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Itinerary of high density lipoproteins in endothelial cells

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ABSTRACT

High density lipoprotein (HDL) and its main protein component apolipoprotein A-I (ApoA-I) have multiple anti-atherogenic functions. Some of them are exerted within the vessel wall, so that HDL needs to pass the endothelial barrier. To elucidate their itinerary through endothelial cells (ECs), we labelled ApoA-I and HDL either fluorescently or with 1.4 nm nanogold and investigated their cellular localization by using immunofluorescent microscopy (IFM) and electron microscopy (EM). HDL as well as ApoA-I is taken up by ECs into the same route of intracellular trafficking. Time kinetics and pulse chase experiments revealed that HDL is trafficked through different vesicles. HDL partially co-localized with LDL, albumin, and transferrin. HDL did not co-localize with clathrin and caveolin-1. Fluorescent HDL was recovered at small proportions in early endosomes and endosome to trans-golgi network vesicles but not at all in recycling endosomes, in late endosomes or lysosomes. EM identified HDL mainly in large filled vesicles which however upon IFM did not colocalize with markers of multivesicular bodies or autophagosomes. The uptake or cellular distribution of HDL was altered upon pharmacological interference with cytochalasine D, colchicine and dynasore. Blockage of fluid phase uptake with Amiloride or EIPA did not reduce the uptake of HDL. Neither did we observe any co-localization of HDL with dextran as the marker of fluid phase uptake. In conclusion, HDL and ApoA-I are internalized and trafficked by endothelial cells through a non-classical endocytic route.

1. Introduction

The endothelium is a dynamic and selective barrier and its permeability is highly regulated under physiological and pathological conditions. In the subendothelial space of arteries, lipids and cholesterol as well as protein aggregates [1] can be stranded and accumulate extracellularly or in macrophages. Eventually, a plaque is formed in the intima leading to atherosclerosis. Many epidemiological studies showed an inverse correlation between high density lipoprotein (HDL) cholesterol concentration and cardiovascular events [2]. Therefore and due to its biological impacts HDL is believed to protect from atherosclerosis. Of note, HDL removes cholesterol from macrophage foam cells of the arterial wall for reverse transport to the liver [3,4]. Beyond this classical function, HDL has many anti-oxidative, anti-inflammatory, and anti-thrombotic activities [5].

Many of these functions happen inside the vascular wall or other peripheral tissues rather than in the blood stream. Therefore, HDL has to pass the endothelium of blood [6] and lymphatic vessels [7,8] to enter and leave the extravascular compartments, respectively.

The endothelium is actively regulating the passage of any molecule or cell. Endothelial cells form the glycocalyx, which is a thick steric filter [9,10] as well as an interaction platform for a variety of proteins in the blood stream. Furthermore, ECs regulate transcellular and paracellular transport by internalizing cargo through endocytic receptors, opening and closing of channels, as well as tight and adherence junctions.

Previously, we showed by biochemical methods and RNA interference that both lipid-rich HDL and lipid free ApoA-I are transported through endothelial cell layers by regulated processes: ApoA-I is first lipidated by the ATP binding cassette transporter (ABC) A1 prior to its transport through the endothelial cells [11]. Transendotelial HDL transport is modulated by ABCG1, scavenger receptor B I (SR-BI), endothelial lipase (EL) [6], and the ectopic beta-ATPase/purinergic receptor axis [12]. None of these proteins is a good candidate to directly mediate holoparticle uptake. They appear rather to indirectly modulate transendothelial transport of HDL by signalling or altering the structure of HDL.

In this study we applied microscopic techniques to better characterize the transendothelial transport of HDL.

2. Materials and methods

2.1. Cell culture

Primary bovine aortic endothelial cells (EC) were cultured in Dulbecco's modified eagle's medium (DMEM) with 5% fetal bovine

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serum albumin (BSA) at 37 °C in 5% CO_2 and passaged every 7–8 days 1:1. A 10 cm cell culture treated petri dish was populated with 2.5–3 × 10^6 cells.

2.2. Preparation and labeling of lipoproteins and marker proteins

HDL and LDL were isolated from normolipidemic human plasma (blood bank of cantonal hospital Schaffhausen) by sequential ultracentrifugation following the protocol described by Tong et al. [13] and stored at 4 °C. Lipid free ApoA-I was generated by delipidation of HDL by ethanol-ether [14] and purification over an anion exchanger column (Mono-Q) on the Äkta FPLC (GE Healthcare). Qualitative purity control was performed by SDS-PAGE, Coomassie-staining verifying the HDL and ApoA-I fractions to be void of visible albumin and ApoB (LDL) bands.

Lipoproteins were labeled with 1.4 nm nanogold (Nanoprobes USA) (Au-HDL) or fluorescent Atto dyes Atto488, Atto594, and Atto655, obtained as succimidyl-ester (Atto-Tec, Germany) and set to pH 8.0 by 1 M $NaHCO_3$ to a final concentration of 0.1 M. The dye to lipoprotein (molar) ratio was 5:1, the lipoprotein concentration in the labeling reaction was 6 g/l. The reaction was performed in the dark at room temperature (rt) for 2 h. The labeled lipoproteins were separated from free label by gel filtration chromatography (NAP5 and PD10 columns GE). Bovine serum albumin (Sigma-Aldrich, CH) and transferrin (Invitrogen) were labeled with fluorescent dyes as described for lipoproteins.

2.3. Binding and uptake studies by fluorescence light (FLM) and electron microscopy (EM)

For both fluorescence light microscopy and electron microscopy experiments, 10,000 cells were seeded 48–96 h before the experiments either onto collagen I coated transwell inserts of 24 well dishes (0.4 μm low density, BD) or round Nr 1.0 glass coverslips of 12 mm diameter (Metzler Glass/Thermo Fisher). To analyze their uptake and subcellular distribution, the labeled lipoproteins were added to the cells for 30 min at 37 °C in 5% CO2 if not otherwise indicated, followed by rinsing with ice cold PBS++ (PBS 7.4 with 1 mM $CaCl_2$ and 0.1 mM $MgCl_2$). For binding experiments, the cells were incubated at 4 °C in a humidified closed box. Subsequently, cells were either chemically fixed for fluorescence microscopy or high pressure frozen (HPF) for EM analysis.

2.4. Fixation for FLM

Chemical fixation for FLM was performed with 3.75% formaldehyde in PBS at rt for 20–30 min for monolayers of cells. The free fixative was then quenched by a brief wash with 0.5 M TrisHCL 8.0 followed by two washes with PBS. Then either additional stainings were performed or the sample was embedded directly for microscopy.

2.5. Indirect immunostainings for FLM

Coverslips or transwell inserts with fixed cells were preincubated with 0.1% saponin in PBS at pH 7.4 at room temperature for 15 min for antibody staining. The primary antibody was incubated for 1 h at rt or 16 h at 4 °C, the cells were then washed tree times with PBS and then incubated with the secondary antibody for 30–45 min at rt in the dark. Antibody dilutions were made in PBS with 1% Donkey Serum (Sigma Aldrich) and 5% BSA. Optimal antibody titers were determined beforehand by testing different dilutions ranging from 1:200 to 1:10,000. Subsequently, the coverslips were directly mounted upside down on glass slides with ProLong Gold antifade solution (Life technologies, USA). Transwells were cut out with a scalpel Nr11 from the bottom side and placed with the cells upwards between a glass slide and a 25 × 50 mm Nr 1.0 coverslip (Metzler Glass/Thermo Fisher) with a drop of ProLong Gold in between.

Images were acquired on a Zeiss Axiovert 200 M with a Plan-Apochromat 40×/1.4 and Hamamatsu ORCA-ER EMCCD camera (C4742) resulting in a pixel size of 0.1577 μm. To be able to quantitatively compare conditions, fixed exposure times were used of 10 to 800 ms to have the signal histogram of the (positive) control in the middle of the 12-bit sensitivity range of the chip and this exposure was kept throughout the acquisition session.

2.6. Image processing and quantification

To quantify the HDL taken up by ECs, we segmented the cells in the monolayer and then measured fluorescence intensities per cell. Segmentation was performed using the open source FIJI is just ImageJ (FIJI) software. First the nucleus was isolated in the DAPI channel by the Otsu thresholding method. Based on the nucleus and using the Voronoi algorithm the cells were approximated. For quantification, only the cells, which were completely in the field of view, were considered. The mean fluorescence signal per cell was calculated for at least 200 cells per condition and experiment.

When co-localization was neither complete nor absent upon visual inspection, partial co-localization was quantified by using FIJI's Coloc2 plugin to calculate the Pearson's correlation coefficient ρ.

2.7. High pressure freezing (HPF) and freeze substitution fixation (FSF) for EM

The membrane of the transwell containing a monolayer of endothelial cells was punched out with a biopsy-punch of 5 mm diameter. The isolated membrane disc was carefully inserted into a 6 mm aluminum specimen carrier with an indentation of 100 μm, sandwiched with a flat carrier wetted with 1-hexadecene (Sigma Aldrich, CH) and immediately high pressure frozen using an automated Leica EM HPM100 high pressure freezing machine (Leica Microsystems, Austria). Samples were stored in liquid nitrogen until further processing.

HPF frozen discs containing membranes with cell monolayers were transferred to 2 ml safe-lock Eppendorf tubes containing anhydrous acetone with 1% OsO4 at −90 °C. Substitution was performed in an automated substitution machine (Leica EM AFS) at −90 °C for 7 h, −60 °C for 6 h, −30 °C for 5 h and at 0 °C for 1 h with transition gradients of 30 °C per hour.

2.8. Silver enhancement

The reagents (Sigma Aldrich, CH) were prepared in individual tubes as saturated solutions of hydroquinone, malic acid, and silver nitrate in anhydrous acetone at room temperature. Arabic gum was dispersed in anhydrous acetone and shaken for 1 h at room temperature. The Arabic gum dispersion was used to protect the colloid from aggregation by inhibiting the self-nucleation of silver.

After slowly cooling the saturated solutions to 0 °C during one hour, equal parts of all saturated solutions and the Arabic gum suspension were mixed at 0 °C. The mixture was centrifuged at 300 *g* for 5 min at 4 °C to remove any precipitates and was always freshly prepared before use.

After FSF the samples were washed three times with anhydrous acetone and the enhancement mixture was added to the reaction tubes for 30 min. Afterwards, the treated samples were washed again three times with anhydrous acetone before embedding in epoxy resin. All steps were performed at 0 °C on ice water.

2.9. Embedding and preparation for TEM and EM image acquisition

Samples in anhydrous acetone were embedded in Epon/Araldite (EA) essentially as described by Hohenberg et al. [15,16] by incubating the samples in 33% and 66% EA in acetone for 8 h each prior to transfer in 100% EA and polymerization at 60 °C for 40 h.

Ultrathin cross sections of cells of 50 nm were cut with a 45° diamond knife (Diatome) using an ultramicrotome (Reichert) and put on Formvar coated single slot grids (Ted Pella inc. USA).