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Effect of Reconstituted Discoidal High-Density Lipoproteins on Lipid Mobilization in RAW 264.7 and CHOK1 Cells

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ABSTRACT

Reconstituted discoidal high-density lipoproteins (rHDL) resemble nascent HDL, which are formed at the early reverse cholesterol transport steps, and constitute the initial cholesterol (Chol) acceptors from cell membranes. We have used different sized rHDL containing or not Chol, to test their abilities to promote cholesterol and phospholipid efflux from two different cell lines: Raw 264.7 macrophages and CHOK1 cells. All rHDL and lipid-free apolipoprotein A-I (apoA-I) were found to be bound to CHO and RAW cells. In RAW cells, a positive correlation between cellular binding and Chol removal was found for 78 and 96 Å rHDL. Chol-free rHDL were more effective than Chol-containing ones in binding to RAW cells and promoting Chol removal. These results were more evident in the 96 Å rHDL. On the other hand, rHDL binding to CHO cells was relatively independent of disc size and Chol content. In spite of the fact that apoA-I and rHDL promoted Chol efflux from both cellular lines, only in CHOK1 cells this result was also associated to decrease Chol esterification. Among choline-containing phospholipids, only phosphatidylcholine (PC) (but not sphingomyelin) was detected to be effuxed from both cellular lines. With the only exception of Chol-free 96 Å discs, the other rHDL as well as apoA-I promoted PC efflux from RAW cells. Chol-containing rHDL were more active than Chol-free ones of comparable size to promote PC efflux from RAW macrophages. Regarding CHO cells, only apoA-I and Chol-free 78 Å rHDL were active enough to remove PC. J. Cell. Biochem. 113: 1208–1216, 2012.

KEY WORDS: RECONSTITUTED HIGH-DENSITY LIPOPROTEIN; INTRACELLULAR CHOLESTEROL MOBILIZATION; APOLIPOPROTEIN A-I; CELL CHOLESTEROL EFFLUX; CELL PHOSPHATYDILCHOLINE EFFLUX

Cholesterol (Chol) is a lipid molecule that performs important cellular functions such as regulation of physicochemical properties of cellular membranes, formation of microdomains to locate several signal-related molecules. It is also a precursor for the biosynthesis of hormones and bile acids. Although most somatic cells synthesize Chol, only liver and a few steroidogenic cells can catabolize it. Consequently, release of cell Chol is a key event in Chol homeostasis. Apolipoprotein A-I (apoA-I) is the major protein component of high-density lipoproteins (HDL) which play a key role in reverse cholesterol transport (RCT). ApoA-I mediated release of phospholipids (PL) and Chol is one of the first essential reactions in RCT and production of plasma HDL. Plasmatic HDL concentration is inversely related to the incidence of coronary disease, and it significantly influences the atherogenic effect of Chol. Macrophages have a leading role in the formation of atherogenic plaques. They accumulate large amounts of cholesteryl ester (CE) in lipid droplets and consequently become foam cells. It has been suggested that the intracellular Chol pool, which is readily available for both esterification and transport to the plasma membrane, plays a central role in macrophage transformation into foam cells [Yamada et al., 1998; Pennings et al., 2006]. Consequently, Chol efflux mediating mechanisms are critical for maintaining cholesterol homeostasis in the macrophage [Smith et al., 2004]. The ATP-binding cassette transporter ABCA1 mediates Chol and PL efflux to lipid-poor apolipoproteins. ABCG1, like ABCA1, has been shown to regulate cellular Chol and PL efflux [Klucken et al., 2000; Venkateswaran et al., 2000; Fu et al., 2001; Gelissen et al., 2005; Vaughan and Oram, 2006]. ABCA1-mediated lipid efflux was found to convert lipid-free apoA-I into lipidated particles which in turn are efficient acceptors for ABCG1-mediated cholesterol efflux [Gelissen et al., 2005; Vaughan and Oram, 2006]. Whether one or both of these interactions also generate an interaction partner for scavenger

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receptor class B (SR-BI) is unknown. Lorenzi et al. [2008] observed that ABCA1, ABCG1, and SR-BI modulate the cellular binding of lipid-free apoA-I and HDL, and that the lipidation degree determines the binding and cholesterol efflux capacities of reconstituted HDL. ABCA1 and ABCG1 are overexpressed in Chol-loaded cells [Sabol et al., 2005].

Recently, Favari et al. [2009] have shown experimental evidence that ABCA1-mediated efflux was efficiently promoted by the 78 nm reconstituted phospholipid/apoA-I-containing discoidal particles (rHDL). This rHDL also promoted ABCG1, but not SR-BI, cholesterol efflux. All large and lipid-rich rHDLs with a bigger diameter of 78 nm only promoted ABCG1 and SR-BI cholesterol efflux.

Efflux capacity of native and reconstituted HDL has been extensively studied [Thuahnai et al., 2004; Wang et al., 2004; Yancey et al., 2004; Kennedy et al., 2005]. It has been shown that cholesterol efflux capacity increases with PL content of particles [Davidson et al., 1995]. The anti-atherogenic property of HDL is probably mediated in part by its capacity to remove cholesterol excess from macrophage-derived foam cells. ApoA-I is composed mainly of amphipathic α-helices; it may undergo large conformational changes during its functional cycle. To accomplish this cycle the protein can adopt either lipid-free or different lipidated forms, as bound to membranes or part of lipoprotein complexes with different size, morphology, and composition. Discoidal HDL are formed at the early steps of RCT, being the initial Chol acceptors from cell membranes [Krimbou et al., 2005]. As they are enriched in Chol and PL, they can be substrate of LCAT being transformed in spherical HDL. Discoidal HDL are found in serum at a low concentration. For this reason, rHDLs obtained from purified apolipoproteins and phospholipids by detergent dialysis [Jonas and Matz, 1982] are used to study their interaction with cells. Discoidal rHDLs resembling nascent HDL [Jonas, 1986] have been extensively characterized. They are good Chol acceptors from cells [Agnani and Marcel, 1993] and excellent LCAT substrates [Jonas and Matz, 1982]. We have previously shown that size and Chol content of discoidal rHDLs affect the apoA-I conformation and its ability to interact with lipid bilayers [Tricerri et al., 1998].

The main objective of this study was to correlate the lipid content and size of rHDL with their ability to promote Chol and PL efflux, comparing their effect on two different Chol-loaded cell lines: Raw 264.7 macrophages and CHOK1cells.

MATERIALS AND METHODS

CELL CULTURE

About 5×10^5 to 6×10^5 (RAW 264.7) or Chinese hamster ovary (CHOK1) cells were seeded on six-well plates and incubated until confluence (1 day) with 2 ml of Dulbecco's Modified Eagle Medium (DMEM) or Minimal Essential Medium (MEM), respectively, supplemented with penicillin/streptomycin (100 units/ml) and 10% FBS at 37°C in a 5% CO_2 atmosphere.

PREPARATION OF SERUM apoA-I

Serum apoA-I was obtained as previously described [Tricerri et al., 1998], using human serum kindly donated by Banco de Sangre, Instituto de Hemoterapia de la Provincia de Buenos Aires, La Plata, Argentina. It showed more than 95% purity as estimated by SDS–polycrylamide gel electrophoresis (SDS–PAGE).

RECONSTITUTION OF HIGH-DENSITY LIPOPROTEIN PARTICLES (rHDL)

The cholate dialysis method was used to obtain apoA-I-containing rHDL. Discoidal rHDL of 96 and 78 Å Stokes diameter, with or without cholesterol, were obtained starting from 95/1/150 and 40/1/65, palmitoyl-oleyl-phosphatidylcholine (POPC)/apoA-I/sodium cholate; or 95/24/1/150 and 40/10/1/65 POPC/Chol/apoA-I/sodium cholate molar ratios, respectively. The rHDL of 120 Å were purified from the 96 Å reconstitution mixture. Particles were purified by molecular size exclusion chromatography by FPLC as described before [Corsico et al., 2001], and their molecular weight was determined by polyacrylamide gel gradient electrophoresis in non-denaturing conditions (PAGGE). The composition was determined by the usual analytical methods.

QUANTIFICATION OF rHDL BINDING TO CELLS

Binding of rHDL to cells was measured by enzyme-linked immunosorbent assay (ELISA) adapting the procedure described by Price et al. [1990]. Briefly, around 2×10^5 to 3×10^5 RAW 264.7 or CHOK1 cells were seeded on 24-well plates. Then, they were stimulated with 6.25 μM hydroxycholesterol (H-Chol) and 10 μM retinoic acid or 0.5 mM 8-bromoadenosine-3-5-cyclic monophosphate (Br-cAMP) for 24 h. In preliminary experiments, cells were incubated with increasing doses of apoA-I (0, 12, 24, 48, and 56 μg/ml) to establish the optimal-binding concentration. Different controls were used: a protein-free control (containing only DMEM), a negative control [DMEM with a non-specific protein (casein)], and a positive control (30% v/v fetal calf serum).

In subsequent experiments, cells were incubated with lipid-free apoA-I and rHDL of different size and composition at the optimal-binding concentration (48 μg/ ml) at 37°C for 1 h. Then, cell monolayers were washed and blocked with PBS-serum albumin (1% w/v) for 90 min, then treated with the primary antibody (polyclonal anti-apoA-I) (1:1,000) for 1 h, washed with PBS and incubated with the secondary commercial antibody (Pierce) anti-rabbit IgG coupled to peroxidase (1:1,500) for 1 h. Polyclonal anti apoA-I were prepared in our laboratory from purified human apoA-I injected to rabbits. Antibodies were purified by affinity chromatography using HiTrap NHS-activated HP columns (Amersham Biosciences). Binding experiments were also performed at 4°C in order to reduce the membrane fluidity and to prevent potential internalization of HDL particles into the cells (data not shown).

Competitive immuno assay as previously described [Tricerri et al., 1998] was used to check the reactivity of the polyclonal anti apoA-I antibody to the different rHDL. In the assayed conditions (with a dilution of the anti-apoA-I antibody of 10^{-4}), the concentration of competing antigen producing 50% of competence was about 0.7 μg/ml and no significant differences were found among rHDL of different size and composition.

After washing with PBS, cells were treated with *o*-phenylenediamine dihydrochloride (OPD) or 2,2'-azino-di-[3-ethylbenzthiazoline-6 sulfonic acid] (ABTS) for 10 min. The OD (492 or 415 nm) of

each experimental point was quantified using a Multimode Detector, DTX 880, Beckman-Coulter.

CHOLESTEROL EFFLUX

For Chol efflux measurements, RAW 264.7 cells were labeled with 50 μg/ml cholesterol in DMEM and 0.05 μCi/ml ^{14}[C] cholesterol (Perkin Elmer) supplemented with 1% fatty acid-free bovine albumin for 24 h. Following the labeling period, cells were washed twice with PBS and allowed to equilibrate in culture medium containing 1 mg/ml BSA in the presence of 0.5 mM Br-cAMP [Smith et al., 1996] or 6.25 μM H-Chol plus 10 μM retinoic acid. After 8 h equilibration, cells were washed and incubated for 12 h in DMEM with increasing doses of apoA-I (0, 10, 15, 20, 25, 30 μg/ml) maintaining the stimulus of Br-cAMP. In other efflux experiments, cells were treated in the presence or in the absence of 12 μg/ml apoA-I either as the lipid-free protein or as rHDL in the presence of Br-cAMP or H-Chol plus retinoic acid. Background efflux was determined in control cells incubated with serum-free medium. Efflux media were collected and centrifuged to remove any detached cells and counted for ^{14}C. Then, cells were scraped and suspended in methanol for lipid analysis. Cellular lipids were extracted by the method of Bligh and Dyer [Bligh and Dyer, 1959]. Cholesterol efflux was calculated as the percentage of radioactivity in the medium relative to total radioactivity in cells plus medium. Cholesterol efflux in CHOK1 cells was performed as previously described [Gonzalez et al., 2008].

EVALUATION OF CELLULAR-FREE AND ESTERIFIED CHOLESTEROL IN RAW AND CHO CELLS

After the analysis of cholesterol removal, sterol species of cellular monolayers were separated by thin layer chromatography (TLC) on silica gel G plates developed in hexane:ethyl ether:acetic acid (80:20:1, v:v:v). Lipid spots corresponding to cholesteryl esters and non-esterified cholesterol were identified by staining with I_2 vapor and co-migration with authentic standards. Then, the radioactivity in each lipid fraction was determined by Phosphor-Image using an ImageQuant TL (Storm) system.

CHOLINE-CONTAINING PHOSPHOLIPID EFFLUX

Cells were treated with medium containing bovine serum albumin (2 mg/ml), unlabeled cholesterol (50 μg/ml), and ^{14}[C]-phosphorylcholine (0.5 μCi/ml) for 24 h. Then, cells were rinsed three times with PBS and equilibrated in appropriate culture medium (DMEM containing 0.5 mM Br-cAMP in RAW cells, or MEM in CHOK1 cells) for 8 h. Then, cells were treated with appropriate culture medium containing 12 μg/ml of lipid-free apoA-I or different rHDL.

ANALYSIS OF INTRACELLULAR PHOSPHATIDYLCHOLINE AND SPHINGOMYELIN

After the analysis of PL removal, polar lipid species of cell monolayers were separated by TLC on silica gel G plates developed in chloroform: methanol:acetic acid:water (50:37.5:3.5 2, v:v:v:v). Lipid spots corresponding to phosphatidylcholine (PC) and sphingomyelin (SM) were identified by staining with I_2 vapor and co-migration with authentic standards. Then, the radioactivity in each lipid fraction was determined by Phosphor-Image using an ImageQuant TL (Storm) system.

OTHER ANALYTICAL METHODS

Quantitative measurement of protein was performed by the method of [Lowry et al., 1951]. Student's *t*-test was used to compare pooled data.

OTHER MATERIALS

Dulbecco's Modified Eagle Medium (DMEM) and Minimal Essential Medium (MEM) were obtained from Invitrogen Corporation. Penicillin–streptomycin was from Parafarm (Bs. As., Argentina) and fetal bovine serum (FBS) was purchased from Bioser (Bs. As., Argentina). [^{14}C] Cholesterol was obtained from Amersham Biosciences (Pittsburgh, PA) and [^{14}C] phosphorylcholine (methyl ^{14}C) from NEN Reasearch Products, DUPONT.

Bovine serum albumin (BSA), Cholesterol, 8-bromoadenosine-3-5-cyclic monophosphate, *o*-phenylenediamine dihydrochloride (OPD), bromide 3-[4,5-dimethyltiazol-2-i]-2,5-diphenyl tetrazolium (MTT), hydroxycholesterol and retinoic acid were obtained from Sigma Co (St. Louis, MI). Dimethylsulphoxide (DMSO) was obtained from Carlo Erba (Milan). POPC and SM were purchased from Avanti Polar Lipids (Alabaster, AL).

RESULTS

BINDING OF FREE AND LIPIDATED apoA-I TO RAW 264.7 AND CHOKI CELL MEMBRANES

Enzyme-Linked Immuno Sorbent Assay (ELISA) applied directly on cell monolayer on the culture plate proved to be an effective and novel test to evaluate the binding of free or lipidated apoA-I to cellular membranes. The use of increasing doses of lipid-free apoA-I (see Material and Methods section) indicated that a concentration of 48 μg/ml resulted optimal to evaluate binding to membranes of both cell lines (Fig. 1A,B). All assayed rHDL and lipid-free apoA-I showed high and significant ($P \leq 0.01$) binding to membranes of RAW (Fig. 2A,B) and CHO (Fig. 3) cells compared to controls (protein-free DMEM or MEM, respectively). In CHO cells, free and lipidated apoA-I bind at the same extent to cellular membranes. In RAW cells treated with H-Chol and retinoic acid, binding of rHDL depends mainly on their composition The rHDL without Chol bind to cells with similar efficiency than lipid-free apoA-I except 96 rHDL which have a higher binding than apoA-I. The presence of Chol in the particles diminished their binding to cells.

A remarkable fivefold increase on apoA-I and rHDL binding to RAW cells was produced by the treatment with Br-cAMP in comparison with the treatment with H-Chol + retinoic acid. (Fig. 2A,B) This effect of Br-cAMP, however, was observed only when binding was measured at 37°C but not when it was measured at 4°C (data not shown). This fact indicates that the membrane physical state is important for the apoA-I and rHDL binding to Br-cAMP-treated macrophagues. Also in Br-cAMP-treated macrophagues, the presence of Chol in rHDL diminishes their binding to the cell membranes.

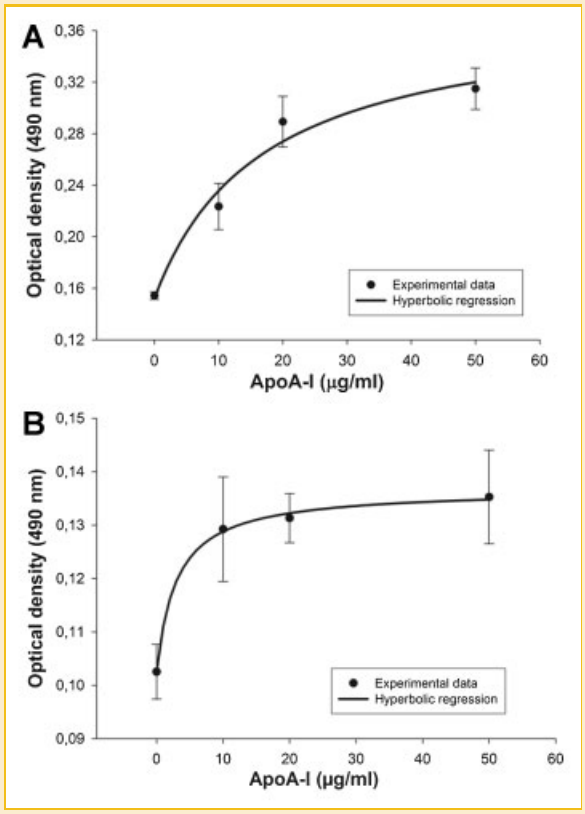

Fig. 1. Evaluation of apoA-I binding to (A) RAW 264.7 and (B) CHOK1 cells. Cells were seeded in plates of 24 wells, they were stimulated with hydroxycholesterol and retinoic acid for 24 h, and then treated with increasing doses of apoA-I (0, 12, 24, 48, and 56 μg/ml) for 1 h at 37°C. ELISA assay was carried out as we described in Materials and Methods section. Each point represents the mean value of three separate samples and the error bars represents SE. The plotted line represents hyperbolic regression.

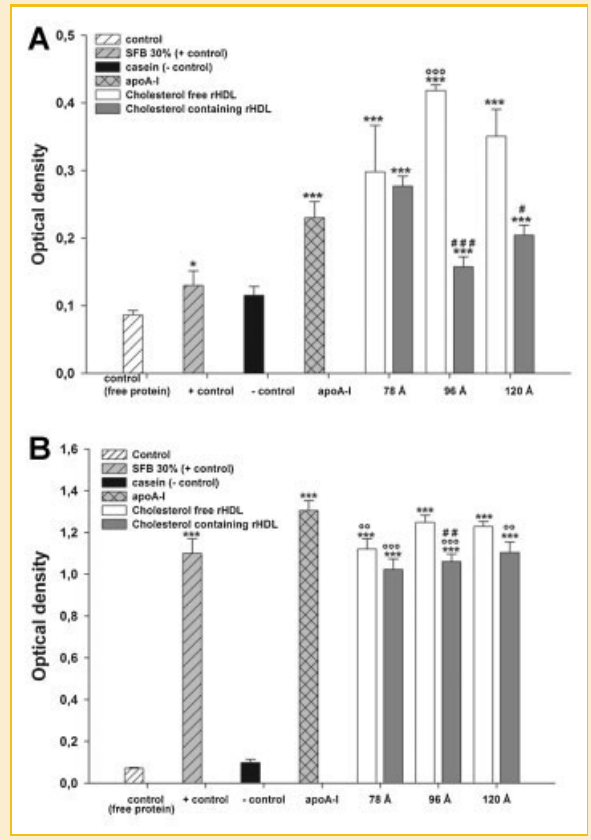

Fig. 2. Effect of lipidation state of apoA-I on binding to RAW cell membranes. Raw 264.7 cells were stimulated with H-Chol + retinoic acid (A) or Br-cAMP (B) for 24 h and incubated in DMEM-containing lipid-free apoA-I and rHDL of different size and composition at the optimal-binding concentration (48 g/ml) for 1 h at 37°C. ELISA assay was carried out as described in Materials and Methods section. The OD was quantified by Multimode Detector, Beckman-Coulter. Each point represents the mean value. Bars represents mean value and the error bars represents SE of three independent experiments. Student's *t*-test was performed. Levels of significance: $^{*}P<0.05$ and $^{***}P<0.001$ with respect to control; $^{\circ\circ}P<0.01$, $^{\circ\circ\circ}P<0.001$ with respect to apoA-I; $\#\#P<0.01$ and $\#\#\#P<0.001$ significance of Chol-containing rHDL with respect to Chol-free rHDL particles of the same size.

EFFECT OF CONCENTRATION AND LIPIDATION STATE OF apoA-I ON CHOLESTEROL EFFLUX IN RAW CELLS

We have chosen 30 μg/ml of free and lipidated apoA-I to evaluate the effect of different particles on Chol efflux in RAW cells (Fig. 4A). All tested rHDL were capable of promoting a significant cholesterol removal from RAW cells treated with Br-cAMP (Fig. 4B), but particles of 78 and 96 Å were more active than free apoA-I and 120 Å rHDL. The inclusion of Chol in 78 and 96 Å rHDL diminished the Chol efflux efficiency (Fig. 4B), particularly for 96 Å discs. Discoidal particles of 120 Å containing Chol showed no difference compared to the same sized discs without Chol. In RAW cells, the highest binding efficiency of rHDL without Chol directly associates with the highest capacity of Chol removal. Only in 96 Å rHDL without Chol the binding efficiency (Fig. 2A and Fig. 3) was directly associated with maximal Chol efflux (Fig. 4B) in both cellular lines [Gonzalez et al., 2008]. These results were in agreement with previous experimental results obtained with artificial vesicles [Toledo et al., 2000]. Concerning 120 Å discs, the presence of cholesterol decreased their binding to RAW cells, although their capacity to remove cellular Chol was similar to that of cholesterol-free particles.

When the cells were treated with H-Chol + retinoic acid all the particles stimulated Chol efflux with respect to control (Fig. 5). Meanwhile, free apoA-I did not show a significant increased Chol efflux with respect to control. In this situation, the control efflux is higher than that corresponding to cells treated with Br-cAMP. The Chol efflux was similar in rHDL with or without Chol. Particles of 96 Å with Chol and 120 Å containing or not Chol showed a significant increase in Chol efflux with respect to free apoA-I.

ANALYSIS OF CELLULAR-FREE AND ESTERIFIED CHOLESTEROL IN RAW CELLS. COMPARISON WITH CHO CELLS

When we analyzed the intracellular EC/Chol ratio in RAW cells, we could not observe any effect caused by the treatment with lipid-free apoA-I (Fig. 6A,B). However, the EC/Chol ratio showed a trend to be increased by the treatment with 96 and 120 Å rHDL, which was statistically significant only in Chol-containing 96 Å discs.

In CHO cells, we have observed that EC/Chol ratio depended on the particle size and cholesterol content in discs. The EC/Chol ratio

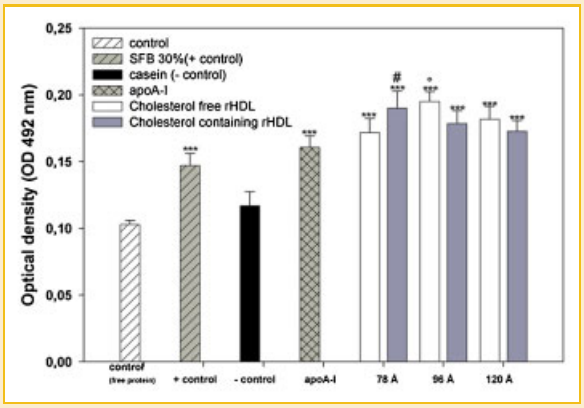

Fig. 3. Effect of lipidation state of apoA-I on binding to CHOK1 cell membranes. CHOK1 cells were stimulated with H-Chol + retinoic acid for 24 h and incubated in MEM-containing lipid-free apoA-I and rHDL of different size and composition at the optimal-binding concentration (48 μg/ml) for 1 h at 37°C. ELISA assay was carried out as described in Materials and Methods section. The OD (492 nm) was quantified by Multimode Detector, Beckman-Coulter. Each point represents the mean value. Bars represents mean value and the error bars represents SE of three independent experiments. Student's *t*-test was performed. Levels of significance: $^{***}P < 0.001$ with respect to control; $^{\circ}P < 0.05$ respect to apoA-I; $\#P < 0.05$ significance of Chol-containing rHDL with respect to Chol-free rHDL particles of the same size. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

diminished only when the cells were incubated with apoA-I and 120 Å rHDL without Chol (Fig. 7).

EFFECT OF rHDL ON PHOSPHOLIPID EFFLUX

The treatment of RAW cells with apoA-I for 18 h increased 4.6 times the phospholipid efflux compared to controls. All the particles were active in promoting phospholipid efflux, although in a lesser extent than apoA-I. Discs of 96 Å were less active for PL efflux, particularly those lacking Chol. Reconstituted particles with Chol seemed to be more active for PL efflux than the corresponding rHDL without the sterol in RAW cells (Fig. 8A). In CHO cells, apoA-I increased PL efflux more than twofold with respect to control. Only small rHDL of 78 Å without Chol-enhanced PL efflux significantly when compared to that of control (Fig. 8B).

ANALYSIS OF PHOSPHOLIPIDS REMAINING IN CELL MONOLAYERS

When we analyzed the lipidic extract of RAW cell monolayers we detected radioactivity mostly in PC and scarcely in SM (Table I). When the cells were treated with apoA-I and discs of different size and Chol content the measured PC/SM radioactivity ratio was significantly diminished compared with non-treated control cells (Table I). When we analyzed the culture medium we could only detect radioactivity in PC (data not shown). This result could explain the lowest PC/SM ratio measured after disc and apoA-I treatment.

In CHO cells, the PC/SM ratio diminished significantly compared to that of control when cells were treated with apoA-I and 78 Å discs without Chol (Table I). These results correlated with the major capacity of apoA-I and 78 Å discs without Chol to promote PL efflux.

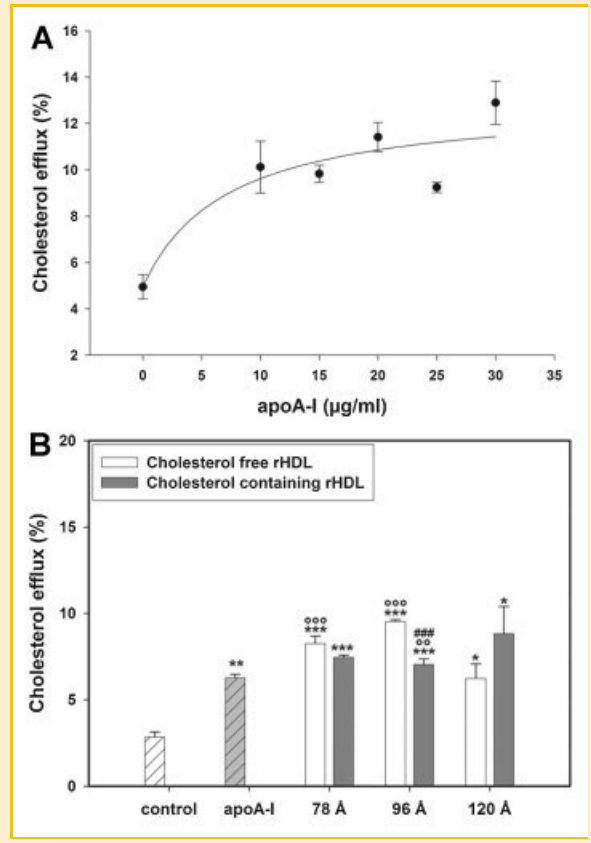

Fig. 4. Cholesterol efflux. Effect of apoA-I concentration (A) and apoA-I lipidation state (B) on cholesterol removal from Raw cells. Cells were labeled for 24 h with 50 μg/ml cholesterol in DMEM and 0.05 μCi/ml 14[C] cholesterol supplemented with fatty acid-free bovine albumin in DMEM and then treated with increasing doses of apoA-I (0, 10, 15, 20, 25, 30 μg/ml) (A) or different rHDL (30 μg/ml) (B) in the presence of Br-cAMP. Chol efflux was calculated as described in Materials and Methods section. Student's *t*-test was performed. The mean value and SE of three separated experiments are represented by each point and bar. The line represents hyperbolic regression of experimental data. Levels of significance: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ with respect to control; $^{\circ\circ}P < 0.01$, $^{\circ\circ\circ}P < 0.001$ with respect to apoA-I; $\#\#\#P < 0.001$ significance of Chol-containing rHDL with respect to Chol-free rHDL particles of the same size.

DISCUSSION

Cholesterol esterification is carried out by ACAT, which catalyses the fatty acid transfer from acyl-CoA to hydroxyl group of Chol generating a more hydrophobic molecule. Many authors have reported that apoA-I has the capacity to diminish the Chol pool accessible to ACAT in various cell lines [Gonzalez et al., 2008; Yamauchi et al., 2004; Li and Yokoyama, 1995; Li et al., 1997]. In the present study, we have observed a decrease in the EC/Chol radioactivity ratio when CHOK1 cells were treated with apoA-I. On the contrary, RAW cells treated with apoA-I did not show the same behavior. This difference could be due to the different metabolic characteristics of both cell lines. Macrophage metabolism is adapted to store considerable amounts of neutral lipids. In certain cellular types as macrophages, there are receptors for modified LDL that are not negatively regulated by high

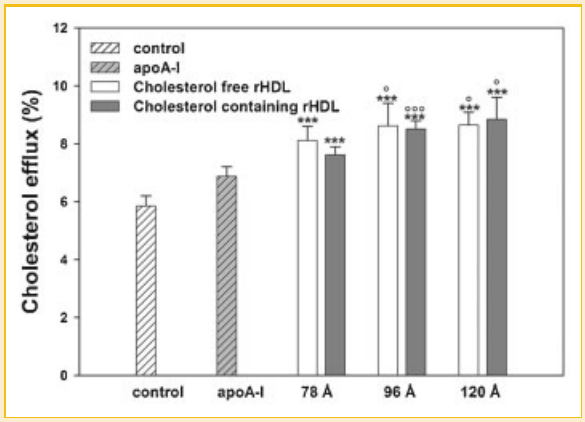

Fig. 5. Cholesterol efflux in macrophages stimulated with H-Chol + retinoic acid. Cells were labeled for 24 h with 50 μg/ml cholesterol in DMEM and 0.05 μCi/ml 14[C] cholesterol supplemented with fatty acid-free bovine albumin in DMEM and then treated with free or lipidated apoA-I in the presence of HChol + retinoic acid. Chol efflux was calculated as described in Materials and Methods section. Student's *t*-test was performed. The mean value and SE of three separated experiments are represented by each point and bar. Levels of significance: $^{***}P < 0.001$ with respect to control; $^{\circ}P < 0.01$ with respect to apoA-I.

levels of cellular Chol. This is probably the reason explaining why these cells accumulate cholesteryl esters and are transformed in foam cells which are characteristic of atheromatous plaque [Glass and Witztum, 2001]. Consequently in RAW cells, the intracellular-free Chol originated from lysosomal hydrolysis of cholesteryl esters contributes to elevate the free Chol level [Tiwari et al., 2008], which can be destined for removal by specific extra cellular acceptors or esterification by ACAT. In that way, Kiss et al. [2005] have observed that some macrophage cell lines (J774) have an elevated EC/Chol ratio correlated to an elevated Chol efflux.

A different behavior between both cell lines is also observed after treatment with rHDLs. While Br-cAMP stimulated RAW cells show a trend to increase Chol esterification when treated with Chol-free rHDLs of increasing size, the same rHDLs evoke an opposite trend to decrease Chol esterification in CHO cells, being the effect highly significant for the case of the largest particles (120 Å) containing three molecules of apoA-I per disc [Favari et al., 2009]. This difference could be attributed to the capacity of macrophages to direct the main part of free Chol to be esterified. The behavior of the largest particles could be due to the variation of apoA-I conformation in the biggest discs. In that way, we have previously demonstrated a different immunoreactivity of monoclonal antibodies directed against epitopes of the central apoA-I region [Tricerri et al., 1998]. We also have shown that the deletion in the central region of apoA-I (ΔK107) diminishes the Chol intracellular mobilization in CHOK1 cells [Gonzalez et al., 2008]. These results support the relevance of the apoA-I central region to mediate the cell responses involved in endogenous Chol mobilization [Corsico et al., 2001; Toledo et al., 2004]. Concerning the Chol-containing rHDLs, we have observed that only 96 Å discs increased the EC/Chol radioactivity ratio in RAW cells, but we have not observed any change of this ratio in CHOK1 cells.

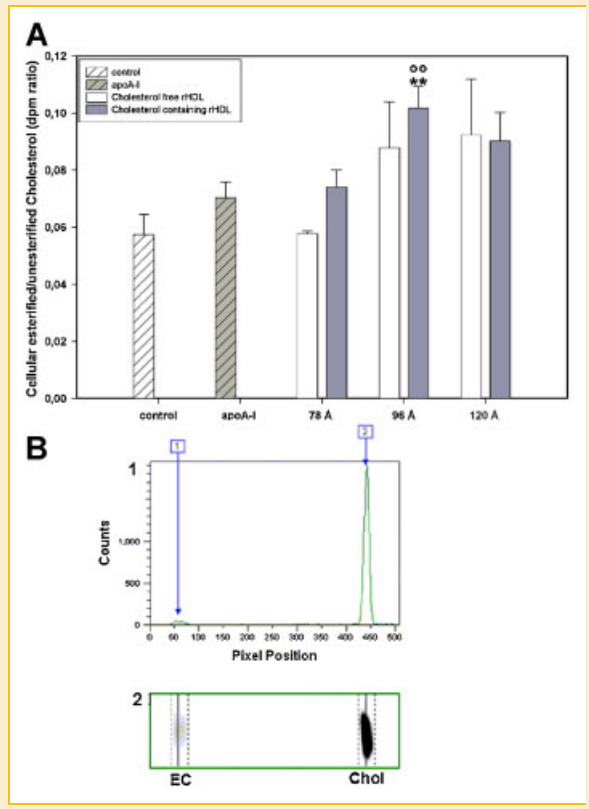

Fig. 6. Analysis of cellular-free and esterified cholesterol in RAW cells treated with Br-cAMP. (A) CE/Chol Cellular ratio: lipids from RAW monolayers were extracted and then separated by TLC as described in Materials and Methods section. The shown data are the mean of triplicate determination ± SE SE of CE/Chol radioactivity ratio. Student's *t*-test was performed. Level of significance: $^{**}P < 0.01$ with respect to control and $^{\circ\circ}P < 0.01$ with respect to apoA-I. (B) Scan of radioactivity distribution profile between Chol and cholesteryl esters. We show an example of the radioactivity profile scan and the quantification of spots corresponding to Chol and EC. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

Besides ACAT-1 and apoA-I [Sniderman and Marcovina, 2006; van der Westhuyzen et al., 2007], macrophage NCEH, ABCA1 [Oram and Vaughan, 2006], and ABCG1 [Baldán et al., 2006] also play an important role in cholesterol transport and macrophage foam cell formation [Oram and Vaughan, 2006; van der Westhuyzen et al., 2007]. Loading of cells with cholesterol increases the expression of ABCA1 [Kiss et al., 2005] and ABCG1 proteins involved in the efflux of phospholipids and cholesterol as shown by Sabol et al. [Sabol et al., 2005] in RAW 264.7 macrophage cells, HepG2 hepatoma cells, and primary mouse hepatocytes. Under the present conditions, previous macrophages Chol loading and further stimulation with cAMP analogue or H-Chol + retinoic acid would stimulate only ABCA1 or ABCG1 + ABCA1 expression, respectively [Smith et al., 1996; Oram et al., 1999; See et al., 2002]. HDL interactions with cells have shown to proceed by ABCG1 [Wang et al., 2004; Sniderman and Marcovina, 2006]. Thus, the 96 and 120 Å particles could interact with RAW cell through ABCG1. Small 78 Å particles and free apoA-I promote cholesterol efflux via ABCA-I

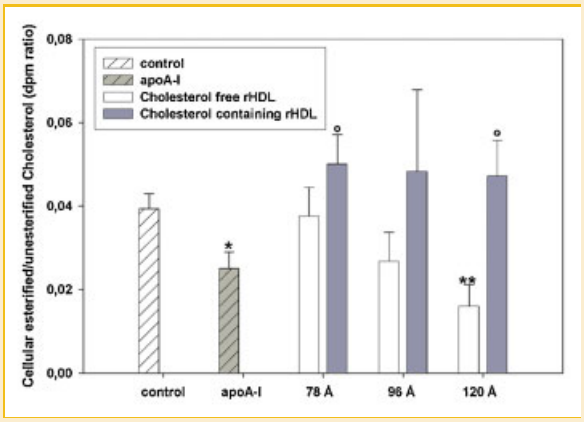

Fig. 7. Analysis of cellular-free and esterified cholesterol in CHOK1 cells. Lipids from CHOK1 monolayers were extracted and then separated by TLC as described in Materials and Methods section. Shown data are the mean of triplicate determination ± SE of CE/Chol radioactivity ratio for each sample. Student's *t*-test was performed. Levels of significance: $^{*}P < 0.05$, $^{**}P < 0.01$ with respect to control and $^{\circ}P < 0.05$ with respect to apoA-I. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

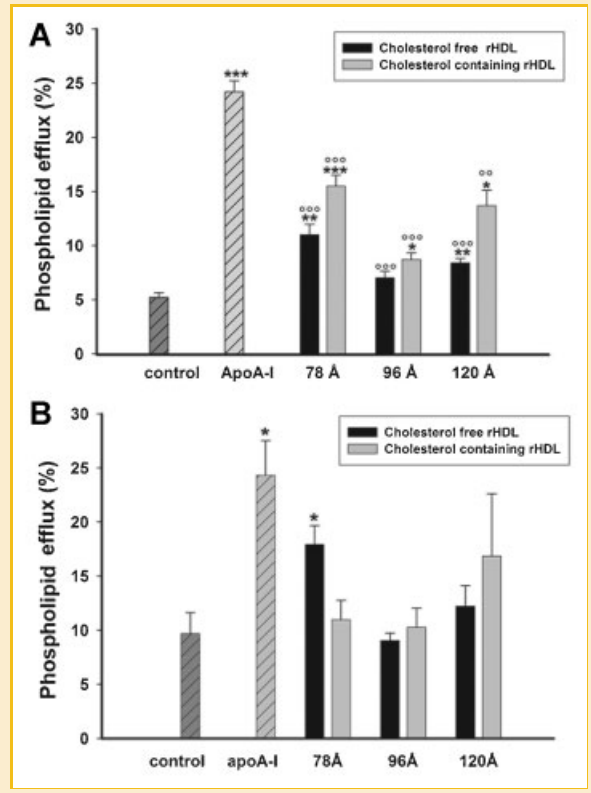

Fig. 8. Effect of lipidation state on Phospholipid efflux in RAW (A) and CHOK1 cells (B). Cells were incubated with medium containing bovine serum albumin unlabeled cholesterol, and 14[C]-phosphorylcholine for 24 h and then treated with free or lipidated apoA-I in the presence of Br-cAMP. Phospholipid efflux was calculated as indicated in Materials and Methods section. Data shown are the mean of triplicate determination ± SE for each sample. Student's *t*-test was performed. Levels of significance: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ with respect to control; $^{\circ\circ}P < 0.01$; $^{\circ\circ\circ}P < 0.001$ with respect to apoA-I.

as demonstrated recently by [Favari et al., 2009]. These facts can explain why the 96 and 120 Å particles showed a significant increased Chol efflux with respect to free apoA-I in H-Chol + retinoic acid-treated macrophages. Despite the difference in the cellular EC/Chol ratio remaining after the treatment with rHDL, all tested particles significantly promoted removal of cholesterol with respect to control in both cell lines.

There is controversy in the literature regarding the Chol removal generated by particles of different size. While some authors reported that the smallest particles (~78 Å) are the most active [Jonas et al., 1994], others reported that the largest ones (~ 120 Å) are the most effective [Agnani and Marcel, 1993]. In addition, the particle size dependence on acceptor may reflect different abilities to access the region very close to cell plasma membrane where most Chol is expected to occur [Davidson et al., 1995]. This apparent contradiction could be due to the different cells used in these studies and may reflect different abilities of the rHDL to access specific regions of the cell membrane where the different proteins involved in Chol efflux are located. Our results with RAW cells have not shown a large influence of rHDL size on Chol removal when the cells were treated with Br-cAMP. However, we have observed an increment in Chol removal promoted by Chol-free discs compared with Chol-containing discs, particularly in 96 Å rHDL. These particles are the largest ones containing two apoA-I molecules per disc. The inclusion of Chol in those particles decreased their acceptor capacity with respect to the same sized discs without Chol.

Binding experiments of free and lipidated apoA-I in different rHDL complexes to cells were carried out in an attempt to correlate cellular lipid removal with binding capacity. All discoidal particles bind to CHO and RAW cells. RAW cells treated with Br-cAMP bind apoA-I and rHDL with a remarkably higher efficiency than H-Chol + retinoic-treated cells. At the same time, the Chol efflux increase produced by apoA-I and particles in Br-cAMP-treated macrophagues was significantly higher (threefold) than the slight increase produced in H-Chol + retinoic acid-treated cells. These results could be due to the phosphorilation of ABCA1 produced

TABLE I. Effect of ApoA-I and rHDL Particles on Choline Radioactivity Ratio in PC and SM Phospholipids in RAW Cells Cellular Lipids were Extracted as Describes in Materials and Methods Section and Polar Lipids were Separated by Thin Layer Chromatography (TLC)

PC/SM ratio	RAW 264.7	CHOK1
Control	3.57 ± 0.04	3.98 ± 0.14
ApoA-I	$2.85 \pm 0.08^{***}$	$2.54 \pm 0.50^{*}$
rHDL 78 A	$2.26 \pm 0.28^{*}$	$2.10 \pm 0.54^{*}$
rHDL 96 A	$2.88 \pm 0.15^{*}$	3.79 ± 0.62
rHDL 120 A	$3.04 \pm 0.15^{*}$	3.96 ± 0.51
rHDL 78 A-Chol	$2.38 \pm 0.14^{**}$	4.32 ± 0.90
rHDL96A-Chol	$2.20 \pm 0.004^{***}$	3.48 ± 0.33
rHDL 120 A-Choi	$2.59 \pm 0.23^{*}$	4.11 ± 0.18

Radioactivity in each lipid fraction was determined by Storage Phosphor Screen using a ImageQuant TL program. Data shown are the mean of triplicate determination ± SE for PC/SM radioactivity ratio. Student's *t*-test was performed. Levels of significance: $^{*}P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.001$ with respect to control.

by Br-cAMP [See et al., 2002]. In macrophages treated with H-Chol + retinoic acid or with Br-cAMP, Chol-free discs bind to cells with higher efficiency than Chol-containing discs. This fact is observed both in experiments performed at 4 (data not show) or 37°C. Particles of 96 Å without Chol have shown a higher binding efficiency than 78 and 120 discs and, at the same time they were optimal to remove Chol from RAW cells treated with Br-cAMP. The apoA-I conformation in these particles is likely to be the optimal one for interacting with RAW cell membrane regions where Chol removal occurs. The rHDL binding to CHO cells, on the other hand, seems to be relatively independent of the particle size and Chol content. These results agree with previous data obtained with CHO cells, showing no influence of size and Chol content on Chol efflux [Gonzalez et al., 2008].

Concerning the cellular efflux of choline-containing phospholipids, only PC but not SM was detected in the culture media for both cellular lines. In RAW cells, PC efflux was significantly incremented by free or lipidated apoA-I in discoidal particles with the only exception of 96 Å Chol-free discs. The 78 and 120 Å particles could be more active to promote PC efflux because they could enlarge size admitting more PL than 96 Å discs which are at their limiting capacity of growth, restricted by the number of apoA-I molecules per disc. The Chol content of discs modified apoA-I conformation in these particles. We have shown a higher immunoreactivity for an N terminal epitope [Tricerri et al., 1998] in Chol-containing particles, compared with Chol-free ones of comparable size. Chol inclusion in these particles seemed to make them more competent to promote PL efflux in RAW cells. When we analyzed the distribution of radioactivity between PC and SM phospholipid fractions remaining at cell monolayers, we observed that PC/SM ratio diminished in those cells treated with lipid-free apoA-I or all kinds of rHDL with respect to control cells. Only in 96 Å particles, the cellular PC/SM ratio was significantly lower for cells treated with Chol-containing discs compared with Chol-free ones.

Regarding CHO cells, only apoA-I and 78 Å discs lacking Chol were active to remove PC compared to controls. These results are in accordance with a significant decrease of intracellular PC/SM ratio produced by apoA-I and Chol-free 78 Å discs. Miyazaki et al. [2009] proposed that small discoidal particles with diameter lower than 96 Å have a suddle form, with a negative curvature radio and minimal surface which could result in an optimal structure to interact with cells.

The results presented in this work suggest that Chol removal by free apoA-I or apoA-I-containing rHDL complexes, is a mechanism related to endogenous Chol mobilization and mainly dependent on Chol availability. The endogenous mobilization and lipid removal are then highly dependent on the cellular type, metabolic circumstances and acceptor particle involved in triggering these mechanisms.

In conclusion, RAW and CHO cells behave differently concerning Chol that is available to be removed or esterified. In RAW cells, the highest Chol availability for esterification could explain the trend to increase EC/Chol ratio by treatment with free or lipidated apoA-I, although the Chol efflux is enhanced at the same time. In CHO cells, the lowest Chol availability for esterification could explain that only the treatment with free apoA-I or Chol-free 120 Å particles produces a lower EC/Chol ratio in parallel with Chol removal from cells. Thus, it is evident that these treatments decrease Chol esterification in CHO cells.

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