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ABCA1-mediated cholesterol efflux generates microparticles in addition to HDL through processes governed by membrane rigidity[S]

Shilpi Nandi,* Loretta Ma,* Maxime Denis,* Joel Karwatsky,* Zhiqiang Li,† Xian-Cheng Jiang,† and Xiaohui Zha[1,*]

Ottawa Health Research Institute and Department of Biochemistry, Microbiology & Immunology,* University of Ottawa, Ottawa, Ontario K1Y 4E9, Canada; and SUNY Downstate Medical Center,† Brooklyn, NY 11203

Abstract ATP-binding cassette transporter A1 (ABCA1) mediates cholesterol efflux to lipid-poor apolipoprotein A-I (apoA-I) and generates HDL. Here, we demonstrate that ABCA1 also directly mediates the production of apoA-I free microparticles. In baby hamster kidney (BHK) cells and RAW macrophages, ABCA1 expression led to lipid efflux in the absence of apoA-I and released large microparticles devoid of apoB and apoE. We provide evidence that these microparticles are an integral component of the classical cholesterol efflux pathway when apoA-I is present and accounted for approximately 30% of the total cholesterol released to the medium. Furthermore, microparticle release required similar ABCA1 activities as was required for HDL production. For instance, a nucleotide binding domain mutation in ABCA1 (A937V) that impaired HDL generation also abolished microparticle release. Similarly, inhibition of protein kinase A (PKA) prevented the release of both types of particles. Interestingly, physical modulation of membrane dynamics affected HDL and microparticle production, rigidifying the plasma membrane with wheat germ agglutinin inhibited HDL and microparticle release, whereas increasing the fluidity promoted the production of these particles. Given the established role of ABCA1 in expending nonraft or more fluid-like membrane domains, our results suggest that both HDL and microparticle release is favored by a more fluid plasma membrane. We speculate that **ABCA1 enhances the dynamic movement of the plasma membrane, which is required for apoA-I lipidation and microparticle formation.**—Nandi, S., L. Ma, M. Denis, J. Karwatsky, Z. Li, X-C. Jiang, and X. Zha. **ABCA1-mediated cholesterol efflux generates microparticles in addition to HDL through processes governed by membrane rigidity.** ***J. Lipid Res.*** **2009.** 50: **456–466.**

Supplementary key words apoA-I • macrophages • wheat germ agglutinin • plasma membrane

ATP-binding cassette transport A1 (ABCA1) has been shown to be anti-atherogenic in human and animal models (1–4). This is thought to be due to its role in facilitating cholesterol/phospholipid efflux to extracellular acceptors such as lipid poor apolipoprotein A-I (apoA-I). ABCA1 expression promotes the interaction of apoA-I with cells, which enables apoA-I to acquire cholesterol/phospholipid and form nascent HDL particles (5). However, neither the intrinsic molecular function of ABCA1 nor the detailed mechanisms that lead to HDL generation are well defined at present.

Because ABCA1 was identified as the key molecular machinery for cholesterol efflux, several models have been proposed. ABCA1 belongs to the ABC transporter superfamily that potentially uses the energy released from hydrolyzing ATP to pump substrates across biological membranes. Mutations in the nucleotide binding domains in many cases inhibit ABCA1-mediated cholesterol efflux. It was speculated that ABCA1 actively flips phospholipids and/or cholesterol to the outer leaflet of the plasma membrane, making them available to apoA-I (6). ABCA1 was also proposed to function as a surface receptor for apoA-I. This would bring apoA-I within proximity of the plasma membrane and facilitate apoA-I/lipid interactions to form nascent HDL. In support of this notion, apoA-I is found to be cross-linked with ABCA1 (7, 8) and mutations in the extracellular loops result in diminished cholesterol efflux (9). However, only 10% of cell-associated apoA-I can be cross-linked to ABCA1 (10), suggesting that ABCA1 may not simply function as a conventional cell-surface receptor. Recently, Vedhachalam et al. (11) proposed a more comprehensive model in which ABCA1 is thought to generate

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[1] To whom correspondence should be addressed.
e-mail: xzha@ohri.ca

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high membrane curvatures on the plasma membrane thereby allowing apoA-I to interact with the cell surface. As a result, apoA-I can then solubilize the membrane to form nascent HDL particles.

In addition to lipidating apoA-I to form HDL, ABCA1 is known to influence cholesterol packing in the plasma membrane. Vaughan and Oram (12) first reported that ABCA1-expressing baby hamster kidney (BHK) cells are more susceptible to cholesterol oxidase, an enzyme that preferentially modifies cholesterol in disordered membrane domains. We have also observed that ABCA1 expression in BHK cells alters the micro-organization of the plasma membrane (13). In the absence of apoA-I, functional ABCA1 on the plasma membrane alone is sufficient to generate more loosely packed membrane microdomains, or nonrafts. Similar alterations of the plasma membrane are also detected in primary macrophages from ABCA1 transgenic animals (14). Together, these findings support the notion that ABCA1 increases the fluidity of the plasma membrane through its intrinsic molecular function. Such increased membrane fluidity may facilitate the generation of high curvature structures on the plasma membrane and perhaps conditions cells for cholesterol efflux.

In this report, we show that, in the absence of apoA-I, ABCA1 expression directly leads to the release of cholesterol/phospholipid-rich, non-HDL microparticles. ABCA1-mediated cholesterol efflux thus produced two distinct classes of particles: apoA-I-containing nascent HDL and apoA-I-free microparticles. Impairing ABCA1 function either by mutation or by protein kinase A (PKA) inhibition abolished both events. We also report that restricting membrane dynamics by rigidifying the plasma membrane prevented the production of both HDL and microparticles.

MATERIAL AND METHODS

Materials and reagents

Cell culture media and reagents were purchased from Invitrogen Canada (Burlington, ON, Canada). Mifepristone, cholesteryl hemisuccinate (CH) and all lectins were purchased from Sigma-Aldrich Canada (Oakville, ON, Canada). ^{3}H cholesterol and ^{3}H choline chloride were purchased from PerkinElmer-Canada Inc. (Vaudreuil-Dorion, QC, Canada). The polyclonal antibody against ABCA1 was from Novus Biological Inc. (Littleton, CO). Purified human apoA-I was purchased from Biodesign International (Saco, ME). ^{125}I was from GE-Healthcare/Amersham (Baie d'Urfé, QC, Canada). The anti-apoA-I monoclonal antibody was from Biodesign and the anti-mouse HSP-70 monoclonal antibody from BD Biosciences (Mississauga, ON. Canada). Complete protease inhibitor cocktail was purchased from Roche Canada (Mississauga, ON, Canada). PKA inhibitor, PKI, was from Calbiochem. Scintillation liquid, chloroform, and methanol were from Fisher Scientific Canada (Ottawa, ON, Canada). The antibody against apoE was purchased from Midland Bioproducts (Boone, IA). The antibodies that recognize hamster apoA-I and apo-B were kind gifts from Drs. Milne and Sparks (University of Ottawa Heart Institute).

Cell cultures

BHK cells were the generous gift from Drs. Oram and Vaughan (University of Washington, Seattle, WA). These cells carry a mifepristone inducible vector with or without an ABCA1 gene insert (12). For some of the experiments, an adherent mouse macrophage cell line, RAW 264.7, was used. The RAW 264.7 cell line was purchased from ATCC (Manassas, VA). This murine macrophage cell line expresses endogenous ABCA1 when treated with cAMP analogs. RAW macrophages can efficiently efflux free cholesterol (FC) and phospholipid (PL) to apoA-I (15). Cells without cAMP induction were used as negative controls. Both BHK cells and macrophage cells were maintained in DMEM + 10% fetal calf serum (FCS) at 37°C in a 5% CO_2 incubator. For BHK cells, induction medium consisted of DMEM with 1 mg/ml BSA and 10 nM mifepristone. In the case of macrophages, induction medium was comprised of DMEM plus 1 mg/ml BSA and 0.5 mM of 8-bromo-cAMP. For all experiments, BHK and macrophage cells were incubated with induction medium for 18–20 h to induce the expression of ABCA1 prior to experiments.

^{125}I-apoA-I cell association

Purified plasma apoA-I (Biodesign) was iodinated with ^{125}Iodine by IODO-GEN (Pierce) to a specific activity of ~2,500 CPM/ng and purified by Sephadex G25 columns followed by extensive dialysis. Mifepristone-induced BHK cells were washed two times with DMEM and incubated for 2 h at 37°C in DMEM containing 10 μg/ml ^{125}I-apoA-I in the presence or absence of wheat germ agglutinin (WGA). The medium was removed, cells were washed three times with PBS, and lysed with 0.5 N NaOH. The protein concentration in each well was determined by Lowry.

Cholesterol efflux

BHK and macrophage cells were grown in DMEM plus 10% FCS with 1 μCi/ml ^{3}H cholesterol for 2 days to label cellular cholesterol to equilibrium. After 2 days, medium was replaced by induction medium containing DMEM, BSA (1 mg/ml) and mifepristone (10 nM) for BHK cells. For macrophages, induction medium contained DMEM, BSA (1 mg/ml) and 8-bromo-cAMP (0.5 mM). After 18–20 h of induction, the medium was replaced by fresh DMEM/BSA without inducing reagents. For most experiments, cholesterol efflux was carried out by incubating cells with 10 μg/ml apoA-I for 2 h at 37°C unless otherwise mentioned. At the end of efflux, medium was collected, and cells were washed twice with PBS. The medium was centrifuged at 3,000 *g* for 5 min to remove cell debris. Then, 0.3 ml of the supernatant was mixed with scintillation liquid and counted for radioactivity. The cells were lysed in 0.5 ml of 0.5 N NaOH overnight with constant shaking. Further, 0.2 ml of cell lysates were mixed with scintillation liquid and counted for radioactivity. For experiments involving WGA, cholesteryl hemisuccinate, benzyl alcohol, hexanol, and LGM, cells were pretreated with the respective reagents for the indicated times. ApoA-I mediated cholesterol efflux was then carried out in the presence of the respective reagents. Recovery after exposure to WGA was conducted as follows: cells were treated with WGA (10 μg/ml) for 2 h followed by recovery in WGA-free medium containing N-acetylglucosamine for an additional 2 h before cholesterol efflux.

Phospholipid efflux

Cells were grown in DMEM plus 10% FCS containing 1 μCi/ml ^{3}H-choline chloride for 2 days to label cellular phospholipids to equilibrium. Cells were then put into induction medium for 18–20 h. This was followed by incubation with apoA-I (10 μg/ml) with or without WGA (10 μg/ml) for 2 h at 37°C. At the end of the experiment, phospholipids in the medium were extracted by adding 5 vols of chloroform /methanol (2:1 v/v) to the glass tube. The lower organic phase was collected and mixed with scintillation fluid to determine its radioactivity. For lipid extraction from

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cells, the cells were washed twice with PBS after removing the efflux medium. Lipids were collected after extraction with a mixture of hexane/isopropanol (3:2 v/v). Scintillation fluid was added and counted. To each well, 0.5 ml of NaOH was added and the protein concentration was determined by Lowry's method. Phospholipid efflux was calculated as a percentage of ^{3}H-choline phospholipid released into the medium relative to the total ^{3}H-choline phospholipid.

Methylthiazol tetrazolium assay

After overnight induction with various concentration of mifepristone, cells in 12-well dishes were rinsed and returned to DMEM + 10% FCS. Twenty microliters of methylthiazol tetrazolium solution (5 mg/ml) was then added directly into each well. Cells were incubated at 37°C for 3 h. Medium was carefully removed with a syringe to avoid disturbing formazan crystals formed during the incubation. One hundred microliters of DMSO was added to dissolve the crystals. The absorbance was then measured at 550 nm.

Rigidity measurements by micropipette aspiration method

To directly measure the effect of WGA, the membrane extensional rigidity on single cells was measured by the micropipette aspiration method. Cells were grown in coverslip-glass-bottom microscopy dishes and ABCA1 expression was induced. Cells were treated with WGA for 2 h before the measurements. A constant negative pressure (−10 mmHg) was applied to the plasma membrane through a micropipette that makes contact with the plasma membrane. The negative pressure forced the plasma membrane flow into micropipette and the rate of the flow was recorded with a video camera.

Microscopic experiments

BHK cells were plated and grown in glass-coverslip-bottom microscopy dishes to 80% confluency followed by induction for 18–20 h with mifepristone. For immunofluorescent staining, cells were treated with or without WGA for 2 h followed by two washes with PBS. Cells were fixed with paraformaldehyde (4%) at room temperature for 10 min and permeabilized with 0.1 mg/ml saponin for 30 min. The cells were blocked with 50 mM NH_4Cl and 5% calf serum solution for 20 min. ABCA1 was visualized using a primary polyclonal antibody against ABCA1 followed by an Alexa488-goat anti-rabbit secondary antibody. Distribution of WGA in BHK cells was observed by incubating cells with 10 μg/ml Alexa488-WGA for 2 h before fixation.

FPLC analysis

Lipoprotein profiles were obtained using fast performance liquid chromatography (FPLC) separation with a Superose 6B column. A 200 μl aliquot of concentrated medium was loaded onto the column, and eluted with TS buffer (50 mM Tris, 0.15 M NaCl, pH 7.4) at a constant flow rate of 0.35 ml/min. An aliquot of 250 μl from each fraction (0.5 ml) was used for the determination of radioactivity. Forty microliters from each fraction was used for detection of apoA-I by immunoblotting.

RESULTS

ABCA1 expression can significantly decrease plasma membrane rigidity by generating more loosely packed membranes (12, 13). We speculated that this increased membrane fluidity may have detectable cellular consequences, even in the absence of apoA-I. Indeed, we observed that basal cholesterol efflux (without apoA-I) from ABCA1 expressing cells was several-fold greater than the efflux from mock-transfected BHK cells or uninduced macrophages. However, it was difficult to judge the accuracy of these observations due to the fact that previous experiments were designed to optimally measure apoA-I-mediated cholesterol efflux for a short duration. To investigate this basal ABCA1 activity, we prolonged apoA-I independent cholesterol efflux from 2 h to 8 h in cells with or without ABCA1 expression. We found that in BHK cells and RAW macrophages, ABCA1 expression alone was sufficient to induce approximately 10-fold greater cholesterol efflux over uninduced control cells in the absence of apoA-I (**Fig. 1A**). Without induction (control), neither BHK nor macrophages expressed significant levels of ABCA1 (13). Furthermore, this apoA-I independent efflux was not limited to cholesterol; a similar result was obtained with phospholipid efflux from both BHK and RAW macrophages in the absence of apoA-I (Fig. 1B).

We next attempted to estimate the contribution of this apoA-I independent efflux to the total cholesterol efflux when apoA-I was present. As shown in Fig. 1C, the amount of cholesterol efflux from ABCA1-expressing BHK cells in the absence of apoA-I was approximately 30% of the total efflux when apoA-I was present. ApoA-I independent cholesterol efflux thus contributed an appreciable amount to the total efflux.

To further confirm the causal relationship between ABCA1 and basal cholesterol efflux, we next tested this apoA-I independent efflux in cells expressing a dysfunctional ABCA1 nucleotide binding domains ($ABCA1^{A937V}$). These cells expressed similar levels of $ABCA1^{A937V}$ on the cell surface as wild type (wt) ABCA1-expressing cells (13, 16), but were unable to efflux cholesterol to apoA-I (see supplementary Fig. IA) (16). We found that $ABCA1^{A937V}$ cells were also defective in apoA-I independent efflux (**Fig. 2A**). Moreover, ABCA1-mediated cholesterol efflux to apoA-I has been shown to depend on PKA activity, potentially through ABCA1 phosphorylation (17, 18). We therefore used a membrane permeable PKA inhibitor, PKI, to determine the importance of PKA on basal cholesterol efflux. PKI encodes a myrisoylated 9 a.a. peptide and binds to the catalytic unit of PKA with high affinity (Kd = 30 nM) to block its activity (19). As expected, PKI completely abolished cholesterol efflux to apoA-I (see supplementary Fig. IB). We found that PKI also significantly impaired apoA-I independent cholesterol efflux (Fig. 2B). Thus, apoA-I independent and dependent cholesterol efflux shared similar requirements for functional ABCA1.

To characterize the particles being released from ABCA1-expressing cells, we analyzed the efflux medium by FPLC. After BHK cells were labeled with ^{3}H-cholesterol for 2 days, efflux medium (4 h) was collected and analyzed. The elution profile of ^{3}H-cholesterol is shown in **Fig. 3A**. In the presence of apoA-I, ABCA1-expressing cells produced two types of particles: a large peak containing apoA-I (fractions 20–29), presumably the nascent HDL particles (20), and a smaller peak with no detectable apoA-I (fractions 16–18). Interestingly, a single peak was detected in the 4 h medium collected from ABCA1 expressing cells without apoA-I

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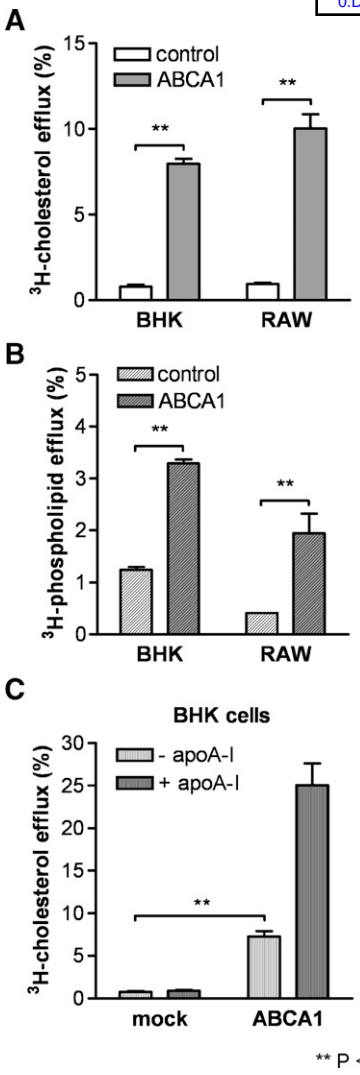

Fig. 1. ATP-binding cassette transport A1 (ABCA1) expression alone induces cholesterol efflux. A: Baby hamster kidney (BHK) and RAW264.7 macrophage cells were labeled with ^{3}H-cholesterol. ABCA1 expression was induced by treating BHK cells with 10 nM mifepristone or RAW macrophages with 0.5 mM 8-bromo-cAMP. Cells were then changed into fresh DMEM medium [no apolipoprotein A-I (apoA-I)] with induction reagents. Medium was collected after 8 h. Medium and cell-associated ^{3}H radioactivity was counted and presented as percentage of cholesterol in the medium relative to the total cholesterol (medium and cell-associated). B: BHK and RAW cells prelabeled with ^{3}H-choline chloride were treated as in A. Phospholipids containing ^{3}H-choline were extracted from both medium and cells to calculate the percentage of ^{3}H-choline containing phospholipids in the medium. C: ^{3}H-Cholesterol efflux was carried out in DMEM (no BSA) for 8 h in the absence (−apoA-I) or in presence of 10 μg/ml apoA-I (+apoA-I).

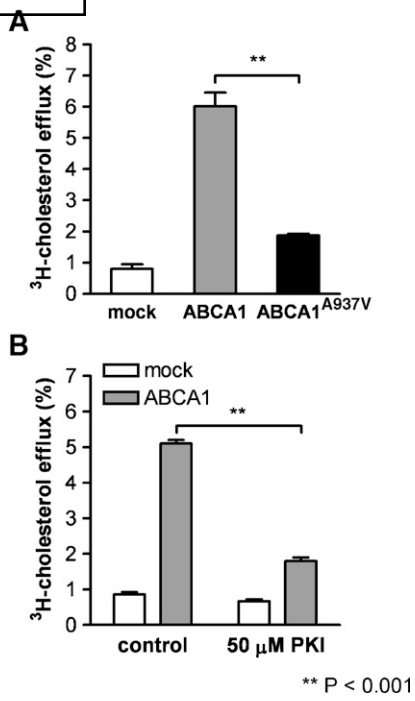

Fig. 2. Functional ABCA1 and protein kinase A (PKA) activity is required for apoA-I independent cholesterol efflux. A: ^{3}H-cholesterol labeled BHK-mock, ABCA1, ABCA1^{-A937V} cells were induced for 20 h and incubated with DMEM without apoA-I for 4 h. B: ^{3}H-cholesterol labeled BHK-mock and ABCA1 were induced and incubated with DMEM with or without 50 μM PKI. Cholesterol efflux was measured as in Fig. 2. Bars represent mean ± standard deviation of triplicate wells.

(Fig. 3B). This peak was identical in size and in quantity to the smaller peak produced by cells exposed to apoA-I. Based on the peak area, it accounted for approximately 30% of the total ^{3}H-cholesterol effluxed from cells incubated with apoA-I. This is in agreement with our efflux results shown in Fig. 1C. These findings therefore demonstrate that there are two parallel components in ABCA1-mediated cholesterol efflux: an apoA-I dependent component (HDL) that accounted for approximately 70% of the overall cholesterol efflux, and an apoA-I independent component (non-HDL) that supplied the remaining 30%.

To further characterize the non-HDL component, the efflux medium from ^{3}H-cholesterol labeled, ABCA1-expressing cells that were not exposed to apoA-I was collected and filtered through a 0.2 μm filter. We found that ^{3}H-cholesterol was 100% recovered after 0.2 μm filtration, indicating that the non-HDL component is smaller than 200 nm in size. This non-HDL component, however, was entirely retained from passing through 10 kDa molecular weight cut filter, suggesting a particle size that is equal to, or greater than 20 nm (21). It was also determined by KBr floatation that this non-HDL component has a density that is greater than 1.063, but less than 1.21, similar to that of HDL. The non-HDL component, however, is slightly larger than HDL in size as indicated by the FPLC profile (Fig. 3A). This non-HDL component thus matches the characteristics of the 24 nm microparticles released by J774 macrophages that are characterized extensively by Duong et al. (22). Interestingly, another study demonstrated that similar microparticles (>20 nm) are released from ABCA1-expressing BHK cells, although the medium from mock-BHK cells was not examined in this study (21).

Macrophages may secrete lipid particles containing endogenous apolipoproteins, such as apoE. As a result, the presence of endogenously secreted lipoproteins may give the appearance of apolipoprotein-independent efflux.

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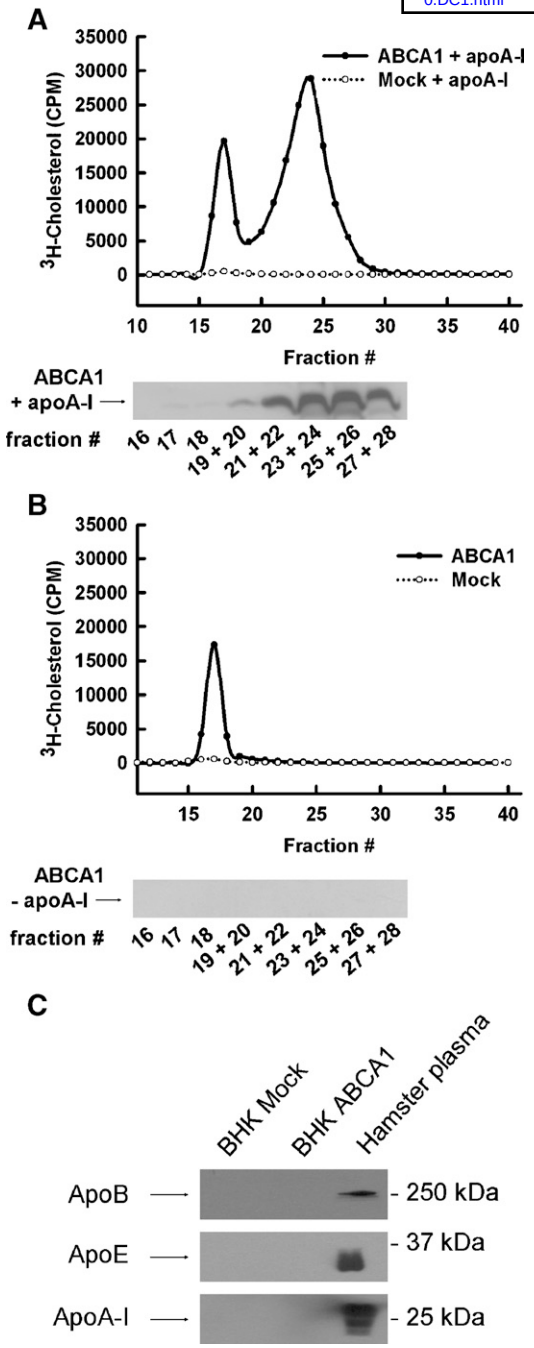

Fig. 3. Characterization of lipid particles shed to the medium in the absence of apoA-I. ^{3}H-cholesterol-labeled BHK cells expressing ABCA1 or not (mock) were incubated in presence of 10 μg/ml apoA-I or without apoA-I for 8 h. Media were collected, concentrated by ultrafiltration. A, B: Concentrated medium from ABCA1 or mock cells was analyzed by fast performance liquid chromatography (FPLC). Radioactivity associated with each fraction was determined. Fractions corresponding to elution peaks were analyzed by immunoblotting to detect apoA-I. C: Concentrated medium from ABCA1 cells was immunoblotted using antibodies against hamster apoB, hamster apoE, and hamster apoA-I to exclude potential production of endogenous apoproteins by BHK cells. Hamster plasma was used as positive control.

However, BHK cells did not secret apolipoproteins, such as apoE, apoB, or apoA-I (Fig. 3C), yet they were still able to produce apoA-I independent efflux identical to macrophages. This suggests that the contribution of endogenous apolipoprotein secretion is negligible to the microparticle formation in these cells.

It is noteworthy that, although the non-HDL component described here is termed microparticle, they are likely different from the circulating immune-microparticles. First, immune-microparticles are generally described as membrane particles with a diameter ranging from a few hundred nm to several μm (23), which is much larger than the non-HDL microparticles described here. Also immune microparticles are known to be released mainly by platelets but also by leukocytes upon activation or apoptosis (23). We however could not detect any increase of non-HDL microparticles in the culture medium when macrophages were activated by lipopolysaccharide (**Fig. 4A**). In addition, the non-HDL microparticles reported here could not be stained by annexin V (data not shown). ABCA1-expressing cells also remain perfectly viable as shown by a methylthiazol tetrazolium test (Fig. 4B), a well-established assay for cell viability (24). This rules out potential involvement of apoptosis in non-HDL microparticle generation. Furthermore, the release of immune-microparticles is known to be blocked by calcium chelating reagents such as EGTA (25). We found no influence of EGTA on non-HDL microparticle release (Fig. 4C). We therefore concluded that the non-HDL microparticles are distinct entities from immune-microparticles. For simplicity, we will refer to non-HDL microparticles as microparticles for the rest of this report.

We have recently shown that wt ABCA1 expression leads to a more fluid plasma membrane and, conversely, ABCA1^{A937V} fails to remodel the plasma membrane (13). Interestingly, PKA inhibition by PKI also prevented membrane remodeling in wt ABCA1 expressing cells (unpublished observation). We therefore reasoned that HDL and microparticle biogenesis could both arise from membrane remodeling by ABCA1. A more fluid plasma membrane is likely a prerequisite for generating both HDL and microparticles. To test this, we selected reagents known for their abilities to acutely modulate membrane rigidity and evaluated their effect on HDL and microparticle release to the medium, respectively. WGA is a lectin that multivalently binds and cross-links polysaccharides on the cell surface (26). This rigidifies the plasma membrane (27) and limits membrane movement (27, 28). We first examined the effects of WGA on the plasma membrane of BHK cells by using the micropipette aspiration method, which measures extensional elasticity (27, 29). Consistent with its reported rigidifying effect, WGA largely prevented the plasma membrane from being aspirated into the micropipette, which was held at a constant negative pressure (see supplementary Fig. III). Thus, WGA completely restricted plasma membrane protrusion or bulging. We then tested the effect of WGA on HDL generation. Strikingly, WGA severely inhibited apoA-I mediated cholesterol efflux in ABCA1-expressing BHK cells (**Fig. 5A**) and RAW macrophages (Fig. 5B). WGA also inhibited ^{3}H-phospholipid efflux to

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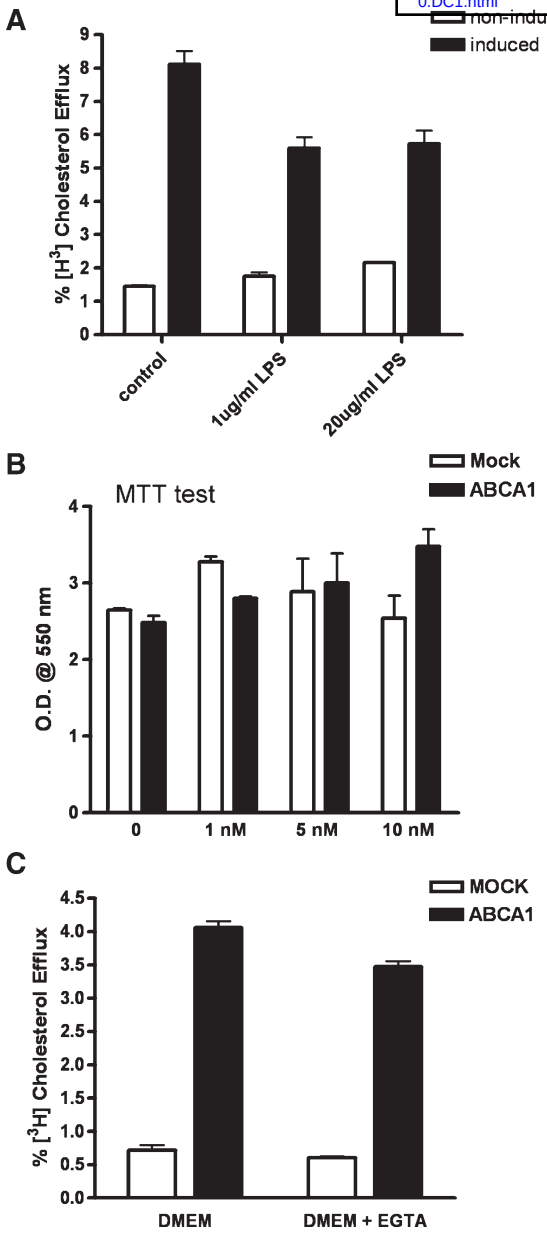

Fig. 4. Non-HDL microparticles are distinct from immune-microparticles. A: ^{3}H-cholesterol-labeled RAW macrophages, either induced or noninduced were treated with the indicated dose of lipopolysaccharide for 6 h. Cholesterol efflux was measured as in Fig. 1A. B: Mock and ABCA1 BHK cells were induced with mifepristone at indicated concentration for 18 h. Methylthiazol tetrazolium toxicity tests were carried as described in Materials and Methods. Results are presented as optical density (550 nm) readings from formazan and bars represent mean ± standard deviation of triplicate wells. C: ^{3}H-cholesterol-labeled BHK cells were induced with 10 nM mifepristone overnight. Cells were then pretreated with DMEM or DMEM + EGTA (2 mM) for 30 min. Cholesterol efflux was then carried out for 2 h with or without EGTA.

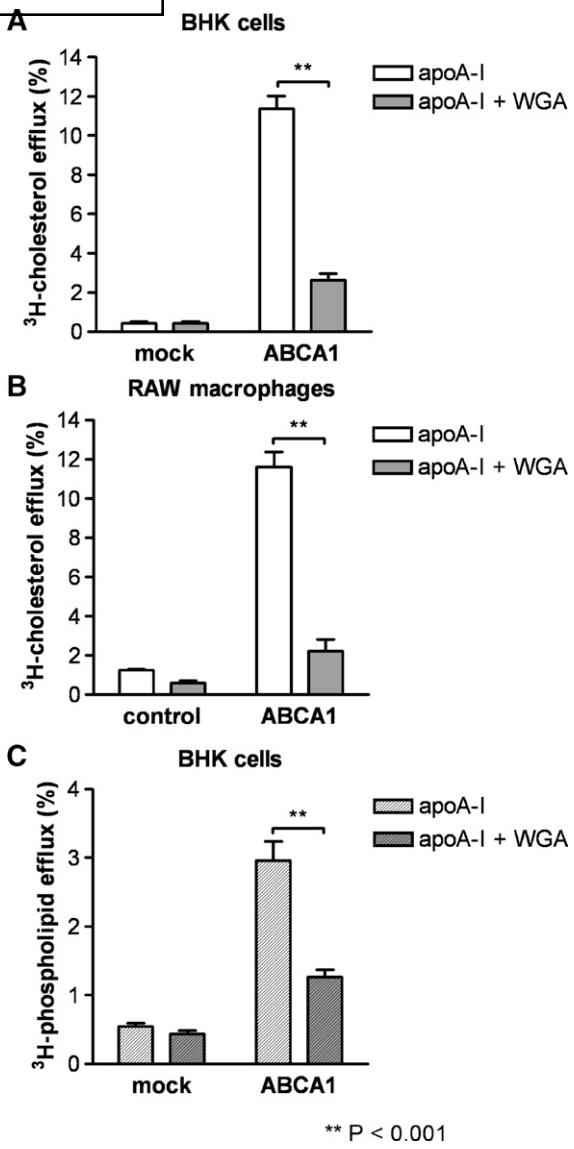

Fig. 5. Wheat germ agglutinin (WGA) inhibits lipid release to medium. A: ^{3}H-cholesterol-labeled BHK cells stably expressing human ABCA1 or an empty vector (mock) were incubated for 20 h in DMEM/BSA containing 10 nM mifepristone to induce ABCA1 expression. The release of ^{3}H-cholesterol to the medium containing apoA-I (10 μg/ml) for 2 h was measured in the presence or absence of WGA (10 μg/ml). B: Cholesterol efflux (2 h) from induced or noninduced RAW macrophages. C: BHK cells prelabeled with ^{3}H-choline chloride were treated as in A. Phospholipids containing ^{3}H-choline were extracted from both medium and cells to calculate the percentage of ^{3}H-choline containing phospholipids. Bars represent mean ± standard deviation of triplicate wells.

apoA-I in a similar manner (Fig. 5C). WGA inhibition of ^{3}H-cholesterol efflux occurred rapidly and its maximum effect was produced at a concentration of 10 μg/ml (see supplementary Fig. III). These results suggest that HDL biogenesis relies on proper membrane fluidity generated by ABCA1.

To determine if plasma membrane rigidity also interfered with ABCA1-mediated microparticle biogenesis, we measured the release of ^{3}H-cholesterol to the medium without apoA-I in WGA-treated cells. WGA also significantly inhibited apoA-I independent cholesterol efflux (**Fig. 6A**). Consistent with this notion, FPLC analysis of the medium showed that WGA inhibited HDL and microparticle production (Fig. 6B, C). Because WGA predominantly restrains membrane movement such as protrusion or bulging (28),

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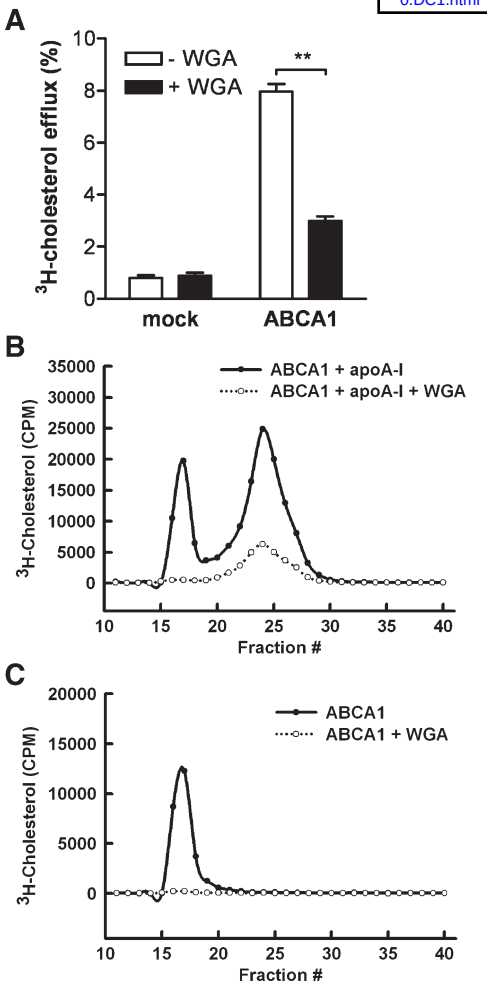

Fig. 6. WGA inhibits HDL and microparticles formation. A: ^{3}H-cholesterol released to the medium from BHK cells incubated without apoA-I for 8 h in the presence or absence of WGA (10 μg/ml). B, C: BHK cells induced to express ABCA1 were incubated with DMEM containing apoA-I (10 μg/ml) in the presence or absence of 10 μg/ml WGA for 4 h. Medium was collected and concentrated before FPLC analysis. B: Cells were treated identically as in A, except without apoA-I.

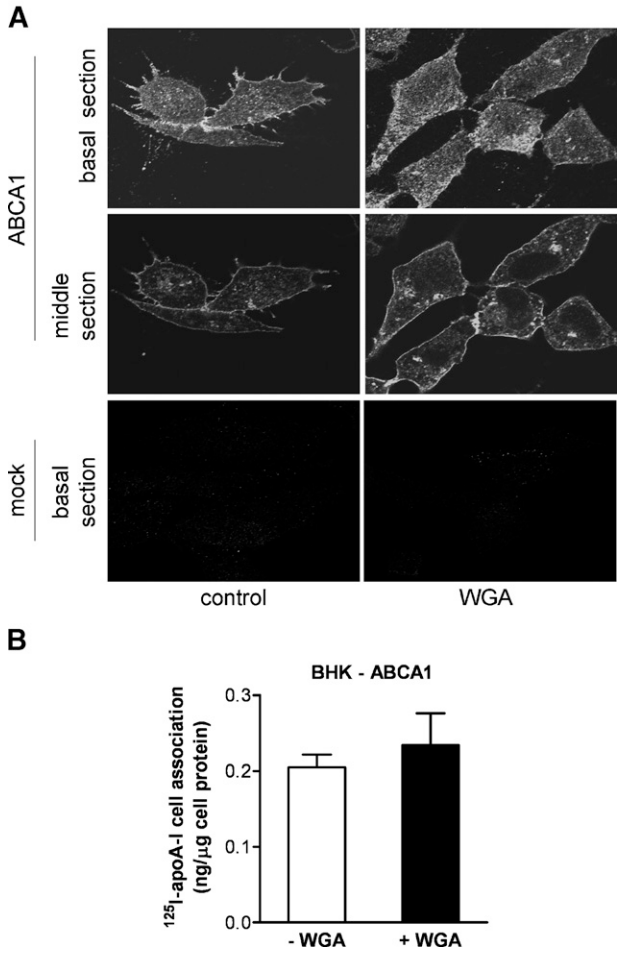

Fig. 7. Effect of WGA on ABCA1 distribution and apoA-I binding. A: BHK cells were treated with and without WGA (10 μg/ml) for 2 h. Cells were then fixed and immuno-stained for ABCA1. Confocal images were taken from both basal section (first row) and middle section (second row) of the cells. Mock cells had no visible staining when the images were taken under identical condition as for ABCA1 cells (third row). B: ABCA1 cells were treated with or without 10 μg/ml of WGA and ^{125}I-apoA-I (10 μg/ml) for 2 h and cell-associated ^{125}I-apoA-I was calculated by counting radioactivity in a γ counter. Data represents average from triplicate samples and error bars expressing standard deviation from the mean.

we concluded that a dynamic membrane is required for effective production of nascent HDL as well as apoA-I-free microparticles.

To further understand the mechanism by which WGA impaired cholesterol efflux, we first examined whether acute manipulations by WGA altered ABCA1 expression or cellular distribution. Immuno-fluorescence detection of ABCA1 in cells treated with WGA was compared with untreated control cells. ABCA1 fluorescent intensity, as determined by confocal microscopy, was unchanged with WGA treatment. Furthermore, ABCA1 was primarily localized to the plasma membrane and the Golgi in control and WGA-treated cells (**Fig. 7A**), suggesting a minimum disturbance of ABCA1 by WGA. We then measured ^{125}I-apoA-I cell association to determine whether WGA influenced apoA-I binding to cells. WGA did not affect apoA-I cell association (Fig. 7B), nor did it influence cross-linking between ABCA1 and apoA-I or apoA-I intracellular distribution (data not shown). Together, these findings indicate that WGA interfered with steps downstream of apoA-I binding to ABCA1 expressing cells.

Consistent with this notion, we found that the inhibition by WGA was readily reversible after WGA was stripped from the plasma membrane. This was illustrated by treating cells with a fluorescently labeled WGA. Alexa488-WGA was localized to the plasma membrane (arrows) and intracellular compartments after 2 h incubation (**Fig. 8A**, left panel). Washing these cells with medium containing excess N-acetylglucosamine specifically stripped Alexa488-WGA from the cell surface (arrows) (Fig. 8A, right panel), while keeping intracellular WGA largely unaltered. N-acetylglucosamine binds to WGA and serves as a competitive decoy for WGA. Importantly, cholesterol efflux was fully restored after re-

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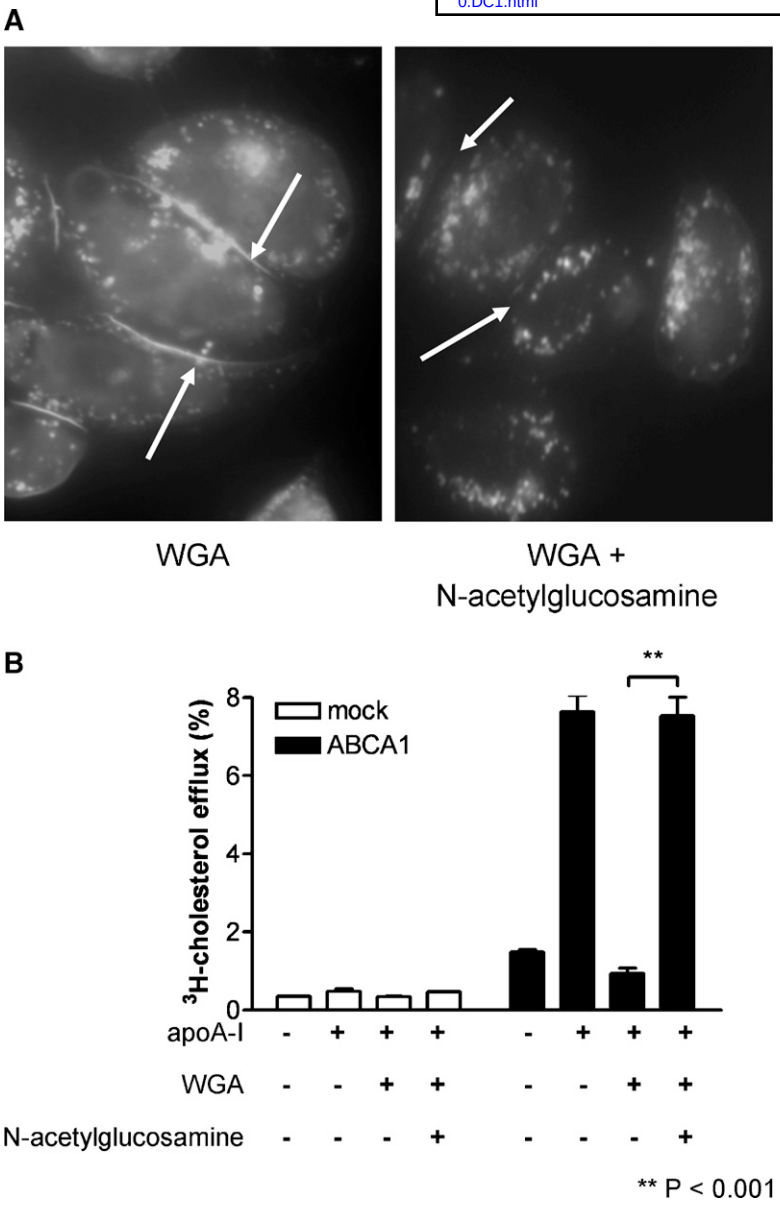

Fig. 8. Removal of WGA from the plasma membrane restores normal cholesterol efflux. A: BHK-ABCA1 cells were incubated with Alexa 488-WGA (10 μg/ml) for 2 h (left panel). Cells were then changed into WGA-free medium containing N-acetylglucosamine (200 μM) for another 2 h (right panel) before fluorescence microscopy observation. B: As indicated, ^{3}H-cholesterol efflux was measured in BHK cells with or without apoA-I (10 μg/ml) for 2 h; or with apoA-I plus WGA (10 μg/ml) for 2 h; or in cells preincubated for 2 h with WGA, followed by 2 h efflux with apoA-I plus N-acetylglucosamine (200 μM).

moving surface-bound WGA (Fig. 8B). N-acetylglucosamine alone had no effect on efflux (see supplementary Fig. IVA).

To control for the possible indirect effects of WGA binding, we also tested another lectin, soy bean agglutinin, that binds to surface glycoproteins but does not rigidify the plasma membrane (30). Soy bean agglutinin had no effect on cholesterol efflux (see supplementary Fig. IVB), indicating that the inhibition of efflux by WGA was not simply due to its glycoprotein-binding capacity.

Aside from WGA, other classes of molecules are also known to decrease or increase the fluidity of the plasma membrane. Cholesteryl hemisuccinate (CH) is slightly more hydrophilic than cholesterol but capable of placing the rigid ring structures of its cholesteryl moiety deeper into the bilayer (31). For this reason, CH increases membrane rigidity (31, 32). The addition of CH to ABCA1 expressing cells significantly inhibited cholesterol efflux (**Fig. 9A**). The cholesterol efflux was fully restored upon removal of CH. (see supplementary Fig. IVC). Together with our observations from WGA, we conclude that rigidifying the membrane has a general inhibitory effect on cholesterol release to the medium.

The effects of compounds that soften the plasma membrane were subsequently evaluated. For this purpose, two membrane fluidizers, benzyl alcohol (33) and hexanol (34), were employed. Benzyl alcohol is known to insert into the bilayer and disturb phospholipid packing in the acyl region of the bilayer membrane due to its short length (3–4 methylene groups) (35). Hexanol belongs to a group of primary alkanols with an intermediate methylene chain length that reduces the phospholipid packing efficiency (36). Both reagents significantly enhanced the release of ^{3}H-cholesterol to the medium in the presence and absence of apoA-I (Fig. 9B, C). Interestingly, membrane fluidizers had little effect on mock-transfected cells, suggesting that a more fluid membrane is necessary but not sufficient to

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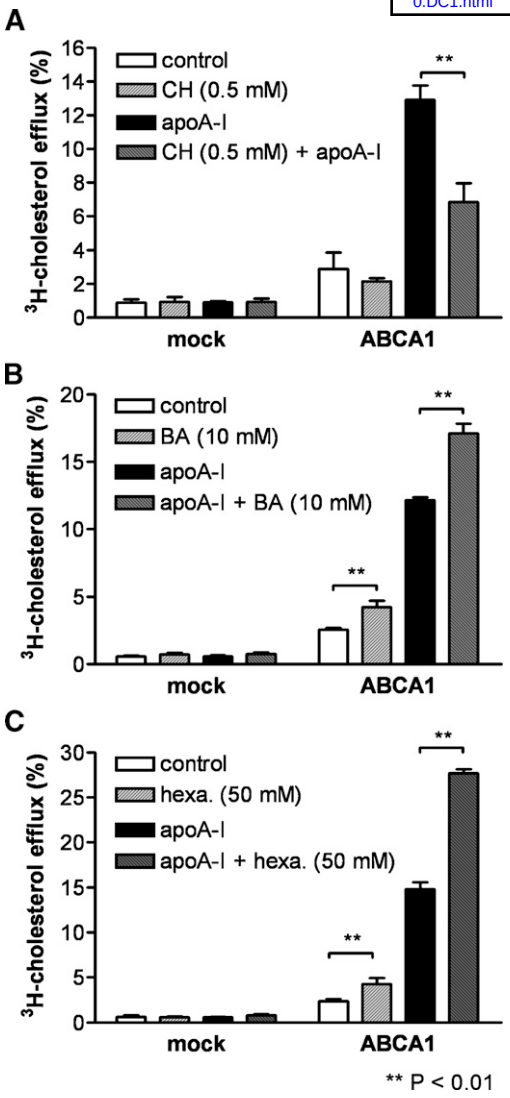

Fig. 9. Membrane rigidifiers and fluidizers modulate cholesterol release to medium. BHK cells were labeled with 1 μCi/ml ^{3}H-cholesterol for 2 days and ABCA1 expression was induced. Cells were pretreated for 2 h, and further coincubated for 2 h in the absence or presence of 10 μg/ml apoA-I in medium containing the following agents: cholesteryl hemisuccinate (CH) (500 μM), a membrane rigidifier (A); benzyl alcohol (10 mM) (B) and hexanol (50 mM) (C), both membrane fluidizers, enhanced cholesterol release to medium in ABCA1 expressing cells in the absence and the presence of apoA-I.

produce HDL or microparticles. Functional ABCA1 and proper membrane dynamics are required to initiate the release of HDL or microparticles.

DISCUSSION

In this study, we report that ABCA1-mediated cholesterol efflux produced HDL and apoA-I free microparticles from macrophages and BHK cells. We provide direct evidence that both processes relied on functional ABCA1. Furthermore, we show that proper membrane dynamics was required for HDL and microparticle formation.

Generating HDL by ABCA1-mediated cholesterol efflux to apoA-I is well established. Non-HDL microparticles, similar to these described here, have also been detected (21) and extensively characterized (22, 37). According to these studies, our non-HDL microparticles are small in size (approximately 24 nm) relative to immune-microparticles, but slightly larger than HDL with a density less than 1.21. This is consistent with the characteristics of non-HDL particles described in this report. However, this is the first study that directly links functional ABCA1 to the production of non-HDL microparticles. We provide several lines of evidence here demonstrating that these microparticles were generated by the intrinsic functions of ABCA1. First, only ABCA1-expressing cells produced measurable amounts of microparticles, independent of apoA-I. Second, a dysfunctional ABCA1 mutant, ABCA1^{A937V}, was defective in producing microparticles. This effectively rules out the possibility that microparticle release was due to nonspecific disturbances on the plasma membrane by ABCA1 through its 12-transmembrane domains. Furthermore, inhibition of PKA by PKI not only abolished cholesterol efflux to apoA-I but also impaired microparticle formation. If phosphorylation by PKA is necessary for ABCA1 function as proposed by others (17, 18), microparticle formation must also rely on properly phosphorylated ABCA1. Together with earlier characterizations by others as previously described (21, 22), we conclude that, through its intrinsic molecular activity, ABCA1 directly mediates the release of cholesterol/phospholipid-rich non-HDL microparticles approximately 20 nm in size, independent of its interaction with apoA-I.

We also provide evidence that the cholesterol/phospholipid-rich microparticles described here are entirely different entities from immune-microparticles in the circulatory system. Interestingly, ABCA1-expressing wt mice were found to have more circulating immune-microparticles upon malaria infection, in comparison with ABCA1-null animals (38). It is not clear whether macrophages contribute to immune-microparticles in circulation (platelets are the most common source of immune-microparticles) and whether smaller non-HDL microparticles can convert into larger immune-microparticles in the event of infection.

It remains to be determined how ABCA1 facilitates microparticle release. We and others (12, 13) have shown that ABCA1 expression alone (i.e., in the absence of apoA-I) can remodel the plasma membrane and shift the cells to a state with more "non-raft" microdomains. The generation of microparticles could be a direct consequence of this membrane "softening." It is noteworthy that neither ABCA1^{A937V} expressing cells nor PKI treated wt ABCA1 cells were able to remodel their plasma membrane (13) (unpublished observation). They also failed to produce microparticles. Consistent with this notion, microparticles did not depend on apoA-I for their production, nor did they contain apoA-I. Interestingly, microparticles released by J774 macrophages are also free of apoA-I (22, 37). It is thus conceivable that ABCA1 generally promotes the release of apoA-I free microparticles. Consequently, ABCA1-mediated cholesterol efflux consists of two events: *1*) lipidation of apoA-I to form nascent HDL, and *2*) generation of microparticles. These

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two events are likely independent of each other, as the presence of apoA-I did not influence the formation of microparticles. Becauase both events are sensitive to membrane rigidity, they must both rely on the dynamic membrane environment produced by ABCA1.

Current advances indicate that ABCA1 is capable of regulating the dynamics of the plasma membrane. For example, ABCA1 expression is associated with a decrease in general endocytosis in all cell models tested so far (39, 40) (our unpublished observations in BHK cells). ABCA1 expressing cells are also characterized by membrane protrusions (8, 41). Moreover, ABCA1 is frequently linked with an increased capacity for phagocytosis (42, 43). Thus, current experimental evidence supports a role for ABCA1 that favors outward bending or bulging of the plasma membrane. Such membrane dynamics could be necessary to release microparticles to the medium.

Interestingly, apoA-I may also utilize similar membrane dynamics to produce nascent HDL. ApoA-I was observed by EM to frequently bind to membrane protrusions in an ABCA1-dependent manner (11, 41). Most recently, the notion that apoA-I preferentially binds to high curvature membranes was elegantly demonstrated by Vedhachalam et al. (11). It was also proposed in the same study that high curvature membrane domains produce an "unstable" membrane environment that favors eventual "microsolubilization", a process likely necessary for apoA-I lipidation. This scenario is consistent with our observation in WGA treated cells. If ABCA1-expressing cells are indeed capable of generating high curvature membrane domains on the plasma membrane, these structures must have been in place prior to acute WGA treatment. WGA should then merely preserve these structures by limiting membrane movements. Also, because "rigidifying" the membrane by WGA is through crosslinking glycolproteins on the plasma membrane, WGA should have little effect on lipid composition in the membrane. It is therefore not surprising that apoA-I retains its favorable binding to the high curvature or loosely packed domains on the plasma membrane in WGA treated cells. WGA, however, would restrain further membrane bulging and impair the subsequent steps in apoA-I lipidation. For example, lipidation of apoA-I could further increase membrane curvature. This apoA-I-induced membrane bulging may be critical for the eventual dissociation of nascent HDL particles from the plasma membrane. We speculate that it is this loss of dynamic plasma membrane movement in WGA-treated cells that jeopardized the lipidation of apoA-I and the final departure of nascent HDL particles. The fact that apoA-I lipidation and HDL release was fully recovered after WGA removal from the plasma membrane supports this notion. Our conclusion is also consistent with recent reports that HDL is primarily generated on the plasma membrane (44, 45).

In summary, we report that ABCA1 directly induced microparticle release in addition to HDL formation. We demonstrate that ABCA1-induced plasma membrane dynamics was necessary for HDL and microparticle generation. We provide evidence indicating that such membrane dynamics were specifically required for apoA-I lipidation. Further investigation is necessary in order to define the physiological function as well as the exact origin of microparticles.

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