTgAACAAgAg-3’), and ABCG1 (5’-TCAACAgTggAgAgCTggTg-3’; 5’-CTgCCCTTCATCCTTTC-3’). All reactions were run at least in triplicate.

Copper-mediated HDL oxidation

Lipoprotein peroxidation was carried out as previously described using copper ions as an oxidizing agent (24).

Conjugated diene and thiobarbituric acid-reactive substance (TBARS) evaluation

HDL peroxidation was continuously monitored at 234 nm to detect the formation of conjugated diene as previously described (17). TBARS, mainly malondialdehyde (MDA), were assayed by high-performance liquid chromatography (HPLC) coupled to fluorescence detection (25).

Apolipoprotein A-I modification

ApoA-I was analysed as described by Laemli (26) on 12.5% SDS-PAGE gels (Mini Gel II from BioRad, Hercules, CA, USA). Each lane was loaded with 8 µg protein of native or oxidized HDL, (for 0, 1, 4 and 8 h). Gels were stained with Coomassie Brilliant Blue.

ApoA-I modifications were also evaluated by measuring the HDL surface charge and for the presence of apoA-I carbonyl groups. Oxidative modification of HDL (apoA-I) results in an increase in net negative charge which can be detected using agarose electrophoresis (titan gel lipoprotein electrophoretic system). Relative electrophoretic mobility was calculated as the ratio of the electrophoretic mobility of the samples to that of native HDL of young subjects (Y-HDL). Ratio value > 1 means an increase of the negative charge compared to native Y-HDL. HDL
Electrophoresis was carried out in barbital buffer at pH 8.6 on 0.6% agarose gels (Helena Lab., Montreal, Québec). The gels were stained with 0.1% (w/v) Fat Red 7B in 95% methanol.

ApoA-I-bound carbonyl content was assayed as described by Levine et al. (27). Briefly, apoA-I was purified from HDL as previously described (27). The carbonyl content were determined by dinitrophenylhydrazine derivatisation. ApoA-I-bound carbonyls were detected in TCA-precipitable materials by absorbance at 370 nm ($\varepsilon=22000 \text{ M}^{-1}\text{cm}^{-1}$).

**HDL fluidity**

Lipoprotein fluidity was determined by steady-state anisotropy of DPH as described previously (28). Fluidity represents the inverse values of anisotropy and is expressed as: $1/r$: steady-state fluorescence anisotropy. $(r)$: was calculated as $[(I_v - GI_p) / (I_v - 2GI_p)]$ where $I_v$ and $I_p$ are the parallel and perpendicular polarized fluorescence intensities and $(G)$ is the monochromator grating correction factor.

**HDL Phospholipid analysis**

Total lipids were extracted using a modified Folch’s method (29). One hundred µl of lipid extract were then injected in a HPLC system coupled to evaporative light scattering detector (ELSD) according to the Becart’s method (30). Solvent eluents was prepared following a binary gradient (A: chloroform/methanol/ammonium hydroxide at 30 %: 80/19.5/0.5. B: chloroform/methanol/water/ammonium hydroxide at 30 %: 60/34/5.5/0.5).

**Statistical analysis**

Values are expressed as the mean ± s.e.m (standard error of the mean). One-way analysis of variance (ANOVA) was used for multiple comparisons. Linear regression analysis was used to assess the association between two continuous variables and the $t$-test was used to assess the comparison between two groups. Statistical analyses were performed using Prism v4.0.
Results

Effect of aging on HDL-mediated cholesterol efflux

The biochemical characteristics of participating subjects are reported in Table I. The two age groups had no significant differences in their body mass index, total as well as LDL and HDL cholesterol. ApoA-I concentrations were in the same range for young and elderly subjects and the acute inflammation phase protein C-reactive protein (CRP) was below detection levels for both age groups (< 3 mg/L).

To examine the effect of aging on the anti-atherogenic activities of HDL and particularly their ability to promote RCT, we assessed the capacity of HDL obtained from young (Y-HDL) and elderly (E-HDL) healthy subjects to enable the cholesterol efflux. A time course (0-48 hours) on cholesterol efflux revealed differences between Y-HDL and E-HDL that were manifested with 50 µg/ml of HDL after 24 hours of incubation (Figure 1). In fact, when Y-HDL and E-HDL were incubated for 24 hours with THP-1 macrophages pre-loaded with radiolabled [3H]-cholesterol, cholesterol efflux promoted by Y-HDL was significantly higher by 14.9 % (p<0.05) than that promoted by E-HDL. The amount of cholesterol efflux promoted by Y-HDL and E-HDL were dependent on the incubation time of incubation with macrophages and reached a maximum after 24 hours of incubation, that is in agreement with previous results published by Nagano et al. (31). The measure of cholesterol mass in the media and in cells also demonstrated a higher significant capacity of Y-HDL to mediate net cholesterol efflux than E-HDL (p=0.023).

To gain more insight into the effect of aging on HDL-mediated cholesterol efflux, we assessed the capacity of different HDL subfractions in promoting cholesterol efflux, in particular HDL2 and HDL3. Under our conditions, HDL2 and HDL3 were isolated by ultra-centrifugation which excluded the presence of preβ1-HDL in our preparations and thus eliminating its possible role in the difference of the RCT capacity between young and elderly (32,33).

We subsequently sought to determine first, which of HDL subfractions (HDL2 or HDL3) were more implicated in the HDL-mediated cholesterol efflux from macrophages; and second, the effect of aging in this process. Whole HDL, HDL2 and HDL3 isolated from both young and elderly subjects were incubated separately for 24 hours with [3H]-cholesterol-THP-1 loaded macrophages. Y-HDL3 induced significantly a higher [3H]-free cholesterol efflux (9.7 % higher; p<0.05) than that induced by Y-HDL2, whereas no differences were apparent between E-HDL3 and E-HDL2 (Figure 1). When regarded as a function of aging, Y-HDL3 showed a higher
capacity in promoting cholesterol efflux than E-HDL₃ (11.1% higher; \(p<0.05\)) with no observed variations between Y-HDL₂ and E-HDL₂.

**Effect of aging on ABCA-1-mediated cholesterol efflux pathway**

To clarify which of the cholesterol efflux pathways were more affected with aging, we investigated the mechanism based on the ABCA-1 protein by using a second macrophage cell line, J774. J774 cells express less ABCA-1 compared to THP-1 macrophages (9) that is confirmed by our results (Figure 2A).

Interestingly, when Y-HDL and E-HDL (50 µg/ml) were incubated separately with \[^3\text{H}]\)-cholesterol-loaded J774 cells during 24 h there was no effect of aging on the capacity of either HDL in promoting cholesterol efflux compared with the THP-1 cells (Figure 2B). Furthermore, we chemically-induced over-expression of ABCA-1 by J774 in order to analyse the effect of aging on the ABCA-1 cholesterol efflux related pathway. Our results indicate that J774 cells stimulated with AMPc have a robust expression of ABCA-1 (> 9-fold) compared to non-stimulated cells (Figure 3A). Interestingly, the modulation of expression of ABCG-1 by AMPc was less than 1.8-fold.

The chemical-induced expression of ABCA-1 in J774 cells by AMPc (21,34,35), increased significantly whole HDL and HDL₃ mediated cholesterol efflux in both young and elderly subjects (Figure 3B), whereas no differences were observed in HDL₂ related cholesterol efflux as a function of aging (results not shown). Moreover, in ABCA-1-enriched J774 cells, Y-HDL increased cholesterol efflux by (21.7 %, \(p<0.05\)) compared to E-HDL, whereas Y-HDL₃ enhanced the cholesterol efflux by (26.1 %, \(p<0.001\)) when compared to E-HDL₃ (Figure 3B).

ApoA-I purified from young (Y-apoA-I) and elderly subjects (E-apoA-I) were also evaluated for their capacity to promote cholesterol efflux (Figure 3C). Our results do not shown an effect of aging on the apoA-I-dependant cholesterol efflux from control J774 cells. However, in J774 ABCA-1-enriched cells, E-apoA-I was significantly less efficient to mediate cholesterol efflux than Y-apoA-I (\(p = 0.0048\)) (Figure 3C).

**ApoA-I structure and HDL cholesterol efflux capacity**

The ABCA-1-mediated cholesterol efflux pathway is dependent on the interaction of apoA-I with the ABCA-1 receptor. Thus, alteration or modification of apoA-I protein could modulate the HDL cholesterol efflux capacity. For this purpose, we used SDS-PAGE to compare the apoA-I modification level, as a function of aging. Interestingly, for the same HDL concentration
expressed in terms of total protein contents, Y-HDL demonstrated a higher apoA-I band intensity than E-HDL (Figure 4A).

Submission of Y-HDL and E-HDL to oxidative stress induced by copper ions and followed by the measurement of apoA-I by electrophoresis resulted in a reduction in the apoA-I band intensity as shown in Figure 4A. At higher oxidative stress conditions (4 and 8 hours), the apoA-I band is dramatically reduced as consequence of the oxidative modifications. The relative electropheretic mobility (Figure 4B and Table II) also demonstrated an alteration of the apoA-I charge as a function of aging and HDL oxidation intensity. In addition, our results show that protein carbonyl content was 38 % higher in E-apoA-I than in Y-apoA-I (1.1 ± 0.1 vs. 1.7 ± 0.1 μM, p=0.04) (see supplementary data).

Oxidative modification of Y-HDL and E-HDL induces a significant reduction of HDL capacity to promote cholesterol efflux (Figure 4C). It is noteworthy that for the same level of oxidation (incubation time of 0 and 1 hour), Y-HDL show has higher capacity to promote cholesterol efflux than E-HDL (p<0.05) (Figure 4C).

Interestingly, comparison of HDL oxidation kinetics as a function of age of donors show an increased susceptibility of HDL to lipid peroxidation with aging as measured by the lag-phase for conjugated diene and MDA formation (Table III).

**Effect of aging on ABCA-1/apoA-I-independent pathway for cholesterol efflux**

To investigate the influence of other HDL components on cholesterol efflux capacity as a function of aging, we evaluated the HDL phospholipid contents and performed fluorescence anisotropy assays as an indirect measurement for the HDL phospholipid bilayer fluidity.

For the phospholipid contents, we focused on the measurement of the HDL phosphatidylcholine/sphingomyelin (PC/SPM) ratio. This parameter was previously shown to influence significantly the HDL-mediated cholesterol efflux capacity (12, 36) as an apoA-I/ABCA-1-independent pathway. Figure 5A describes a significant decrease in the PC/SPM ratio in whole HDL obtained from elderly subjects compared to those obtained from young subjects (p=0.029). Similar results were obtained for E-HDL3 compared to Y-HDL3 (p=0.026) (Figure 5A).

With fluorescence anisotropy Y-HDL was found to have a higher and significant phospholipidic-layer fluidity than E-HDL (p<0.001). The same results were obtained for Y-HDL2 (p=0.035) and Y-HDL3 (p=0.023) when compared to the same HDL subfractions from elderly subjects (Figure 5B).
Discussion

The severity of atherosclerosis and the incidence of its clinical manifestations increase dramatically with aging and are responsible for the majority of cardiovascular morbidity and mortality in the elderly. High plasma level of HDL is associated with a decreased risk of developing atherosclerosis, an effect commonly attributed to their central role in RCT. Indeed, efflux of free cholesterol from cell membranes to extra-cellular acceptors is considered to represent one of the first steps in the process of RCT.

Aging is characterized by the occurrence of several physical and biochemical modifications, which affect HDL structure and functions (18,37,38). Previous studies from our laboratory have shown an increase of HDL susceptibility to lipid peroxidation and a decrease of the HDL antioxidant activity with aging (18,19). We investigated the effect of aging on the RCT process by studying the anti-atherogenic properties of HDL. Our results showed a significant reduction of the whole HDL capacity to promote cholesterol efflux from macrophages. To determine which HDL sub-fractions were more affected, HDL2 and HDL3 were isolated and evaluated for their capacity to mediate cholesterol efflux. Interestingly, the reduction in the cholesterol efflux, with aging, was significant only for HDL3. Moreover, in the young subject group, a difference in the capacity of HDL subfractions (Y-HDL2 and Y-HDL3) to promote cholesterol efflux was demonstrated (Y-HDL3, \( p=0.019 \)). However, no significant difference was observed between E-HDL2 and E-HDL3. The ability of HDL3 to increase cellular cholesterol efflux has been attributed to the increased presence of lecithin cholesterol acyl transferase (LCAT) (39), greater phospholipidic bilayer-fluidity, higher cholesteryl ester content, elevated linoleic to linolenic acid ratio in phospholipids and smaller size (40). Moreover, ABCA-1-transporters by binding to and cross-link lipid-poor apoA-I is another parameter that might explain the difference, between the Y-HDL and E-HDL cholesterol promoting efficiencies. The idea of carrying out our experiments in more than one cell types was to confirm the implication of ABCA-1 receptor in the dissimilarity between Y-HDL and E-HDL and especially between E-apoA-I and Y-apoA-I to promote cholesterol efflux as a function of aging. Indeed, when J774-control cells were used no difference was observed between Y-HDL and E-HDL and between E-apoA-I and Y-apoA-I in RCT activity, which is not the case with THP1 macrophages. This could be attributed to the difference in the expression of ABCA-1 receptors between the two cell lines or to differences in the release of lipid-free apoA-I from the two HDL samples. Moreover, evidence indicates that ABCA-1 forms a high affinity complex with apoA-I by binding amphipathic helices within the
apolipoprotein (14, 41). Oxidative modifications of apoA-I that might occur with aging may affect the ABCA-1/apoA-I interaction leading to the reduction of cholesterol efflux.

ApoA-I availability, which can be affected by a reduction in its concentration as well as a structural alteration, may affect the cholesterol efflux capacity. SDS-PAGE analysis showed a reduction in the apoA-I band intensity in E-HDL compared to Y-HDL, that suggests a reduction in apoA-I concentration in the elderly as has been recently demonstrated (37). However, for the electrophoresis experiments, Y-HDL as well as E-HDL were used at the same protein concentration (50 µg/ml), which exclude the hypothesis attributing the reduction in the apoA-I band intensity to a reduction of its concentration. Indeed, no significant change in the apoA-I concentration was noted between young and elderly subjects (Table I). Interestingly, HDL incubation with copper ions induces a significant reduction of apoA-I band intensity (Figure 4A) and an increase in the apoA-I negative charge. For the same incubation time, apoA-I oxidative modifications with copper ions were more pronounced for E-HDL than Y-HDL. Taken together, these results, suggest that the basal alteration of apoA-I structure as reflected by the decreased band intensity and charge as well as the increase in bound carbonyls, might be reflective of age-related oxidative stress conditions. Moreover, they might explain, at least in part, the difference as a function of aging in the ABCA-1-mediated cholesterol efflux capacity of HDL.

Furthermore, our results show that in both young and elderly subjects the capacity of HDL to remove free cholesterol excess from macrophages decreases significantly with HDL higher levels of HDL oxidation. Interestingly, for the same level of HDL peroxidation, cholesterol efflux capacity was higher for Y-HDL than E-HDL. Moreover, HDL oxidation induces an apoA-I alteration and decreases HDL-phospholipidic layer fluidity.

Besides the expression levels of ABCA-1, cholesterol efflux from macrophages is also dependent on the biophysical and biochemical structure of HDL subfractions. Contrarily to the ABCA-1-mediated cholesterol efflux, the other implicated mechanisms, the ABCG1 and ABCG4, the spontaneous transfer and the SR-BI pathways all involve cholesterol transfer to phospholipids-containing species principally to HDL2 (42). One might thus expect that alteration or change in the HDL phospholipid composition or structure would affect RCT (42-44). Analysis of the HDL, HDL2 and HDL3 phospholipid composition as a function of donor age of showed a significant decrease in the PC/SPM ratio in the elderly. The increase of phosphatidylcholine was demonstrated to modulate positively the RCT (45). These results corroborated by the measure of
reduced HDL-fluidity that might be attributed in part to the oxidative modifications, which occur with aging as previously shown for apoA-I.

HDL-mediated cholesterol efflux capacity has been demonstrated to be reduced in diabetes (46), in subjects with familial HDL deficiency (47) and in Tangier disease (48) and has been associated with an alteration in HDL concentrations, distribution or composition and structure. In vitro studies have shown that HDL-mediated cholesterol efflux is impaired by whole cigarette smoke extracts through lipid peroxidation (49). Furthermore, cholesterol efflux could be affected by the inactivation of enzymes contained within HDL particularly paraoxonase 1 and LCAT. Rosenblat et al. (35), have demonstrated a function of paraoxonase 1 in reverse cholesterol efflux from macrophages. Interestingly, a study from our laboratory has shown a significant reduction of PON1 activity in the elderly (19).

Moreover, unlike ABCA-1, the ABCG1 and ABCG4 stimulate cholesterol efflux especially to both HDL$_2$ and HDL$_3$ but not to lipid-poor apoA-I (50). In this case, the HDL physico-chemical changes that occur during aging may directly affect the interaction between HDL subfractions and ABCG1/4 leading to an attenuation of cholesterol efflux. The possible relationship between ABCG1/4 and cholesterol efflux during aging warrants a further investigation. In addition to these ATP-dependent transporters, Ji et al. (51), have showed that SR-BI promotes a bi-directional flux between cells and HDL, and also facilitates net cholesterol efflux to phospholipid-rich and cholesterol-poor HDL, but not to lipid-poor apoA-I. However, Zhang et al. (52), demonstrated that SR-BI knockout macrophages display no difference in cholesterol efflux to HDL compared with wild type macrophages suggesting that SR-BI dose not have a major role in cholesterol efflux to HDL.

In summary, our results have shown an impairment of HDL-mediated RCT capacity with aging which has been attributed to a reduction in ABCA-1 pathway. Based on these data, we postulate that the apoA-I/ABCA-1-dependant cholesterol efflux is the more affected pathway with aging and is du principally to the oxidative modifications of apoA-I that might occur during aging. However, the HDL biophysical and biochemical changes are also contributing factors in the reduction of its capacity to mediate cholesterol efflux in the elderly. Although, the obtained results do not lead us to determine the proportion of the cholesterol efflux which could be attributed to ABCG1/4 or SR-BI, we hypothesize that HDL structure and composition alterations could also affect these pathways of cholesterol efflux. In conclusion, the reduced capacity of
elderly-derived HDL to promote cholesterol efflux may explain the increase of the incidence of cardiovascular diseases observed during aging.
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References


12. Yancey, P.G., de la Llera-Moya, M., Swarnakar, S., Monzo, P., Klein, M.S., Connelly, A.,
phospholipid composition is a major determinant of the bi-directional flux and net
movement of cellular free cholesterol mediated by scavenger receptor BI. *J. Biol. Chem.*
**275:** 36596-36604.

T.G., Eggerman, T.L., Patterson, A.P., Duverger, N.J., Santamarina-Fojo, S and Brewer,

inducible apolipoprotein receptor that mediates cholesterol secretion from macrophages. *J.
Biol. Chem.* **275:**34508-11.


16. Sola, R., Baudete, M.F., Motta, C., Maille, M., Boisnier, C., Jacotot, B. 1990. Effects of
dietary fats on the fluidity of human high-density lipoprotein: influence of the overall

17. Bennefont-Rousselot, D., Motta, C., Khalil, A., Sola, R., La Ville, A.K., Delattre, J., Gardés-
Albert, M. 1995. Physiochemical changes in human high density lipoproteins (HDL)

high-density lipoproteins (HDL) to in vitro oxidation induced by gamma-radiolysis of water.
*FEBS Lett.* **435:** 153-158.

19. Jaouad, L., de Guise, C., Berrougui, H., Cloutier, M., Isabelle, M., Fulop, T., Payette, H and
Khalil, A. 2006. Age-related decrease in high-density lipoproteins antioxidant activity is due
to an alteration in the PON1's free sulfhydyl groups. *Atherosclerosis.* **185:** 191-200.

their peroxidation by high-performance liquid chromatography postcolumn

phosphorylation activity and promote cholesterol efflux from fibroblasts. *J. Lipid Res.*
**43:**2087-2094.


Legends

Figure 1.
Effect of aging on HDL mediated cholesterol efflux. Whole HDL, HDL2 and HDL3 (50 µg/ml) were isolated from young and elderly subjects and were incubated with [3H]-cholesterol loaded THP-1 macrophages for 24 h. Cholesterol efflux was determined by measuring [3H]-cholesterol recuperated by HDL. Results are expressed as the mean ± SEM (n>4; * denotes p<0.05).

Figure 2.
A- ABCA-1 protein expression level in THP-1 and J774 macrophage. Protein lysate(20 µg) of each lysed cells line were loaded onto SDS-PAGE (7.5 %) in reduced conditions and immunobloted with ABCA-1 specific antibodies. Quantitative analysis was determined using densitometry from 3 independent experiments. ** denotes p<0.01.
B- Comparison of HDL-mediated cholesterol efflux from THP-1 and J774 macrophages. THP-1 and J774 macrophage either loaded with [3H]-cholesterol were incubated for 24 h with Y-HDL and E-HDL (50 µg/ml). Results are expressed as the mean ± SEM of triplicate determinations (n = 4). [Y-HDL in THP-1 cells vs. Y-HDL in J774 cells. *** denotes p<0.001]; [E-HDL in THP-1 cells vs. E-HDL in J774 cells. ### denotes p<0.001]

Figure 3.
Involvement of the ABCA-1 receptor in HDL-mediated cholesterol efflux in J774 cells. J774 macrophages were incubated for 12 h at 37 °C without (control cells) or with 0.3 mM of 8-Br-cAMP. (A) AMPc regulates mRNA expression of ABCA-1 and ABCG-1 in J774 cells. One representative PCR analysis of ATP-binding cassette A1 (ABCA-1) and G1 (ABCG-1), and β-actin. Graph representing transcriptional expression of ABCA-1 and ABCG1. β-actin was used as an internal control. Expression of ABCG-1 is slightly regulated following AMPc stimulation (<2-fold) compared to ABCA-1 that is highly regulated (>9-fold), as compared to control cells (non-stimulated). Asterisks denote significance for ABCA-1 (* p=0.034) and ABCG-1 (*** p=0.0009).
(B) Stimulated or non-stimulated J774 cells were incubated during 24 h with whole (HDL) or HDL subfraction (HDL3) isolated from young (Y-HDL) or elderly (E-HDL) subjects (50 µg/ml).
ApoA-I-mediated cholesterol efflux between ABCA-1-enriched and control cells represents the specific ABCA-1-related cholesterol efflux. Cells were treated apoA-I (50 μg/ml) purified from young (Y-apoA-I) and elderly (E-apoA-I) subjects. Results represent the mean ± SEM of more than three different experiments.

**Figure 4.**

A- SDS-PAGE profile of apoA-I at basal and during HDL-oxidation. Y-HDL and E-HDL (200 μg/ml) were incubated with 10 μM CuSO₄ for (0, 1, 4 and 8 h). Experiments were realised in triplicate and the gel shown is typical of the results obtained. Results are also analyzed by densitometry and represented as the mean ± SEM of three-separated experiments. (Densitometrical-data of Young and elderly for the same time of oxidation were compared). **p<0.01, ***p<0.001.

B- Relative electrophoretic mobility analysis of HDL. Y-HDL and E-HDL (200 μg/ml) were incubated with 10 μM CuSO₄ for (0, 1, 4 hours). HDL electrophoresis was carried out in barbital buffer at pH 8.6 on 0.6% agarose gels. The gels were stained with Fat Red 7B, 0.1 in 95% methanol.

C- Involvement of apoA-I protein in HDL-mediated CE. [³H]-cholesterol loaded-THP-1 macrophages were incubated for 24 h with 50 μg of native or oxidised Y-HDL or E-HDL. Cholesterol efflux was determined as mentioned above. Results are expressed as the mean ± SEM of triplicate determinations. *p<0.05.

**Figure 5.**

A- Effect of aging on the phospholipid composition of whole HDL and HDL subfractions (HDL₂ and HDL₃) isolated from young and elderly patients. Results are given as a phosphatidylcholine (PC) and sphingomyeline (SPM) ratio (PC/SPM).

B- Effect of aging on the fluidity of phospholipidic bi-layer of whole HDL, HDL₃ and HDL₂, isolated from young and elderly subjects. Results are expressed as the mean ± SEM (n = 4 - 7). *p<0.05, ***p<0.001.
Table legends

Table I

Demographic and biochemical parameters of the participating subject in the present study.

Table II

HDL relative electrophoretic mobility as a function of aging (Y-HDL vs. E-HDL) and HDL oxidation intensity (0, 1, 2 and 4 hours). Data are expressed as the relative electrophoretic mobility of each band compared to that of Y-HDL band (HDL obtained from young subjects). Experimental conditions are the same as in Figure 4B.

Table III

Effect of aging on the susceptibility of HDL to lipid peroxidation. HDL were isolated from young and elderly subjects and were oxidised by time course incubation with 10 µM CuSO₄. Lag-phase was expressed as the time below which little or no conjugated diene or MDA formation was detected. Significance is calculated in comparison of young subjects. Results are expressed as the mean ± S.E.M. of triplicate or more determinations.
Figure 1

Cholesterol efflux (%) for different HDL fractions:
- Y-HDL
- E-HDL
- Y-HDL$_2$
- Y-HDL$_3$
- E-HDL$_2$
- EHDL$_3$

Significance indicated with asterisks (*), suggesting statistical differences among the groups.
Figure 2

A

ABCA-1

Densitometry Analysis

**

B

THP-1

J774

HDL-Mediated cholesterol efflux (%)

***

Y-HDL E-HDL Y-HDL E-HDL

0 10 20 30 40 50 60 70

# # #

THP1

J774

Y-HDL E-HDL Y-HDL E-HDL

0 10 20 30 40 50 60 70
**Figure 3**

**A**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>AMPc</th>
</tr>
</thead>
<tbody>
<tr>
<td>rt-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rt+</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**ABCA1 >**

**ABCG1 >**

**β-actin >**

**Fold expression**

- **Control**
- **Stimulated (AMPC)**

**B**

**HDL-Mediated Cholesterol efflux (%)**

- **Y-HDL**
- **E-HDL**

**C**

**Cholesterol efflux (%)**

- **Y-apoA-I**
- **E-apoA-I**

**P-values**

- **P=0.024**
- **P=0.3**
- **P<0.001**
- **P=0.0048**
- **ns**
Figure 4

A

Densitometry Analysis

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Young (Y-apoA1)</th>
<th>Elderly (E-apoA1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>75</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>50</td>
<td>0</td>
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<tr>
<td>8h</td>
<td>25</td>
<td>0</td>
</tr>
</tbody>
</table>

B

E-HDL

Y-HDL

Hours

0 1 2 4

0 1 2 4
Figure 4

C

HDL-Mediated cholesterol efflux (%)

Time (hours)

Young
elderly

*
Figure 5

A

Ratio (PC/SPM)

Y-HDL  E-HDL  Y-HDL₃  E-HDL₃  Y-HDL₂  E-HDL₂

P=0.029

P=0.027

P=0.056

B

Fluidity

(1/Fluorescence anisotropy)

Y-HDL  E-HDL  Y-HDL₃  E-HDL₃  Y-HDL₂  E-HDL₂

***

*
<table>
<thead>
<tr>
<th></th>
<th>Young</th>
<th>Elderly</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Subjects</strong> n, (M/F)</td>
<td>8 (4/4)</td>
<td>9 (4/5)</td>
</tr>
<tr>
<td>Average age (year)</td>
<td>25.7 ± 0.89</td>
<td>67.9 ± 1.63</td>
</tr>
<tr>
<td>BMI, Kg/m2</td>
<td>22.7 ± 3.57</td>
<td>23.7 ± 4.61</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>4.56 ± 0.22</td>
<td>5.11 ± 0.23</td>
</tr>
<tr>
<td>HDL, mmol/l</td>
<td>1.33 ± 0.14</td>
<td>1.42 ± 0.11</td>
</tr>
<tr>
<td>Chol/HDL</td>
<td>3.57 ± 0.32</td>
<td>3.47 ± 0.32</td>
</tr>
<tr>
<td>LDL, mmol/l</td>
<td>2.85 ± 0.23</td>
<td>2.83 ± 0.2</td>
</tr>
<tr>
<td>TG, mmol/l</td>
<td>1.11 ± 0.13</td>
<td>1.42 ± 0.13</td>
</tr>
<tr>
<td>ApoA-I g/l</td>
<td>1.23 ± 0.29</td>
<td>1.20 ± 0.25</td>
</tr>
<tr>
<td>CRP mg/l</td>
<td>&lt;3.0</td>
<td>&lt;3.0</td>
</tr>
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</table>

Values are mean ± SEM.
Table II

<table>
<thead>
<tr>
<th>HDL relative electrophoretic mobility (compared to Y-HDL)</th>
<th>Y-HDL</th>
<th>E-HDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>At basal conditions (0 hour)</td>
<td>1</td>
<td>1.19 ± 0.02</td>
</tr>
<tr>
<td>Incubation with Cu(^{2+}) for 1 h</td>
<td>1.15 ± 0.01</td>
<td>1.45 ± 0.05</td>
</tr>
<tr>
<td>Incubation with Cu(^{2+}) for 2 h</td>
<td>1.28 ± 0.01</td>
<td>2.19 ± 0.04</td>
</tr>
<tr>
<td>Incubation with Cu(^{2+}) for 4 h</td>
<td>3.31 ± 0.03</td>
<td>3.66 ± 0.04</td>
</tr>
</tbody>
</table>
Table III.

<table>
<thead>
<tr>
<th></th>
<th>Young</th>
<th>Elderly</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lag Phase (CD)</td>
<td>0.608 ± 0.05</td>
<td>0.375 ± 0.025</td>
<td>0.007</td>
</tr>
<tr>
<td>Lag Phase (MDA)</td>
<td>0.841 ± 0.03</td>
<td>0.521 ± 0.025</td>
<td>&lt;0.0001</td>
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</tbody>
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