Age-related impairment of HDL-mediated cholesterol efflux

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Abstract Our aim in this study was to investigate the effect of aging on the capacity of HDLs to promote reverse cholesterol transport. HDLs were isolated from plasma of young (Y-HDL) and elderly (E-HDL) subjects. HDL-mediated cholesterol efflux was studied using THP-1 and J774 macrophages. Our results show that E-HDLs present a lower capacity to promote cholesterol efflux than Y-HDLs (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 ABCA1-mediated cholesterol efflux is the more affected pathway in terms of cholesterol-removing capacity. Interestingly, the composition and structure of HDL revealed a reduction in the phosphatidylcholine-sphingomyelin ratio (E-HDL, 32.7 ± 2.7 vs. Y-HDL, 40.0 ± 1.9; $P = 0.029$) and in the phospholipidic layer membrane fluidity in E-HDL compared with Y-HDL as well as an alteration in the apolipoprotein A-I structure and charge. In conclusion, our results shown that E-HDLs present a reduced capacity to promote cholesterol efflux, principally through the ABCA1 pathway, and this may explain the increase of the incidence of cardiovascular diseases observed during aging.—Berrougui, H., M. Isabelle, M. Cloutier, G. Grenier, and A. Khalil. **Age-related impairment of HDL-mediated cholesterol efflux.** ***J. Lipid Res.*** **2007.** 48: **328–336.**

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

The concept of RCT, as suggested by Glomset (7), consists of the 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 preβ-migrating lipid-poor or lipid-free apolipoprotein A-I (apoA-I) and to HDL_3 (7–9). The process of FC efflux occurs by three known pathways. *1*) Aqueous diffusion. This process involves the 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 class B type I (SR-BI)-mediated FC flux. The movement of FC via SR-BI is bidirectional, and like the aqueous diffusion mechanism, the net movement of FC via SR-BI depends on the direction of the cholesterol gradient (10). *3*) ATP binding cassette-mediated cholesterol efflux. ABCA1 and ABCG1/4 are members of a large family of ATP-dependent 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 ABCA1 and ABCG1/4 is unidirectional, and net efflux of cellular FCs would always occur via this mechanism (12). The preferred cholesterol acceptors for ABCA1 are lipid-poor apolipoproteins and all of the exchangeable apolipoproteins, such as apoA-I, apoA-II, apoA-IV, apoE, and apoC (13). ABCA1 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 was shown that another transporter, ABCG1, promotes mass cholesterol efflux from cells to mature HDL particles (HDL_2 and HDL_3) but not to lipid-poor apoA-I (5,6). Both ABCA1 and ABCG1 are abundant in macrophages, especially after cholesterol loading, suggesting their importance for the cholesterol efflux process.

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Cholesterol efflux was also correlated to 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 the lipoprotein 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, the formation of peroxidation-derived lipid products is associated with changes of the physicochemical properties of HDL and especially a decrease in the fluidity of the HDL phospholipid layer (17).

In previous studies, we have demonstrated that HDLs 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 with the aim of elucidating the 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 cardiovascular disease.

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, Québec, Canada). 1,1,3,3,-Tetraethoxypropane, D-α-tocopherol, γ-tocopherol, butylated hydroxytoluene, $CuSO_4$, EDTA, lithium perchlorate, 1,6-diphenyl-1,3,5-hexatriene, PC, SPM, 8-(4-chlorophenylthio)adenosine 3′,5′-cyclic monophosphate (cAMP), and [^{3}H]cholesterol were obtained from Sigma (St. Louis, MO). THP-1 and J774 cells were purchased from the American Type Culture Collection (Manassas, VA).

Subjects

Plasma was obtained from healthy volunteers (eight young, aged 20–30 years, and nine elderly, aged 65–70 years). Their demographic data are shown in **Table 1**. All subjects were considered healthy because they were normolipidemic and had 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 antioxidant supplementation was used. They were all nonsmokers, and none of the women was currently taking estrogen replacement therapy for menopause. The elderly subjects were living at home, functionally independent, and cognitively intact (Mini Mental State Examination > 28). The Ethics Committee of the Sherbrooke Geriatric University Institute approved the study, and all subjects gave written informed consent.

TABLE 1. Demographic and biochemical parameters of the study subjects

Parameters	Young	Elderly
Subjects, n (male/female)	8 (4/4)	9 (4/5)
Average age, years	25.7 ± 0.89	67.9 ± 1.63
Body mass index, kg/m^2	22.7 ± 3.57	23.7 ± 4.61
Total cholesterol, mmol/l	4.56 ± 0.22	5.11 ± 0.23
HDL, mmol/l	1.33 ± 0.14	1.42 ± 0.11
HDL cholesterol, mmol/l	3.57 ± 0.32	3.47 ± 0.32
LDL, mmol/l	2.85 ± 0.23	2.83 ± 0.2
Triglycerides, mmol/l	1.11 ± 0.13	1.42 ± 0.13
Apolipoprotein A-I, g/l	1.23 ± 0.29	1.20 ± 0.25
C-reactive protein, mg/l	<3.0	<3.0

Values are means ± SEM.

Lipoprotein isolation

Human plasma was collected in citrate tubes, and HDL isolations were performed immediately according to the method of Sattler, Mohr, and Stocker (20). Isolated lipoproteins were dialyzed overnight at 4°C in 10^{-2} M sodium phosphate buffer (pH 7.0), and then the protein concentrations were measured by commercial assay (Bio-Rad, Mississauga, Ontario, Canada).

ApoA-I purification

ApoA-I was purified from HDLs obtained from young and elderly subjects according to the method of Haidar et al. (21). Briefly, HDLs were delipidated in acetone-ethanol solution, and the protein fractions were subjected to chromatographic separation using two Sephacryl S-200 columns (Amersham Biosciences AB, Uppsala, Sweden). ApoA-I pure fractions were pooled and dialyzed in 100 mM NH_4HCO_3, 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. The media were supplemented with 10% heat-inactivated FBS, 2-β-mercaptoethanol (only for THP-1; 50 μM), 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 1.0×10^5 cells/cm^2 in the presence of 100 nM phorbol myristate acetate 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. Labeled cells were washed and then incubated in serum-free medium containing 1% BSA for 24 h of equilibration. The cells were then washed and subjected to different treatments: *1*) fresh medium without HDL (control); *2*) fresh medium containing 50 μg/ml HDL; and *3*) fresh medium containing 50 μg/ml HDL_3 or HDL_2, obtained from young and elderly subjects.

In another series of experiments, J774 macrophages were labeled 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 cAMP for 12 h to yield ABCA1-enriched cells (22). Then, the cells were washed and further incubated for 4 h at 37°C in the presence of whole HDL, HDL subfractions (HDL_3 and HDL_2), or apoA-I obtained from young and elderly subjects.

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

Net cholesterol efflux was also assayed by measuring cell cholesterol mass content. Briefly, cellular lipids were extracted from 2×10^6 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 were measured by HPLC according the method of Katsanidis and Addis (23).

Western blotting and PCR analysis

Twenty five micrograms of cell lysate proteins was loaded per line and separated by 7.5% SDS-PAGE. After transfer, membranes were incubated with specific antibodies against ABCA1 (Santa Cruz Biotechnology, Santa Cruz, CA). Anti-goat IgG-HRP antibody (Santa Cruz Biotechnology) 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 using the GeneAmp kit (Applied Biosystems). Reverse-transcribed RNA was used for PCR analysis with the following primer sequences (F, forward; R, reverse): β-actin (F, 5′-CACCCTGTGCTGCTCACCGAGGCC-3′; R, 5′-ACCGCTCGTTGCCAATAGTGATGA-3′); ABCA1 (F, 5′-GCCCTACTGTCGGTTGAC-3′; R, 5′-TGGGCAACACTGAACAAGAG-3′); and ABCG1 (F, 5′-TCAACAGTGGAGAGCTGGTG-3′; R, 5′-CTGCCTTCATCCTTCTC-3′). All reactions were run at least in triplicate.

Copper-mediated HDL oxidation

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

Conjugated diene and thiobarbituric acid-reactive substance evaluation

HDL peroxidation was monitored continuously at 234 nm to detect the formation of conjugated diene, as described previously (17). Thiobarbituric acid-reactive substances, mainly malondialdehyde, were assayed by HPLC coupled to fluorescence detection (25).

ApoA-I modification

ApoA-I was analyzed as described by Laemmli (26) on 12.5% SDS-PAGE gels (Mini Gel II; Bio-Rad, Hercules, CA). Each lane was loaded with 8 μg of 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 values of >1 indicate an increase of the negative charge compared with native Y-HDL. HDL electrophoresis was carried out in barbital buffer at pH 8.6 on 0.6% agarose gels (Helena Laboratories, Montreal, Québec, Canada). 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 described previously (27). The carbonyl content were determined by dinitrophenylhydrazine derivatization. ApoA-I-bound carbonyls were detected in TCA-precipitable materials by absorbance at 370 nm ($\varepsilon = 22{,}000\ M^{-1}\ cm^{-1}$).

HDL fluidity

Lipoprotein fluidity was determined by steady-state anisotropy of 1,6-diphenyl-1,3,5-hexatriene as described previously (28). Fluidity represents the inverse values of anisotropy and is expressed as 1/r (for 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 method of Folch, Lees, and Sloane-Stanley (29). One hundred microliters of lipid extract was then injected into an HPLC system coupled to an evaporative light-scattering detector according to the method of Becart, Chevalier, and Biesse (30). Solvent eluents were prepared according to 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 means ± SEM. One-way 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 version 4.0.

RESULTS

Effect of aging on HDL-mediated cholesterol efflux

The biochemical characteristics of participating subjects are reported in Table 1. The two age groups had no significant differences in their body mass index, total cholesterol, or 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 was below detection levels for both age groups (<3 mg/l).

To examine the effect of aging on the antiatherogenic 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 cholesterol efflux. A time course (0–48 h) on cholesterol efflux revealed differences between Y-HDL and E-HDL that were manifested with 50 μg/ml HDL after 24 h of incubation (**Fig. 1**). In fact, when Y-HDL and E-HDL were incubated for 24 h with THP-1 macrophages preloaded with radiolabled [^{3}H]cholesterol, cholesterol efflux promoted by Y-HDL was significantly higher by 14.9% ($P < 0.05$) than that promoted by E-HDL. The amounts of cholesterol efflux promoted by Y-HDL and E-HDL were dependent on the incubation time with macrophages and reached a maximum after 24 h of incubation, this is in agreement with previous results published by Nagano, Arai, and Kita (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 to promote cholesterol efflux, in particular HDL_2 and HDL_3. Under our conditions, HDL_2 and HDL_3 were isolated by ultracentrifugation, which

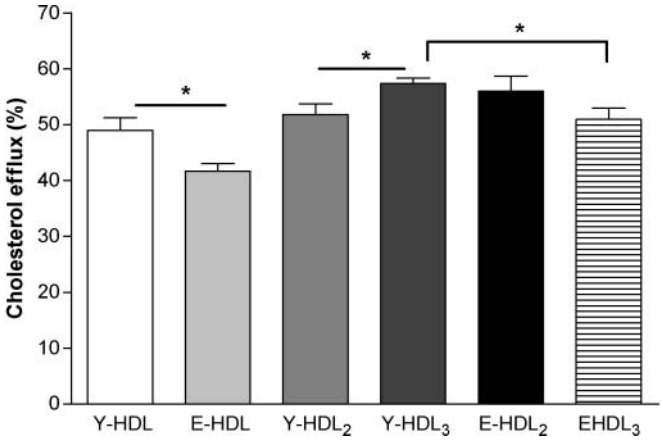

Fig. 1. Effects of aging on HDL-mediated cholesterol efflux. Whole HDL, HDL_2, and HDL_3 (50 μg/ml) were isolated from young (Y-HDL) and elderly (E-HDL) subjects and incubated with [^{3}H]cholesterol-loaded THP-1 macrophages for 24 h. Cholesterol efflux was determined by measuring [^{3}H]cholesterol recuperated by HDL. Results are expressed as means ± SEM (n > 4). * $P < 0.05$.

excluded the presence of preβ_1-HDL in our preparations, 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 HDL subfraction (HDL_2 or HDL_3) was more implicated in the HDL-mediated cholesterol efflux from macrophages, and second, the effect of aging in this process. Whole HDL, HDL_2, and HDL_3 isolated from both young and elderly subjects were incubated separately for 24 h with [^{3}H]cholesterol-loaded THP-1 macrophages. Y-HDL_3 induced significantly a higher [^{3}H]FC efflux (9.7% higher; $P < 0.05$) than that induced by Y-HDL_2, whereas no differences were apparent between E-HDL_3 and E-HDL_2 (Fig. 1). When regarded as a function of aging, Y-HDL_3 showed a higher capacity to promote cholesterol efflux than E-HDL_3 (11.1% higher; $P < 0.05$), with no observed variations between Y-HDL_2 and E-HDL_2.

Effect of aging on the ABCA1-mediated cholesterol efflux pathway

To clarify which of the cholesterol efflux pathways was more affected by aging, we investigated the mechanism based on the ABCA1 protein using a second macrophage cell line, J774. J774 cells express less ABCA1 compared with THP-1 macrophages (9), as confirmed by our results (**Fig. 2A**).

Interestingly, when Y-HDL and E-HDL (50 μg/ml) were incubated separately with [^{3}H]cholesterol-loaded J774 cells during 24 h, there was no effect of aging on the capacity of either HDL to promote cholesterol efflux compared with the THP-1 cells (Fig. 2B). Furthermore, we chemically induced overexpression of ABCA1 by J774 to analyze the effect of aging on the ABCA1 cholesterol efflux-related pathway. Our results indicate that J774 cells stimulated with cAMP have a robust expression of ABCA1 (>9-fold) compared with nonstimulated cells (**Fig. 3A**). Interestingly, the modulation of expression of ABCG1 by cAMP was <1.8-fold.

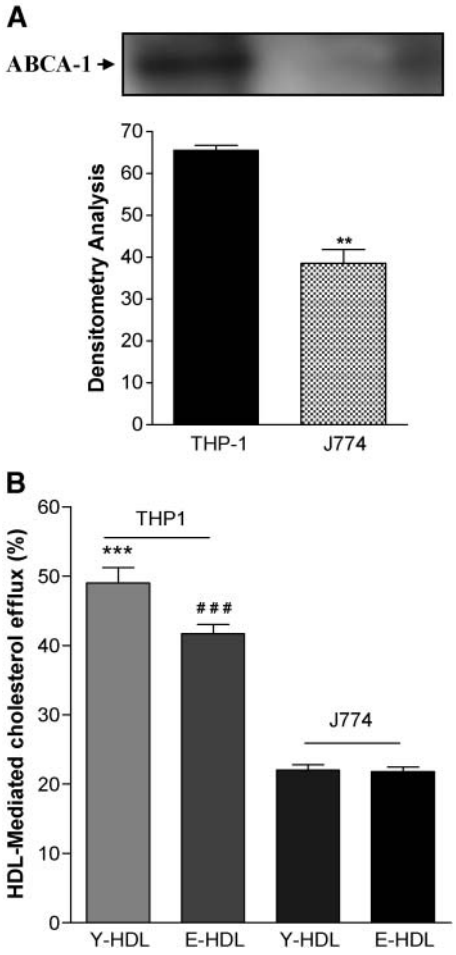

Fig. 2. A: ABCA1 protein expression level in THP-1 and J774 macrophages. Protein lysates (20 μg) of each lysed cell line were loaded onto SDS-PAGE gels (7.5%) in reduced conditions and immunoblotted with ABCA1-specific antibodies. Quantitative analysis was determined using densitometry from three independent experiments. Results are expressed as means ± SEM. ** $P < 0.01$. B: Comparison of HDL-mediated cholesterol efflux from THP-1 and J774 macrophages. THP-1 and J774 macrophages loaded with [^{3}H]cholesterol were incubated for 24 h with Y-HDL and E-HDL (50 μg/ml). Results are expressed as means ± SEM of triplicate determinations (n = 4). *** $P < 0.001$, Y-HDL in THP-1 cells versus Y-HDL in J774 cells. ### $P < 0.001$, E-HDL in THP-1 cells versus E-HDL in J774 cells.

The chemically induced expression of ABCA1 in J774 cells by cAMP (21, 34, 35) significantly increased whole HDL- and HDL_3-mediated cholesterol efflux in both young and elderly subjects (Fig. 3B), whereas no differences were observed in HDL_2-related cholesterol efflux as a function of aging (results not shown). Moreover, in ABCA1-enriched J774 cells, Y-HDL increased cholesterol efflux by 21.7% ($P < 0.05$) compared with E-HDL, whereas Y-HDL_3 enhanced cholesterol efflux by 26.1% ($P < 0.001$) compared with E-HDL_3 (Fig. 3B).

ApoA-I purified from young subjects (Y-apoA-I) and elderly subjects (E-apoA-I) was also evaluated for its capacity to promote cholesterol efflux (Fig. 3C). Our results do not shown an effect of aging on the apoA-I-dependent

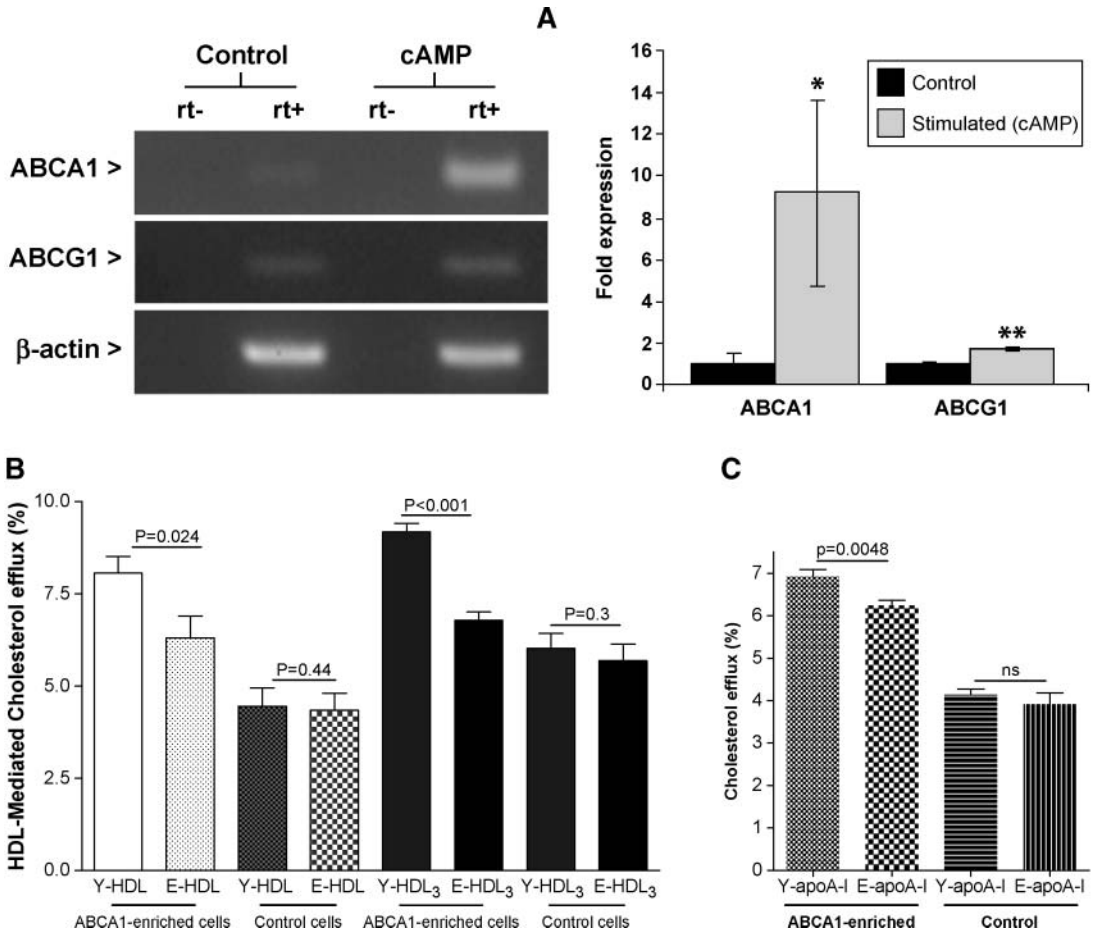

Fig. 3. Involvement of the ABCA1 receptor in HDL-mediated cholesterol efflux in J774 cells. J774 macrophages were incubated for 12 h at 37°C without (control cells) or with 0.3 mM 8-Br-cAMP. A: 8-(4-Chlorophenylthio)adenosine 3′,5′-cyclic monophosphate (cAMP) regulates mRNA expression of ABCA1 and ABCG1 in J774 cells. One representative PCR analysis is shown for ABCA1, ABCG1, and β-actin. The graph at right represents the transcriptional expression of ABCA1 and ABCG1. β-Actin was used as an internal control. Expression of ABCG1 is slightly regulated after cAMP stimulation (<2-fold) compared with ABCA1, which is highly regulated (>9-fold), compared with control cells (nonstimulated). Asterisks denote significance for ABCA1 (* $P = 0.034$) and ABCG1 (** $P = 0.0009$). B: Stimulated or nonstimulated J774 cells were incubated during 24 h with HDL or HDL_3 isolated from young (Y-HDL) or elderly (E-HDL) subjects (50 μg/ml). C: Apolipoprotein A-I (apoA-I)-mediated cholesterol efflux between ABCA1-enriched and control cells. Cells were treated with apoA-I (50 μg/ml) purified from young (Y-apoA-I) and elderly (E-apoA-I) subjects. Results represent means ± SEM of more than three different experiments.

cholesterol efflux from control J774 cells. However, in ABCA1-enriched J774 cells, E-apoA-I was significantly less efficient at mediating cholesterol efflux than Y-apoA-I ($P = 0.0048$) (Fig. 3C).

ApoA-I structure and HDL cholesterol efflux capacity

The ABCA1-mediated cholesterol efflux pathway is dependent on the interaction of apoA-I with the ABCA1 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 content, Y-HDL demonstrated a higher apoA-I band intensity than E-HDL (**Fig. 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 Fig. 4A. At higher oxidative stress (4 and 8 h), the apoA-I band was reduced dramatically as consequence of the oxidative modifications. The relative electrophoretic mobility (Fig. 4B, **Table 2**) 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% greater 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 (Fig. 4C). It is noteworthy that for the same level of oxidation (incubation times of 0 and 1 h), Y-HDLs show a higher capacity to promote cholesterol efflux than E-HDLs ($P < 0.05$) (Fig. 4C).

Interestingly, comparison of HDL oxidation kinetics as a function of the age of donors shows an increased susceptibility of HDL to lipid peroxidation with aging,

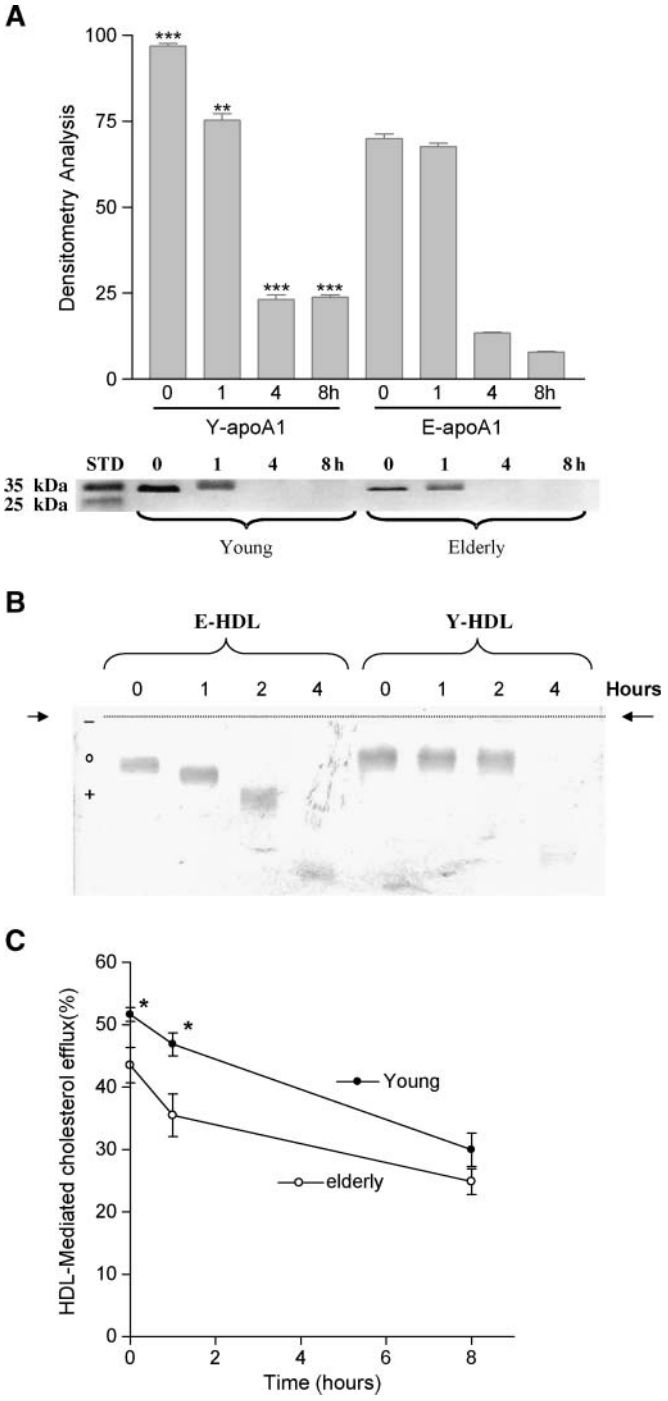

Fig. 4. A: SDS-PAGE profiles of apoA-I in basal conditions and during HDL oxidation. Y-HDL and E-HDL (200 μg/ml) were incubated with 10 μM $CuSO_4$ for 0, 1, 4, and 8 h. Experiments were performed in triplicate, and the gel shown is typical of the results obtained. Results were also analyzed by densitometry and represented as means ± SEM of three-separate experiments (densitometric 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 $CuSO_4$ for 0, 1, and 4 h. HDL electrophoresis was carried out in barbital buffer at pH 8.6 on 0.6% agarose gels. The gels were stained with 0.1% Fat Red 7B in 95% methanol. C: Involvement of apoA-I protein in HDL-mediated CE. [^{3}H]cholesterol-loaded THP-1 macrophages were incubated for 24 h with 50 μg of native or oxidized Y-HDL or E-HDL. Cholesterol efflux was determined as mentioned above. Results are expressed as means ± SEM of triplicate determinations. * $P < 0.05$.

TABLE 2. HDL relative electrophoretic mobility as a function of aging (Y-HDL vs. E-HDL) and HDL oxidation intensity (0, 1, 2, and 4 h)

	HDL Relative Electrophoretic Mobility (Compared with Y-HDL)	
Variable	Y-HDL	E-HDL
At basal conditions (0 h)	1	1.19 ± 0.02
Incubation with Cu^{2+} for 1 h	1.15 ± 0.01	1.45 ± 0.05
Incubation with Cu^{2+} for 2 h	1.28 ± 0.01	2.19 ± 0.04
Incubation with Cu^{2+} for 4 h	3.31 ± 0.03	3.66 ± 0.04

Y-HDL and E-HDL, HDLs isolated from young and elderly subjects, respectively. Data are expressed as the relative electrophoretic mobility of each band compared with that of the Y-HDL band. Experimental conditions are as described for Fig. 4B.

as measured by the lag phase for conjugated diene and malondialdehyde formation (**Table 3**).

Effect of aging on the ABCA1/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 HDL phospholipid bilayer fluidity.

For the phospholipid contents, we focused on the measurement of the HDL PC/SPM ratio. This parameter was shown previously to significantly influence HDL-mediated cholesterol efflux capacity (12, 36) as an ABCA1/apoA-I-independent pathway. **Figure 5A** demonstrates a significant decrease in the PC/SPM ratio in whole HDLs obtained from elderly subjects compared with those obtained from young subjects ($P = 0.029$). Similar results were obtained for E-HDL_3 compared with Y-HDL_3 ($P = 0.0027$) (Fig. 5A).

With fluorescence anisotropy, Y-HDL was found to have higher and significant phospholipidic layer fluidity than E-HDL ($P < 0.001$). The same results were obtained for Y-HDL_2 ($P = 0.035$) and Y-HDL_3 ($P = 0.023$) compared with the same HDL subfractions from elderly subjects (Fig. 5B).

DISCUSSION

The severity of atherosclerosis and the incidence of its clinical manifestations increase dramatically with aging

TABLE 3. Effect of aging on the susceptibility of HDL to lipid peroxidation

Phase	Young	Elderly	*P*
Lag phase (conjugated diene)	0.608 ± 0.05	0.375 ± 0.025	0.007
Lag phase (malondialdehyde)	0.841 ± 0.03	0.521 ± 0.025	<0.0001

HDLs were isolated from young and elderly subjects and were oxidized by time-course incubation with 10 μM $CuSO_4$. Lag phase was expressed as the time below which little or no conjugated diene or malondialdehyde formation was detected. Significance was calculated with reference to young subjects. Results are expressed as means ± SEM of triplicate or more determinations.

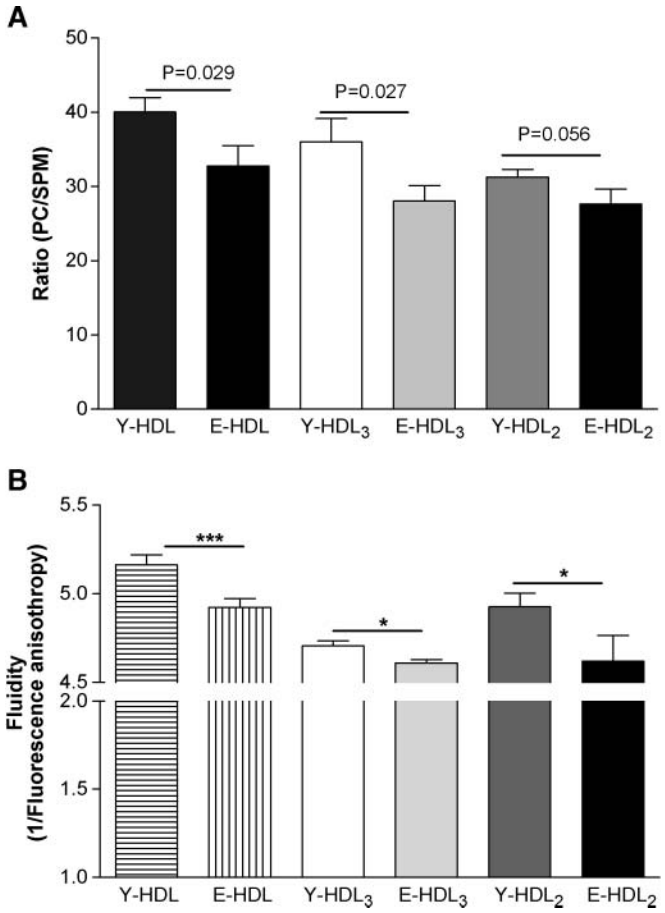

Fig. 5. A: Effects of aging on the phospholipid composition of whole HDL and HDL subfractions (HDL_2 and HDL_3) isolated from young and elderly patients. Results are given as phosphatidylcholine (PC) and sphingomyelin (SPM) ratio (PC/SPM). B: Effect of aging on the fluidity of the phospholipidic bilayer of whole HDL, HDL_3, and HDL_2, isolated from young and elderly subjects. Results are expressed as means ± SEM (n = 4–7). * $P < 0.05$, *** $P < 0.001$.

and are responsible for the majority of cardiovascular morbidity and mortality in the elderly. High plasma levels of HDLs are associated with a decreased risk of developing atherosclerosis, an effect commonly attributed to their central role in RCT. Indeed, efflux of FC from cell membranes to extracellular 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 that 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 HDL antioxidant activity with aging (18, 19). We investigated the effects of aging on the RCT process by studying the antiatherogenic properties of HDL. Our results showed a significant reduction of the whole HDL capacity to promote cholesterol efflux from macrophages. To determine which HDL subfraction was more affected, HDL_2 and HDL_3 were isolated and evaluated for their capacity to mediate cholesterol efflux. Interestingly, the reduction in the cholesterol efflux with aging was significant only for HDL_3. Moreover, in the young subject group, a difference in the capacity of HDL subfractions (Y-HDL_2 and Y-HDL_3) to promote cholesterol efflux was demonstrated (Y-HDL_3; $P = 0.019$). However, no significant difference was observed between E-HDL_2 and E-HDL_3. The ability of HDL_3 to increase cellular cholesterol efflux has been attributed to the increased presence of LCAT (39), greater phospholipidic bilayer fluidity, higher cholesteryl ester content, increased linoleic-to-linolenic acid ratio in phospholipids, and smaller size (40). Moreover, ABCA1 transporters, by binding to and cross-linking with lipid-poor apoA-I, represent another parameter that might explain the difference between the Y-HDL and E-HDL cholesterol-promoting efficiencies. The purpose of carrying out our experiments in more than one cell type was to confirm the notion of the ABCA1 receptor's role 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 or between E-apoA-I and Y-apoA-I in RCT activity, which is not the case with THP-1 macrophages. This could be attributable to the differences in the expression of ABCA1 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 ABCA1 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 ABCA1-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 by a structural alteration, may affect cholesterol efflux capacity. SDS-PAGE analysis showed a reduction in the apoA-I band intensity in E-HDL compared with Y-HDL, which suggests a reduction in apoA-I concentration in the elderly, as was demonstrated recently (37). However, for the electrophoresis experiments, Y-HDL as well as E-HDL were used at the same protein concentration (50 μg/ml), which excludes attributing the reduction in apoA-I band intensity to a reduction of its concentration. Indeed, no significant change in apoA-I concentration was noted between young and elderly subjects (Table 1). Interestingly, HDL incubation with copper ions induced a significant reduction of apoA-I band intensity (Fig. 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 for Y-HDL. Together, these results suggest that the basal alteration of apoA-I structure, as reflected by the decreased band intensity and charge as well as by 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 ABCA1-mediated cholesterol efflux capacity of HDL.

Furthermore, our results show that in both young and elderly subjects, the capacity of HDL to remove FC excess

from macrophages decreases significantly with higher levels of HDL oxidation. Interestingly, for the same level of HDL peroxidation, cholesterol efflux capacity was higher for Y-HDL than for E-HDL. Moreover, HDL oxidation induces an apoA-I alteration and decreases HDL phospholipidic layer fluidity.

Besides the expression levels of ABCA1, cholesterol efflux from macrophages is also dependent on the biophysical and biochemical structures of HDL subfractions. Contrary to the ABCA1-mediated cholesterol efflux, the other implicated mechanisms, ABCG1 and ABCG4, the spontaneous transfer, and the SR-BI pathways, all involve cholesterol transfer to phospholipid-containing species, principally HDL_2 (42). Thus, one might expect that alteration or change in the HDL phospholipid composition or structure would affect RCT (42–44). Analysis of the HDL, HDL_2, and HDL_3 phospholipid composition as a function of donor age showed a significant decrease in the PC/SPM ratio in the elderly. The increase of PC was demonstrated to positively modulate the RCT (45). These results, corroborated by the measure of reduced HDL fluidity, might be attributed in part to the oxidative modifications that occur with aging, as shown previously 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 concentration, 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, Karry, and Aviram (34) demonstrated a function of paraoxonase 1 in reverse cholesterol efflux from macrophages. Interestingly, a study from our laboratory has shown a significant reduction of paraoxonase 1 activity in the elderly (19).

Moreover, unlike ABCA1, ABCG1 and ABCG4 stimulate cholesterol efflux, especially to HDL_2 and HDL_3 but not to lipid-poor apoA-I (50). In this case, the HDL physicochemical 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 further investigation. In addition to these ATP-dependent transporters, Ji et al. (51) have showed that SR-BI promotes a bidirectional 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 does 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 that has been attributed to a reduction in the ABCA1 pathway. Based on these data, we postulate that the ABCA1/apoA-I-dependent cholesterol efflux is the more affected pathway with aging and is attributable principally to the oxidative modifications of apoA-I that might occur during aging. However, HDL biophysical and biochemical changes are also contributing factors in the reduction of its capacity to mediate cholesterol efflux in the elderly. Although the results obtained did not allow us to determine the proportion of cholesterol efflux that 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 E-HDL to promote cholesterol efflux may explain the increase in the incidence of cardiovascular diseases observed during aging.

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