High-Density Lipoprotein-Associated α-Tocopherol Uptake by Human Retinal Pigment Epithelial Cells (ARPE-19 Cells): the Irrelevance of Scavenger Receptor Class B, Type I

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Received January 14, 2009; accepted March 18, 2009; published online March 19, 2009

The purpose of this study was to determine the high-density lipoprotein (HDL)-associated α-tocopherol (α-tocopherol-HDL) transport and clarify the contribution of scavenger receptor class B, type I (SR-BI) to the uptake in the human retinal pigment epithelial cell line (ARPE-19 cells). [^{3}H]α-Tocopherol-HDL uptake into ARPE-19 cells seeded onto a transwell from both the apical (apical-to-cell) and basal compartment (basal-to-cell) exhibited a time-dependent increase for 90 min and there was a reduction at 4 °C. These results indicate the involvement of carrier-mediated process in α-tocopherol-HDL uptake in ARPE-19 cells. Immunoblot analysis revealed that SR-BI protein was expressed in ARPE-19 cells. However, the uptake of [^{3}H]α-tocopherol from the apical or basal compartment was hardly inhibited by block lipid transport-1 (BLT-1), a specific inhibitor of the SR-BI-mediated lipid transfer. In conclusion, ARPE-19 cells have a carrier-mediated transport mechanism of [^{3}H]α-tocopherol-HDL regardless of any SR-BI-mediated process.

Key words α-tocopherol; outer blood-retinal barrier; scavenger receptor class B type I

Vitamin E is one of the essential micronutrients composed of lipid-soluble tocopherols and tocotrienols which play a key role in protecting the body from reactive oxygen species.[1,2] The retina is an ideal environment for the generation of reactive oxygen species because light is always focused on it.[3] Hence, an efficient vitamin E supply to the retina is important for protecting it from oxidative damage. α-Tocopherol is the most abundant vitamin E in the mammalian body and it has the highest biological activity.[1] In addition, α-tocopherol in plasma is associated with lipoproteins because it is hydrophobic.[4] Therefore, it is important to identify the transport mechanism of lipoprotein-associated α-tocopherol for efficient α-tocopherol supply to the retina.

The nonspecific passage of substances to the retina from the circulating blood is restricted by the blood-retinal barrier (BRB), which consists of retinal pigment epithelial cells (RPE, outer BRB) and retinal capillary endothelial cells (inner BRB).[5] In contrast, the BRB expresses various transporters which play essential roles in supplying nutrients to the retina.[6—9] Such cumulative evidence suggests that the BRB acts as an active pathway for supplying nutrients to the retina. We recently reported that the transport of high-density lipoprotein (HDL)-associated α-tocopherol (α-tocopherol-HDL) is mediated by scavenger receptor class B, type I (SR-BI) at the inner BRB.[10] In addition, α-tocopherol distribution of RPE cells is highest in the ocular tissues of the animals receiving a vitamin E-containing diet.[11] Therefore, we hypothesized that the outer BRB has an α-tocopherol-HDL transport mechanism in addition to the inner BRB.

The purpose of this study was to determine the α-tocopherol-HDL transport and clarify the contribution of SR-BI to the uptake in a cultured human RPE cell line (ARPE-19 cells).

MATERIALS AND METHODS

Reagents α-Tocopherol [6,7-^{3}H] ([^{3}H]α-tocopherol, 60 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO, U.S.A.). [^{3}H]α-Tocopherol-HDL was generated in all experiments by incubation of an ethanolic α-tocopherol solution with HDL from human plasma (1.5 mg protein; Merck, Darmstadt, Germany) in 2 ml extracellular fluid (ECF) buffer (122 mM NaCl, 25 mM $NaHCO_3$, 3 mM KCl, 1.4 mM $CaCl_2$, 1.2 mM $MgSO_4$, 0.4 mM K_2HPO_4, 10 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES), pH 7.4) at 37 °C for 3 h. Non [^{3}H]α-tocopherol-HDL was removed by size-exclusion chromatography on a PD-10 column (GE Healthcare, Little Chalfont, U.K.). All other chemicals used were commercially available and of reagent grade.

Cell Culture ARPE-19 (American Type Culture Collection, Manassas, VA, U.S.A.), the human retinal pigment epithelial cell line,[12] was used to characterize the α-tocopherol-HDL transport at the outer BRB. The ARPE-19 cell line was derived from the globes of a 19-year-old male donor. ARPE-19 cells express the mRNAs of typical RPE markers such as cellular retinaldehyde-binding protein and RPE-specific protein 65 kDa.[12] In addition, the cells seeded onto the transwell show the polarity.[12] ARPE-19 cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2. Cells were grown in a rat tail collagen I-coated 100 mm dish (BD Biosciences, Bedford, MA, U.S.A.) and maintained in Dulbecco's modified Eagle's medium (DMEM) : nutrient mixture F12 [1 : 1] (GIBCO, Grand Island, NY, U.S.A.), supplemented with 10% fetal bovine serum (Moregate, Bulimba, Australia).

Uptake Study of α-Tocopherol-HDL in ARPE-19 Cells The uptake study was performed as described previously with minor modifications.[10,13] Cells (3×10^5 cells/cm^2) were cultured at 37 °C for 6 d on 12 mm transwell fitted with 0.4

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μm pore polyester membrane inserts (Corning, Lowell, MA, U.S.A.) and washed with ECF buffer at 37 °C. Subsequently, ECF buffer containing [^{3}H]α-tocopherol-HDL (0.5 μCi/ml, 10 μM) was added to either the apical (500 μl) or basal compartment (1.5 ml) at 37 °C or on ice (4 °C). The study of [^{14}C]α-tocopherol-HDL uptake using a conditionally immortalized rat retinal capillary endothelial cell line (TR-iBRB2 cells), as an *in vitro* inner BRB model indicated the time-dependent uptake for 90 min.[10] Hence, the velocity of [^{3}H]α-tocopherol uptake in ARPE-19 cells was calculated by the uptake amount at 20 min and 90 min. For the inhibition study, ARPE-19 cells were preincubated with ECF buffer in the presence or absence of block lipid transport-1[14] (BLT-1; Chembridge, San Diego, CA, U.S.A.) at 37 °C for 60 min and the uptake was subsequently measured in the presence or absence of BLT-1 at 37 °C for 90 min. After a predetermined time period, uptake was terminated by removing the solution and cells were immersed in ice-cold ECF buffer. The cells were then solubilized in 1 N NaOH and subsequently neutralized with 1 N HCl. Radioactivity was measured by liquid scintillation counting and the protein content was determined using a kit (DC; Bio-Rad, Hercules, CA, U.S.A.) with bovine serum albumin as a standard.

Immunoblot Analysis Protein preparation and immunoblot analysis was performed as described previously.[10] In brief, the crude membrane fraction was prepared from ARPE-19 cells and liver from six-week-old male ddY mice (Nippon SLC, Hamamatsu, Japan; this investigation conformed to the provisions of the Animal Care Committee, University of Toyama (#2008P-2)). Protein samples (25 μg) were separated by 7.5% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (SDS-PAGE, Bio-Rad) and subsequently electrotransferred to nitrocellulose membranes (Bio-Rad). These membranes were incubated with a blocking agent solution (Block Ace; Dainippon Sumitomo Pharma, Osaka, Japan), prior to incubation with rabbit polyclonal anti-SR-BI antibody (1 : 2000, Novus Biologicals, Littleton, CO, U.S.A.) or mouse monoclonal anti-β-actin antibody (1 : 1000, Sigma) at 4 °C for 16 h. The membranes were subsequently incubated with horseradish peroxidase conjugated anti-rabbit or mouse immunoglobulin (Ig)G. The bands were visualized using an enhanced chemiluminescence kit (GE healthcare).

SR-BI siRNA Transfection into ARPE-19 Cells The short interfering RNA (siRNA) targeted to human SR-BI mRNA was designed according to the report of Voisset *et al.*[15] The siRNA sequence containing 3′-dTdT extensions (5′-GCAGCAGGUCCUUAAGAACTT-3′) was chemically synthesized by Japan Bio Services (Saitama, Japan). Double-stranded siRNA was generated according to the manufacturer's instructions. According to the manufacturer, non-specific control siRNA Duplex VIII (Dharmacon, Lafayette, CO, U.S.A.) exhibits no RNA interference (RNAi) effect. ARPE-19 cells were plated on a rat tail collagen type I-coated six-well plate (Asahi Techno Glass, Tokyo, Japan) at 4×10^5 cells/well, and grown for 24 h at 37 °C in medium. SR-BI and non-specific control siRNAs were then transfected using Lipofectamine 2000 and Opti-MEM I reduced serum medium (Invitrogen, Carlsbad, CA, U.S.A.). Protein expression was examined 32 h after transfection. Protein samples (25 μg) of whole cell lysate were prepared from siRNA-treated ARPE-19 cells. Immunoblot analysis was performed as described above.

Data Analysis The uptake of radiolabeled α-tocopherol-HDL was expressed as the cell/medium ratio (μl/mg protein). All data represent the mean±S.E.M. Statistical significance of differences was determined using an unpaired two-tailed Student's *t*-test.

RESULTS AND DISCUSSION

To characterize the α-tocopherol uptake in the outer BRB, [^{3}H]α-tocopherol-HDL uptake was investigated using ARPE-19 cells seeded onto transwell. As shown in Fig. 1A, [^{3}H]α-tocopherol-HDL uptake into ARPE-19 cells from both the apical (apical-to-cell) and basal compartment (basal-to-cell) exhibited a time-dependent increase for 90 min at 37 °C. Moreover, [^{3}H]α-tocopherol-HDL uptake was reduced by 85% from apical compartment (Fig. 1B) and by 92% from basal compartment (Fig. 1C) for 90 min at 4 °C compared with that at 37 °C. These results suggest that a carrier-mediated process is involved in α-tocopherol-HDL uptake into ARPE-19 cells. In addition, it is suggested that α-tocopherol-HDL in circulating blood is taken up into RPE cells because α-tocopherol-HDL uptake from the basal compart-

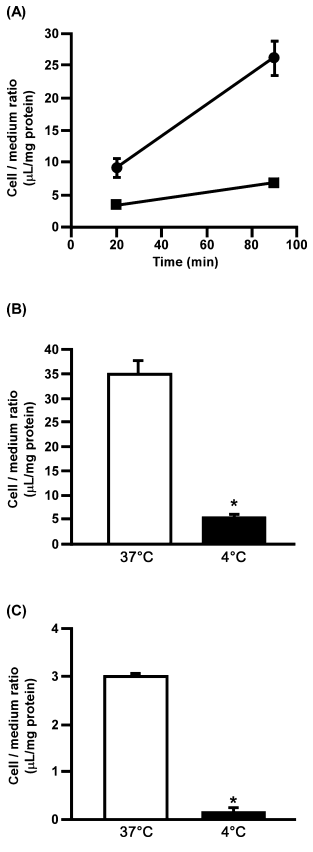

Fig. 1. The Assessment of High-Density Lipoprotein-Associated [^{3}H]α-Tocopherol ([^{3}H]α-Tocopherol-HDL) Uptake Directionality and Characteristics

(A) [^{3}H]α-Tocopherol-HDL (10 μM of [^{3}H]α-tocopherol) uptake into ARPE-19 cells seeded on transwell was performed at 37 °C. Time-dependent [^{3}H]α-tocopherol-HDL uptake from the apical (closed circle) or basal compartment (closed square) was exhibited. Each point represents the mean±S.E.M. (n=3). (B, C) [^{3}H]α-Tocopherol-HDL uptake from the apical (B) or basal compartment (C) was performed at 4 °C for 90 min. Each column represents the mean±S.E.M. (n=3—4). $*p<0.01$, significantly different from that at 37 °C.

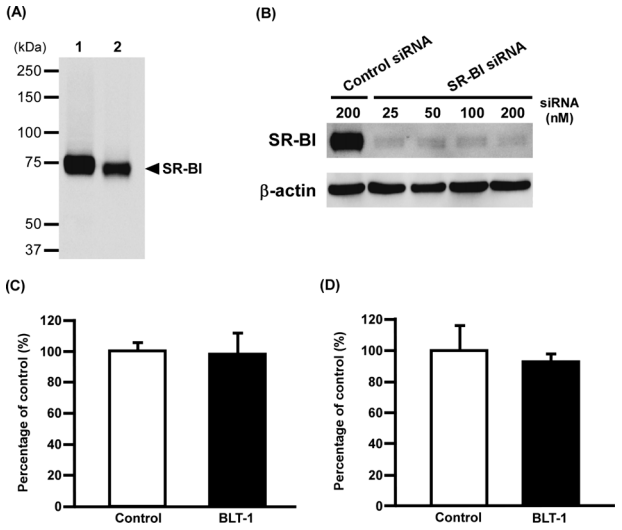

Fig. 2. Scavenger Receptor Class B, Type I (SR-BI) Expression and Effect of SR-BI Inhibition on [^{3}H]α-Tocopherol-HDL Uptake into ARPE-19 Cells

(A) Immunoblot analysis of SR-BI was performed using the protein sample (25 μg) of the crude membrane fraction of mouse liver (lane 1) and ARPE-19 cells (lane 2). Mouse liver was used as a positive control. (B) Immunoblot analysis of SR-BI and β-actin in whole lysate of ARPE-19 cells. ARPE-19 cells were treated with different concentrations of SR-BI specific siRNA or non-specific siRNA (control siRNA). (C, D) Effect of block lipid transfer-1 (BLT-1), a specific SR-BI inhibitor, on [^{3}H]α-tocopherol-HDL uptake into ARPE-19 cells from the apical (C) or basal compartment (D). Each column represents the mean$\pm$S.E.M. (n=3).

ment into ARPE-19 cells was found to take place (Figs. 1A, C).

The velocity of [^{3}H]α-tocopherol-HDL uptake from the apical and basal compartment was $2.42\times10^{-1}\pm0.52\times10^{-1}$ μl/(min·mg protein) and $4.49\times10^{-2}\pm1.05\times10^{-2}$ μl/(min·mg protein), respectively (Fig. 1A). This result suggests that α-tocopherol is eliminated from the neural retina *via* the outer BRB. However, [^{3}H]α-tocopherol-HDL applied to the apical chamber does not appear to the basal chamber (data not shown). Therefore, the most of α-tocopherol-HDL taken up from the apical side is probably stored in ARPE-19 cells. Apolipoproteins involved in the maturation of HDL are expressed in the mammalian retina.[16] In addition, the mRNA expression of ATP binding cassette transporter A1, which is involved in the generation of the α-tocopherol-HDL *via* an apolipoprotein A-I-mediated process,[17] was detected in the human neural retina.[16] Therefore, α-tocopherol-HDL generated in the retina could be taken up into RPE cells to protect them against oxidative stress or for storage if necessary. These findings suggest that the human outer BRB controls α-tocopherol-HDL uptake from circulating blood by a carrier-mediated process and also regulates the amount of the α-tocopherol in the retina.

SR-BI is related to α-tocopherol-HDL transport in brain capillary endothelial cells.[18] Accordingly, we tested the hypothesis that α-tocopherol-HDL was taken up into ARPE-19 cells by SR-BI. The expression of SR-BI protein was determined in ARPE-19 cells by immunoblot analysis. The single band of SR-BI protein was detected in all samples at about 75 kDa (Fig. 2A). This result is inconsistent with the previous report that very little SR-BI protein was expressed in ARPE-19 cells.[16] In order to validate the band of SR-BI protein, human SR-BI specific siRNA treatment was performed using ARPE-19 cells. After SR-BI siRNA treatment from 25 to 200 nM for 36 h, the band intensity at about 75 kDa was markedly attenuated (Fig. 2B). These results show that SR-BI proteins are indeed expressed in ARPE-19 cells.

We next examined the effect of BLT-1, which is a specific inhibitor of SR-BI-mediated lipid transport,[14] on [^{3}H]α-tocopherol-HDL uptake into ARPE-19 cells. The uptake of [^{3}H]α-tocopherol-HDL in the presence of 1 μM BLT-1 into ARPE-19 cells from both the apical (Fig. 2C) and basal compartment (Fig. 2D) was not significantly reduced compared with the uptake in the absence of BLT-1. This concentration of BLT-1 was higher than the reported IC_{50} value for the uptake of [^{14}C]α-tocopherol-HDL using TR-iBRB2 cells, used as an *in vitro* model of the inner BRB.[10] Therefore, it appears that ARPE-19 cells are able to perform α-tocopherol-HDL transport regardless of the SR-BI-mediated pathway, although SR-BI proteins were found to be expressed in ARPE-19 cells (Figs. 2A, B). The plasma membrane localization of SR-BI proteins was regulated by many factors such as anchor proteins.[19] Although the expression level of these proteins in ARPE-19 cells is still not fully understood, it has a probability that SR-BI is not localized at the plasma membrane in the cells because anchor proteins are little expressed, so that SR-BI-mediated α-tocopherol-HDL uptake was not shown.

It was reported that α-tocopherol transfer protein is related to α-tocopherol transport. However, this protein is not responsible for the α-tocopherol-HDL transport. In addition, to our knowledge, there is no information about the molecules which transport or bind to the α-tocopherol-HDL. Thus, unidentified molecule(s) in ARPE-19 cells could be carried out the α-tocopherol-HDL transport. Further studies are needed to characterize unidentified molecules involved in HDL-associated lipids transport including α-tocopherol-HDL at the outer BRB.

In conclusion, ARPE-19 cells carry out α-tocopherol transport regardless of any SR-BI-mediated process. We have reported that the transport of α-tocopherol-HDL is mediated by SR-BI at the inner BRB. Our present study suggests that the outer BRB exhibits different mechanisms of α-tocopherol-HDL uptake from the inner BRB. For an effective α-tocopherol supply to the retina to protect it from oxidative stresses, it is important to identify the molecules involved in α-tocopherol-HDL transport at the outer BRB.

Acknowledgement This study was supported by a Grant-in-Aid for Scientific Research from Japan Society for the Promotion of Science (JSPS).

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