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ABCA1 modulates the oligomerization and Golgi exit of caveolin-1 during HDL-mediated cholesterol efflux in aortic endothelial cells

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ABSTRACT

Previously, the authors have shown that the molecular interaction between caveolin-1 and ATP-binding cassette transporter A1 (ABCA1) is associated with the high-density lipoprotein (HDL)-mediated cholesterol efflux pathway in aortic endothelial cells (ECs). This study analyzed the role ABCA1 plays in caveolin-1-mediated cholesterol efflux in aortic ECs. Knockdown of ABCA1 by siRNA in primary rat aortic ECs after cholesterol treatment did not affect caveolin-1 expression but led to the retention of caveolin-1 in the Golgi apparatus, impaired caveolin-1 oligomerization, and reduced cholesterol efflux. Immunoblotting assay and immunofluorescence microscopy demonstrated that HDL transiently up-regulated ABCA1 expression, induced caveolin-1 oligomerization, and promoted its Golgi exit, thereby enhancing cholesterol efflux. These HDL-induced events, however, were inhibited by down-regulation of ABCA1. It is concluded that HDL up-regulates ABCA1 expression, which in turn modulates the oligomerization and Golgi exit of caveolin-1 to enhance cholesterol efflux in aortic ECs.

Accumulation of excess cholesterol in the arterial wall is a major factor in the development of atherosclerosis [1]. Vascular endothelial cells (ECs) interact continuously with plasma proteins and express surface receptors for the uptake of oxidized lipoproteins. They are, however, the most resistant to cholesterol accumulation among the cells that comprise atherosclerotic plaque [2]. Cholesterol efflux from ECs may involve various mechanisms or may occur via undiscovered pathways [3].

Accelerated efflux of cholesterol is mediated by several prominent proteins [4]. Among them, caveolin-1, the main structural protein of caveolae, plays an important role in the regulation of lipid uptake as well as efflux [5,6]. Overexpression of caveolin-1 increases the number of caveolae and enhances high-density lipoprotein (HDL)-mediated cholesterol efflux in aortic ECs [7]. In addition to caveolin-1, ATP-binding cassette transporter A1 (ABCA1) also affects cellular lipid efflux in the presence of apolipoprotein A-I (apoA-I). Fibroblasts in patients with Tangier disease, a rare genetic condition caused by loss-of-function mutations in the ABCA1 transporter, show a marked defect in apoA-I- and HDL-mediated cholesterol efflux [8,9]. Orso et al. reported that cellular lipid transport from the Golgi to the plasma membrane is defective in patients with Tangier disease and in Abca1$^{-/-}$ mice, resulting in retention of caveolin-1 in the Golgi complex [10]. It has been reported that caveolin-1 and ABCA1 are expressed coordinately in differentiated THP-1 cells and that both of them promote cellular cholesterol efflux [11]. In a previous study, the authors demonstrated that caveolin-1 positively regulated the expression of ABCA1, as well as cholesterol efflux in aortic ECs. Immunoprecipitation analysis indicated an interaction between caveolin-1 and ABCA1 in the cytoplasm and in the plasma membrane after HDL incubation. The study also found that caveolin-1 was colocalized with ABCA1 and cholesterol in the perinuclear region/Golgi and on the cell surface. Blocking of intracellular lipid transport by the inhibitors monensin and brefeldin disrupted the interaction between caveolin-1 and ABCA1 and reduced cholesterol efflux to HDL [7]. From these results, it can be hypothesized that the molecular interaction between caveolin-1 and ABCA1 is associated with the HDL-mediated cholesterol efflux pathway in aortic ECs.

Oligomerization of caveolin-1 is a critical step for its exit from the Golgi to the plasma membrane. Disrupting the organization of caveolin-1 can coordinately affect oligomerization and Golgi exit of caveolin-1 [12]. Several factors have been found to mediate oligomerization of caveolin-1 [13]. Caveolin-2 and flotillin-1 can also interact with caveolin-1 to form a heteroligomer [14–16]. The relationships between caveolin-1 oligomerization and ABCA1 in cellular cholesterol efflux, however, are still unclear. In this study, small interfering RNA (siRNA) targeting ABCA1 was used to knock down ABCA1 in cholesterol-loaded aortic ECs and the effects of ABCA1 on the expression, the distribution, and the oligomerization of caveolin-1 in cellular cholesterol efflux after HDL stimulation was evaluated.

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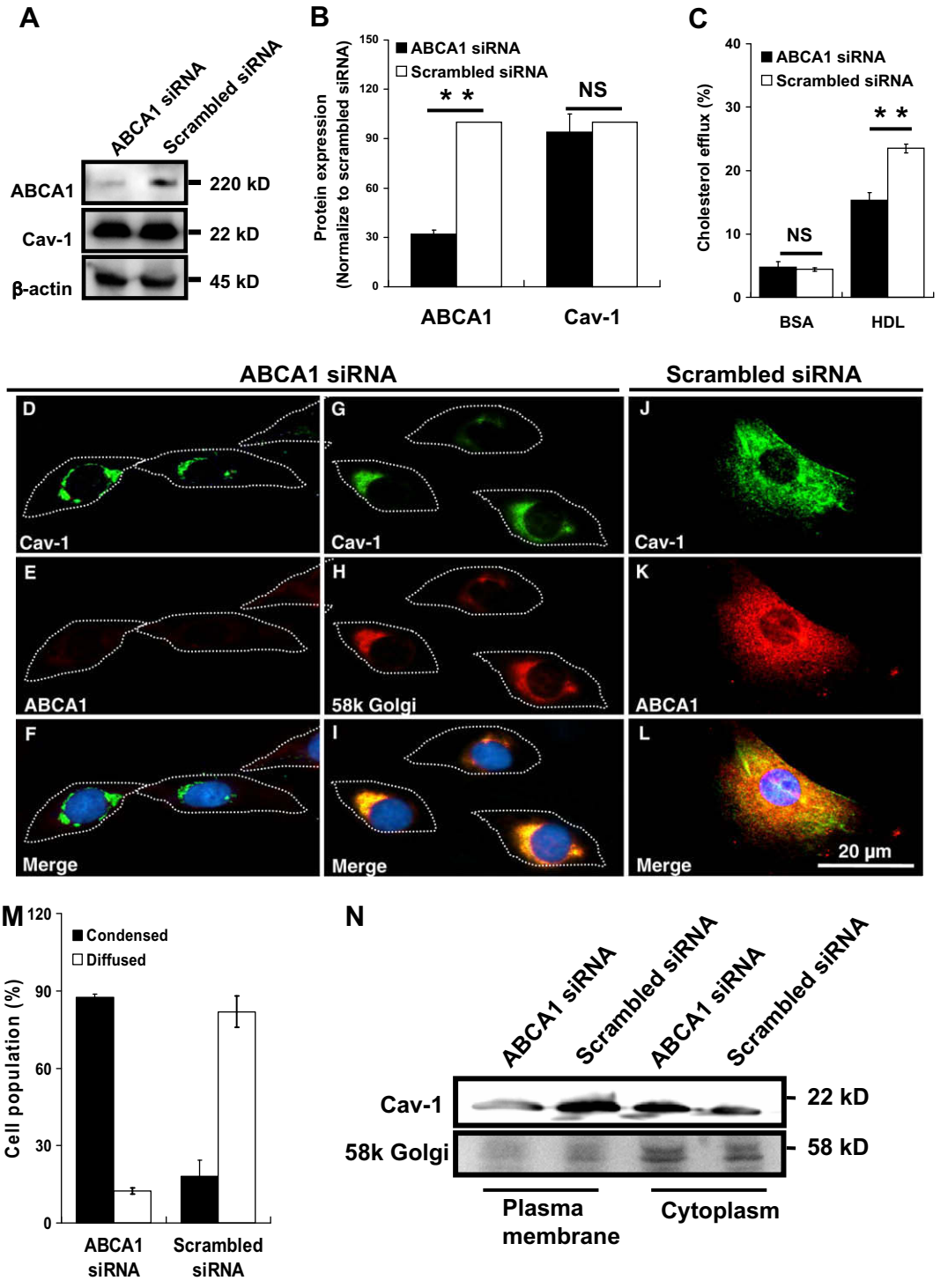

Fig. 1. ECs were transfected with ABCA1 siRNA and scrambled siRNA in the presence of cholesterol (50 μg/mL) or [^{3}H] cholesterol (0.5 μCi/mL) for 24 h. Cholesterol efflux was initiated by incubating ECs with HDL or BSA for 24 h. (A) Immunoblot analysis showing the expression levels of ABCA1 and caveolin-1 in ABCA1 siRNA and scrambled siRNA cells. (B) The quantitative data for the expression levels of ABCA1 and caveolin-1 from ABCA1 siRNA cells and scrambled siRNA cells. (C) The levels of cholesterol efflux with HDL and BSA in ABCA1 siRNA cells and scrambled siRNA cells. Results are means ± SDs from three independent experiments. $^{**}P < 0.01$ for ABCA1 expression as well as cholesterol efflux between ABCA1 siRNA group versus scrambled siRNA group. NS (non-significant difference). ECs were transfected with ABCA1 siRNA and scrambled siRNA in the presence of cholesterol (50 μg/mL) for 24 h followed by incubation with HDL (50 μg/mL) for 30 min. (D–L): Caveolin-1 (green signal) is seen as a condensed pattern in the perinuclear region of ABCA1 siRNA transfected cells (D–F). Caveolin-1 is colocalized with the 58K Golgi protein marker (red signal) in the perinuclear region of ABCA1 siRNA transfected cells (G–I). Caveolin-1 (green signal) (J) or ABCA1 (red signal) (K) in scrambled siRNA transfected cells show a diffused pattern in the cytoplasm and the plasma membrane (J–L). Nucleus (blue signal). (M) Results are presented as percentages of each caveolin-1 pattern for ABCA1 siRNA cells and scrambled siRNA cells. Results are means ± SDs from three independent experiments. (N) Immunoblotting indicates that ABCA1 siRNA cells have a lower amount of caveolin-1 in the plasma membrane and a higher amount of caveolin-1 in the cytoplasm compared with the scrambled siRNA control group. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

Materials and methods

Cell culture. Sprague–Dawley rats (4 weeks-old) were sacrificed. One-mm-thick rings were cut from the thoracic aorta and cultured at 37 °C in Dulbecco's minimum essential medium (DMEM) supplemented with 10% fetal bovine serum and penicillin–streptomycin (50 U/mL, Sigma, St. Louis, MO) in a 5% CO_2/95% air atmosphere. To establish pure endothelial cell cultures, ring explants were removed after 3–4 days of culture. Cultures exhibiting pure ECs, maintained for 2–7 passages, were used in the experiment. To establish purity, cultures were tested for the presence of factor VIII antigen using the indirect immunofluorescent technique [17]. Prior to the experiment, subconfluent monolayers of ECs were washed twice with phosphate buffered saline (PBS) containing 2 mg/mL fatty acid-free albumin (FAFA, Sigma) and incubated with DMEM containing 2 mg/mL FAFA and 50 μg/mL cholesterol in ethanol (10 mg/mL) for 48 h at 37 °C [7].

Transient transfection of siRNA. SiRNA targeting ABCA1 and rat scrambled siRNA (Dharmacon, Lafayette, CO) were used to modulate ABCA1 expression in the ECs. Twenty-four hours before transfection, 3×10^4 cells were seeded per 24-well plate. On the day of transfection, 50 nM siRNA or scrambled RNA was diluted in 25 μL of serum-free DMEM (Invitrogen, Carlsbad, CA). In a separate tube, 1 μL of lipofectamine was diluted in 25 μL of serum-free DMEM. The diluted DNA and the lipofectamine were then gently mixed and incubated at 25 °C for 20 min. After the incubation, 250 μL of serum-free DMEM was added to the DNA/lipofectamine mixture. The final mixture was added to cultured cells which had been grown on 24-well plate. After incubation at 37 °C for 5 h, the cells were added to 250 μL of DMEM containing 20% serum and 100 μg/mL cholesterol and grown for an additional 24 h.

Immunoblot analysis. Cholesterol-loaded ECs were grown in a T25 flask and incubated with DMEM containing HDL (50 μg/mL, Intracel) or BSA alone (2 mg/mL, Sigma) at 37 °C for 0, 5, 15, 30, 60, and 180 min. For detection of the oligomer protein, Dithiobis (DSP) treatment was performed as described by Denis et al. [18]. Dithiobis (DSP, Sigma) was dissolved in dimethyl sulfoxide (DMSO) immediately before use. Cells were then incubated with DSP solution for 1 h at room temperature. After incubation, the cells were washed with PBS and lysed with RIPA lysis buffer (Pierce, Rockford, IL) with protease inhibitor (1 mM phenylmethysulfonyl fluoride, 20 μg/mL leupeptin, and 20 μg/mL protein) overnight at 4 °C. The protein concentration was measured with a bicinchoninic acid (BCA) protein assay (Pierce); the proteins were then separated by 4–15% gradient sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). After electrophoresis, the proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA). Blots were probed with mouse anti-ABCA1 (1:1000, v/v, Abcam, Cambridge, UK), rabbit anti-caveolin-1 (1:5000, v/v, Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-β-actin (1:10,000, v/v, Sigma), and mouse anti-58K Golgi protein (1:3000, v/v, Abcam) primary antibodies. The appropriate horseradish peroxidase (HRP)-conjugated secondary immunoglobulin G (IgG) antibodies were visualized using an enhanced chemiluminescence reagent (PerkinElmer, Boston, MA). The intensity of reaction bands was analyzed by an Image Gauge system (Fuji, Japan).

Immunofluorescence staining. Endothelial cells grown on a cover slip in a 24-well plate were incubated with DMEM containing 2 mg/mL FAFA and 50 μg/mL cholesterol in ethanol for 48 h, then washed and fixed in 4% paraformaldehyde for 45 min at room temperature. After fixation, the cells were blocked with PBS-albumin and incubated with rabbit anti-caveolin-1 (1:200, v/v, Santa Cruz), mouse anti-ABCA1 (1:50, v/v, Abcam), and mouse anti-58K Golgi protein (1:50, v/v, Abcam) primary antibodies for 1 h at room temperature. The cells were then incubated with FITC-conjugated donkey anti-rabbit IgG (1:200, v/v, Invitrogen), and tetramethylrhodamine isothiocyanate (TRITC)-conjugated donkey anti-mouse IgG (1:200, v/v, Invitrogen) for 30 min at room temperature. The slides were mounted and visualized using a Nikon E400 immunofluorescence microscope (Nikon, Japan).

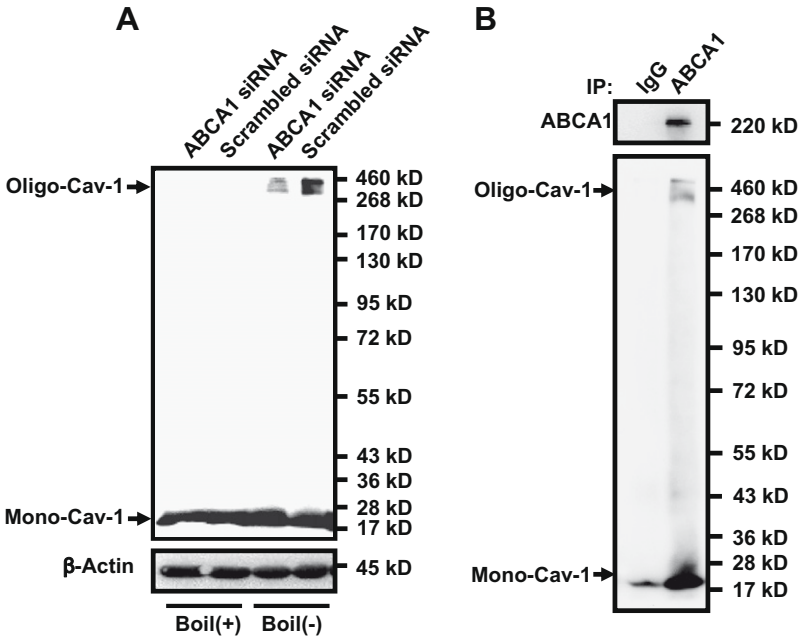

Fig. 2. ECs were transfected with ABCA1 siRNA and scrambled siRNA in the presence of cholesterol for 24 h, and then incubated with HDL for 30 min. (A) Cell lysates were treated with or without boiling. The high-molecular-weight oligomer-caveolin-1 (~460 kDa) and monomer-caveolin-1 (22 kDa) were detected using immunoblot. (B) Cell lysates were immunoprecipitated by ABCA1 antibody and non-immune IgG, respectively, and were subjected to immunoblot analysis without boiling treatment. A faint band for IgG with the same electrophoretic mobility as mono-caveolin-1 is a non-specific band. This result shows that down-regulated of ABCA1 reduces the oligomerization of caveolin-1.

Subcellular membrane fractionation. Subcellular membrane fractionation was performed as described by Lin et al. and Wu et al. and [7,19]. The scrambled siRNA transfected cells and the ABCA1 siRNA transfected cells incubated with HDL (50 μg/mL) for 30 min were harvested and lysed with cold extract solution (0.02 M boric acid, 0.3 mM EDTA, 1 mM phenylmethysulfonyl fluoride, 1 mM benzamidine, pH 10) for 15 min. The cell debris and nuclei were discarded after centrifugation at 650*g* for 10 min at 4 °C, and the supernatant was centrifuged at 12,000*g* for 1 h at 4 °C. The plasma membrane fraction was harvested as the pellet and the supernatant was concentrated using a Centriprep YM-10 centrifuge ultrafiltration apparatus (Amicon).

Immunoprecipitation analysis. Immunoprecipitation analysis was performed as described by Lin et al. [7] with minor modifications. After incubation with DSP (250 μM, Sigma) at 25 °C for 1 h, cholesterol-loaded cells were lysed with a lysis buffer (1% NP-40, 0.25% deoxycholic acid, and 15 mM imidazole) overnight at 4 °C. Immunoprecipitation was performed using a reversible immunoprecipitation system kit (Upstate Biotechnology Charlottesville, VA). The cell lysates (500 μg) were incubated with mouse anti-ABCA1 (4 μg), non-immune IgG, and antibody capture affinity ligand (1 μg) on a rocking platform at 4 °C overnight. The lysates and antibody mixture were transferred to an affinity spin column, washed three times with a wash buffer and eluted with an IP elution buffer. The eluted proteins were determined by immunoblot analysis.

Cholesterol efflux. Endothelial cells grown in a 24-well plate were incubated with DMEM containing 2 mg/mL FAFA and 0.5 μCi/mL [^{3}H] cholesterol for 24 h. Before the efflux experiment, the cells were washed with DMEM–FAFA and then incubated with DMEM–FAFA containing HDL (50 μg/mL, Intracel) or BSA (2 mg/mL) or at 37 °C for 0, 5, 15, 30, 60, or 180 min. After incubation, the medium was collected and the cells were solubilized in 0.5 N NaOH. The radioactivity of the medium and the cell extract were measured using TOPcount machinery (PerkinElmer, Boston, MA). The results represent radioactivity in the medium as a percentage of the total radioactivity (medium plus cell lysate) [3,7].

Statistics. Data are shown as means ± standard deviations (SDs). Treatment groups were compared using a 2-tailed *t*-test with SAS software.

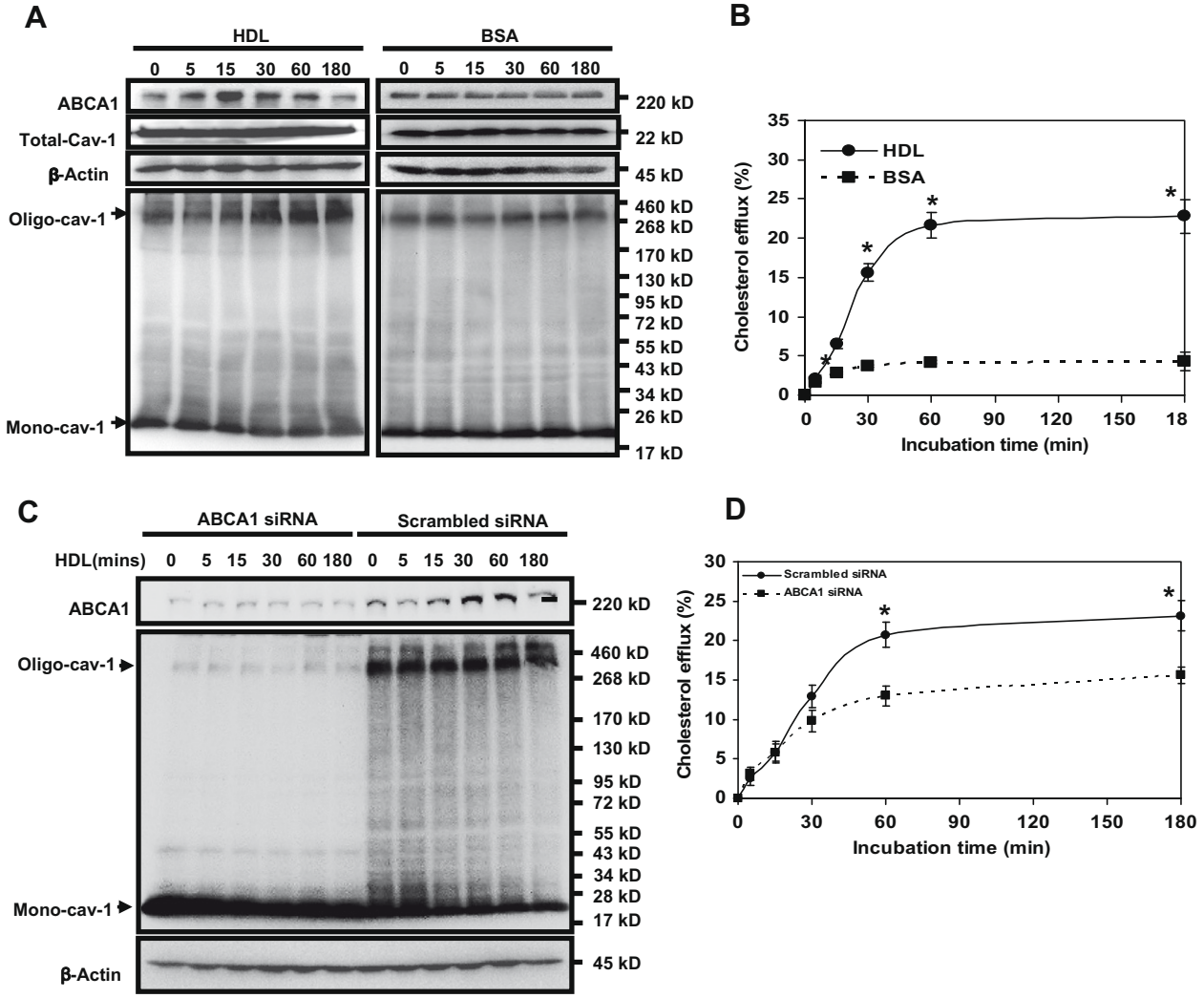

Fig. 3. Cholesterol-loaded ECs were incubated with HDL or BSA for various times followed by immunoblot analysis. (A) The results showed that ABCA1 expression increased significantly 15 min after HDL incubation and then decreased to the basal level after 60 min of incubation. Oligomer-caveolin-1 was elevated at 30 min after HDL incubation. (B) In a parallel experiment, cholesterol efflux increased rapidly during the first 30 min and reached a plateau from 60 to 180 min of HDL incubation. These HDL-induced events, however, were inhibited in ABCA1 siRNA cells (Fig. 3C,D). Results are means ± SDs from three independent experiments. $^*P < 0.05$ for HDL- versus BSA-treated group, and for ABCA1 siRNA group versus scrambled siRNA group.

Results

Caveolin-1 expression, cholesterol efflux, and cellular distribution in down-regulated ABCA1 cells

The level of ABCA1 protein expression decreased in the ABCA1 siRNA transfected cells (Fig. 1A,B). There was no significant difference in the level of caveolin-1 expression between ABCA1 siRNA transfected cells and control cells. Down-regulation of ABCA1 significantly reduced the level of cholesterol efflux in the presence of HDL, but not in the presence of BSA alone (Fig. 1C). Knockdown of ABCA1 led to retention of caveolin-1 in the perinuclear region (Fig. 1D–F). Colocalization of caveolin-1 and the 58K Golgi protein marker indicated that a large amount of the caveolin-1 was retained in the Golgi apparatus in ABCA1 siRNA transfected cells (Fig. 1G–I). Caveolin-1 was present in the cytoplasm and in the plasma membrane in the scrambled siRNA transfected cells after HDL incubation (Fig. 1J–L). Quantitative data indicated that approximately 88% of the ABCA1 siRNA transfected cells showed a condensed pattern of caveolin-1 in the perinuclear region and approximately 85% of the control cells showed a diffused pattern of caveolin-1 in the cytoplasm (Fig. 1M). Western blot analysis also showed that less caveolin-1 existed in the plasma membrane and more caveolin-1 in the cytoplasm of ABCA1 siRNA transfected cells than in the control cells (Fig. 1N). According to these data, ABCA1 is necessary for the exit of caveolin-1 from the Golgi in aortic ECs.

Down-regulation of ABCA1 reduces the oligomerization of caveolin-1

The large-molecular-weight oligomer-caveolin-1 (~460 kDa) and monomer-caveolin-1 (22 kDa) were detected without boiling treatment; only the monomer-caveolin-1 was detected after boiling treatment (100 °C for 6 min) (Fig. 2A). The amount of oligomer-caveolin-1 was significantly lower in ABCA1 siRNA transfected cells than in control cells. These results indicate that knockdown of ABCA1 inhibits the oligomerization of caveolin-1. The immunoprecipitation assay results showed that the ABCA1 protein interacted with both oligomer-caveolin-1 and monomer-caveolin-1 (Fig. 2B). It appears that ABCA1 modulated caveolin-1 oligomerization and physically interacted with oligomer-caveolin-1.

HDL-induces ABCA1 expression, caveolin-1 oligomerization, and cellular cholesterol efflux

After finding that HDL is effective in inducing cholesterol efflux in aortic ECs (Fig. 1C), the relationship between ABCA1 expression and caveolin-1 oligomerization in HDL-mediated cholesterol efflux was analyzed. The results showed that ABCA1 expression increased significantly 15 min after HDL incubation and then decreased to the basal level after 60 min of incubation. (Fig. 3A). There was no significant difference in the levels of total caveolin-1 expression after HDL treatment. Oligomer-caveolin-1 was elevated at 30 min after HDL incubation (Fig. 3A). In a parallel experiment, cholesterol efflux increased rapidly during the first 30 min after HDL incubation and reached a plateau from 60 to 180 min of HDL incubation

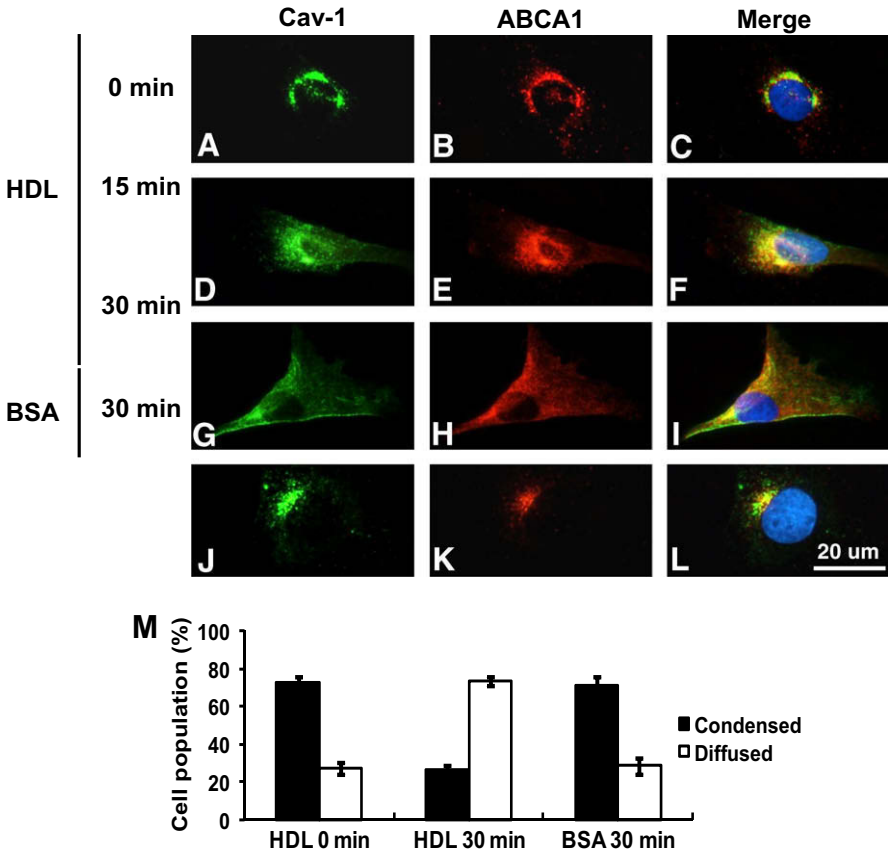

Fig. 4. Cholesterol-loaded ECs were incubated with HDL or BSA for various times. (A–C) Before HDL treatment, caveolin-1 was colocalized with ABCA1 in the Golgi apparatus. (D–F) After 15 min of HDL incubation, diffuse patterns of caveolin-1 and ABCA1 were revealed in the cytoplasm. (G–I) After 30 min of HDL incubation, caveolin-1 colocalized with ABCA1 in the cytoplasm and plasma membrane. (J–L) Caveolin-1 and ABCA1 were retained in the perinuclear region after 30 min of BSA treatment. (M) The percentages of caveolin-1 patterns after HDL and BSA treatments for various times are shown. Results are means ± SDs from three independent experiments.

(Fig. 3B). These data indicate that HDL was effective in inducing ABCA1 expression, promoting caveolin-1 oligomerization, and enhancing cholesterol efflux in cholesterol-loaded aortic ECs. These HDL-induced events, however, were inhibited in siRNA transfected cells (Fig. 3C,D).

HDL promotes caveolin-1 and ABCA1 translocation from Golgi to the plasma membrane

The locations of caveolin-1 and ABCA1 after HDL incubation were examined using immunofluorescence microscopy. Caveolin-1 was predominantly colocalized with ABCA1 in the perinuclear region before HDL incubation (Fig. 4A–C). The Golgi apparatus was confirmed in the perinuclear region using the 58K Golgi protein marker (data not shown). Diffuse patterns of caveolin-1 and ABCA1 began to appear in the cytoplasm after incubation with HDL for 15 min (Fig. 4D–F) After a 30-min incubation with HDL, diffuse patterns of caveolin-1 and ABCA1 were seen in the cytoplasm and in the plasma membrane (Fig. 4G–I). However, caveolin-1 remained in the Golgi apparatus after BSA treatment (Fig. 4J–L). Quantitative data indicated that caveolin-1 and ABCA1 were concentrated in the perinuclear region in approximately 73% of the ECs before HDL or BSA treatment (Fig. 4M). After 30 min of HDL incubation, a diffuse pattern of caveolin-1 was seen in approximately 74% of the ECs. Caveolin-1 and ABCA1 were still concentrated in the perinuclear region in 71% of the ECs after 30 min of BSA treatment (Fig. 4M). These data indicate that HDL promotes caveolin-1 and ABCA1 translocation from the Golgi to the plasma membrane.

Discussion

Accelerated efflux of cellular cholesterol is mediated by several proteins [3,20]. In this study, we show that ABCA1 plays a role in the oligomerization and Golgi exit of caveolin-1 during cholesterol efflux in aortic ECs after HDL stimulation. In addition, HDL is an effective stimulator of cholesterol efflux in ECs.

The results of the present study indicate that cholesterol efflux rapidly increased and reached a maximum within the first 30 min after HDL incubation. The underlying mechanism by which cholesterol is translocated from the cytoplasm to the plasma membrane after HDL incubation remains unclear. Mendez et al. have demonstrated that HDL regulates the efflux of cholesterol by elevating the level of protein kinase C activity, which in turn stimulates the translocation of intracellular cholesterol to the plasma membrane [21]. Recent studies have reported that PKC signaling stabilizes ABCA1 and increases ABCA1 protein expression [22]. The present data indicate that ABCA1 expression was inhibited by a PKC inhibitor, sphingosine, after HDL stimulation (Supplemental Fig. 1), suggesting that HDL may increase ABCA1 expression via the PKC signaling pathway.

Previous studies indicate that caveolin-1 oligomerization is important for its intracellular transport from the Golgi to the plasma membrane [15,23,24]. Several factors have been found to modulate the oligomerization of caveolin-1. The addition of cholesterol [13] and LDL (data not shown) can accelerate the process of oligomerization. Glycosphingolipid depletion can inhibit oligomerization [25]. This study demonstrated that ABCA1 interacts with caveolin-1 to form the heteroligomer. Whether the caveolin-1/ABCA1 association occurs in the caveolae and how ABCA1 affects caveolin-1 oligomerization in the caveolae remains to be investigated.

The present results show that the level of ABCA1 expression was highest at 15 min after HDL incubation and then gradually decreased (Fig. 3A,B). The level of oligomer-caveolin-1 was elevated at 30 min and remained at that level throughout the experiment. It appears that ABCA1 expression is important in the initiation of caveolin-1 oligomerization. ABCA1 expression reached its highest level 30 min after HDL incubation in cells treated with scrambled siRNA (Fig. 3C). This delay may be due to the transfection process. Further investigation of the mechanism of ABCA1-dependent caveolin-1 oligomerization is necessary to better understand HDL-mediated cholesterol efflux in aortic ECs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.03.005.

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