Published in final edited form as:

Curr Pharm Biotechnol. 2012 February ; 13(2): 331–340.

Combined Light and Electron Microscopy using Diaminobenzidine Photooxidation to Monitor Trafficking of Lipids Derived from Lipoprotein Particles

Clemens Röhrl¹, Claudia Meisslitzer-Ruppitsch², Robert Bittman³, Zaiguo Li³, Georg Pabst⁴, Ruth Prassl⁴, Witta Strobl¹, Josef Neumüller², Adolf Ellinger², Margit Pavelka², and Herbert Stangl^{1,*}

¹Institute of Medical Chemistry, Center for Pathobiochemistry and Genetics, Medical University of Vienna, Vienna, Austria

²Department of Cell Biology and Ultrastructure Research, Center for Anatomy and Cell Biology, Medical University of Vienna, Vienna, Austria

³Department of Chemistry and Biochemistry, Queens College of the City University of New York, Flushing, New York, USA

⁴Institute of Biophysics and Nanosystems Research, Austrian Academy of Sciences, Graz, Austria

Abstract

Diaminobenzidine (DAB) photooxidation is a method for conversion of fluorescent signals into electron-dense precipitates that are visible in the electron microscope. Recently, we have applied this method to analyze organelles involved in holo-high density lipoprotein (HDL) particle uptake at the ultrastructural level. In the present work we extended the spectrum of molecules visualized via photooxidation to monitor the uptake of HDL-derived lipids in HepG2 cells. By the combined light-electron microscopic method and with the aid of the DAB photooxidation technique, it became possible for the first time to visualize different intracellular pathways of lipoprotein particle-derived lipids and analyze the compartments involved at the ultrastructural level. HDL-Alexa 568 was used to visualize holo-HDL particle uptake. Reconstituted HDL particles containing the fluorescent cholesterol analogues Bodipy-cholesterol, Bodipy-cholesteryl oleate, or cholesteryl Bodipy-ester were used to visualize uptake of the HDL-associated sterol. In Bodipy-cholesteryl oleate and cholesteryl Bodipy-ester, the cholesterol moiety or the fatty acid moiety is fluorescently labeled, respectively; in contrast, Bodipy-cholesterol is an analogue of free cholesterol. The cellular compartments involved in their intracellular routes after uptake were analyzed in the fluorescence and electron microscope after DAB photooxidation. Bodipy-cholesterol was found to be localized in tubular endosomes and multivesicular bodies (MVBs), in the *trans*-Golgi network, and in stacked Golgi cisternae. In contrast, HepG2 cells incubated with HDL containing Bodipy-cholesteryl oleate or cholesteryl Bodipy-ester gave an uptake pattern comparable to that of holo-HDL particles, with MVBs being involved. Bodipy-cholesteryl oleate was also found in lysosomes. These results indicate that HDL-derived cholesterol and cholesteryl ester are transported by different intracellular pathways in HepG2 cells. Thus, the DAB photooxidation method enables the analysis of intracellular transport of lipoprotein particle-derived lipids at the light and at the ultrastructural level.

*Corresponding Author: Herbert Stangl, Institute of Medical Chemistry, Center for Pathobiochemistry and Genetics, Medical University of Vienna, Währingerstr. 10, 1090 Vienna, Austria. Tel.: + 43 1 4277 60823; Fax: + 43 1 4277 60881, Herbert.stangl@meduniwien.ac.at.

Keywords

Bodipy; cholesterol; cholesteryl ester; diaminobenzidine (DAB) photooxidation; HepG2; high density lipoprotein (HDL); reconstituted HDL particles; transmission electron microscopy

Introduction

Cholesterol is essential for mammalian cell membrane rigidity and dynamics and is also an important metabolite, serving as the precursor for hormone and bile acid synthesis. Cellular cholesterol homeostasis is tightly regulated and many redundant pathways are used to control the cholesterol status. Cholesterol is synthesized, mobilized from storage depots, or internalized from lipoprotein particles and then distributed to areas depleted in cholesterol through a variety of transport pathways via endosomes, lysosomes, the endoplasmic reticulum (ER), the endosomal recycling compartment, and the Golgi apparatus [1]. Ultrastructural analysis is required to identify the compartments and substructures involved in cholesterol uptake and trafficking. Therefore, methods to visualize lipids in the electron microscope are needed for a further understanding of lipoprotein and lipid trafficking.

Cholesterol surrogates

Several methods to visualize intracellular cholesterol trafficking have been established, all of which use cholesterol surrogates. Dehydroergosterol (DHE), a fluorescent cholesterol analogue, has been used in several studies to monitor intracellular cholesterol movement at the resolution of light microscopy [2-5]. However, DHE has unfavorable spectroscopic properties such as UV excitation and rapid bleaching. Thus sophisticated instrumentation (i.e., two-photon microscopy, which is not readily available in many laboratories) is necessary to visualize DHE. Most fluorescent sterol analogues that can be excited with visible light have limitations related to the steric bulk of the fluorophore moiety. For example, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino (NBD)-cholesterol and some Bodipy (a dipyrrometheneboron difluoride chelate (4,4-difluoro-4-bora-3a,4a-diaza-s-indacene))-tagged sterol analogues show a different membrane partitioning compared to cholesterol itself [2]. It was shown that 22-NBD-cholesterol adopts a reverse orientation within phospholipid bilayers [6].

Many probes have been employed for electron microscopic visualization of cholesterol: Möbius et al. [7, 8] used a biotinylated cholesterol-binding toxin (perfringolysin O) to demonstrate the localization of cholesterol at the ultrastructural level. However, perfringolysin O detects cholesterol mainly in cholesterol-rich membranes [9]. Second, cholesterol oxidase was used to visualize free cholesterol in Leydig cells [10]. Similarly to perfringolysin, cholesterol in some endocytic membranes was resistant to oxidation [11, 12]. Third, filipin, a sterol-binding polyene antibiotic, was used to stain cholesterol-rich membranes that can be visualized by electron microscopy in negatively stained membranes, thin sections, and by freeze-fracture. This procedure was used to show cholesterol in the epidermis of *Tenebrio molitor* [13]. However, filipin is cytotoxic, and high autofluorescence levels occur as it is excited using UV light [14, 15]. All of these methods demonstrate the localization of endogenous free (unesterified) cholesterol; however, other methods are needed to analyze the lipid cargo being transferred from lipoprotein particles to cells.

Recently, Bodipy-cholesterol, in which carbon-24 of the cholesterol side chain is linked covalently to a Bodipy moiety, was synthesized [16]. Bodipy-cholesterol was shown to behave in a similar manner to free cholesterol, and its photostable characteristics allowed the visualization of sterol movement in living cells in the light microscope [17]. Thus, we sought to determine if this sterol analogue is suitable for 3,3'-diaminobenzidine (DAB)

photooxidation, which permits visualization of fluorescent signals in the electron microscope. Our findings indicate that DAB photooxidation of fluorescent cholesterol analogues provides an effective method to investigate lipid transfer from lipoprotein particles to cells.

Diaminobenzidine (DAB) photooxidation

The principle of DAB photooxidation was established in 1982 by Maranto [18] to visualize neurons injected with Lucifer yellow by electron microscopy; the fluorescent signals of neurons were converted into electron-dense precipitates visible at the ultrastructural level [18]. Fluorophores are employed to oxidize DAB, affording insoluble granular precipitates that are osmiphilic and can thus be visualized in the electron microscope. The combination of light microscopical techniques with electron microscopy allows a detailed analysis of the uptake and intracellular pathways of labeled proteins and lipids; for a review, see [19]. Using DAB photooxidation, Tabas et al. [20] demonstrated that DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate)-LDL and DiI- β -VLDL exhibited different uptake patterns in macrophages. LDL was degraded more rapidly, whereas β -VLDL appeared in a distinct set of widely distributed vesicles [20]. Furthermore, Bodipy-ceramide was used to delineate ceramide transport to the Golgi apparatus [21]; after 30 min of incubation, DAB deposits were visualized in the luminal space of cisterns of the *trans*-Golgi side [19, 22].

We have successfully applied correlative microscopy using HDL-Alexa 568 driven DAB photooxidation to demonstrate HDL particle uptake and intracellular movement in HepG2 cells at the ultrastructural level [23]. HDL-positive compartments were mainly identified as multivesicular bodies (MVBs). Their luminal content but not the internal vesicles were DAB-positive. Labeled MVBs increased in numbers and changed their shapes over time; polymorphic organelles with multiple surface domains and differently shaped protrusions dominated at earlier time points, while more compact bodies with tubular appendices and densely packed vesicles occurred at later time points [23]. Such detailed structural investigations require the resolution of electron microscopy. Here we show that the combination of light and electron microscopical methods provides structural information ranging from life cell microscopy to high resolution electron microscopy.

In the present work, we describe the steps of DAB photooxidation involved in visualization of fluorescent cholesterol surrogates in the electron microscope, making high resolution analysis of intracellular lipid trafficking possible. We used Bodipy-cholesterol as an analogue of free cholesterol and two differently labeled analogues of cholesteryl esters in HDL (for structures, see Fig. 1): On the one hand, we used cholesteryl Bodipy-ester, in which the fatty acid is the carrier of the label; this compound is commercially available and is widely used. We also synthesized and used Bodipy-cholesteryl oleate, in which the cholesterol moiety bears the label.

Methods

Fluorescent cholesterol and cholesteryl ester analogues

Green fluorescent free cholesterol with boron dipyrromethene difluoride linked to sterol carbon-24 (Bodipy-cholesterol) was synthesized as described [16]; for structure see Fig. 1, left. Bodipy-cholesteryl oleate was synthesized as follows: To a solution of BODIPY-cholesterol (2.9 mg, 5 μ mol) and oleic acid (2.1 mg, 7.5 μ mol) in dry dichloromethane (2 ml) dicyclohexylcarbodiimide (4.1 mg, 20 μ mol) and 4-(*N,N*-dimethylamino)pyridine (0.5 mg, 4.1 μ mol; all from Sigma, St. Louis, MO) were added. The solution was stirred at room temperature until the disappearance of Bodipy-cholesterol (monitored by TLC, using

hexane/ethyl acetate 30/1 as the mobile phase). Water (3 ml) was added, and the organic layer was dried with anhydrous sodium sulfate. The solvent was removed, and the residue was purified by flash chromatography on silica gel (mobile phase: a gradient of hexane (100%) to hexane/ethyl acetate 30/1), affording 3.4 mg of Bodipy-cholesteryl oleate (yield, 81%). The structure (Fig. 1, middle) was confirmed by ^1H and ^{13}C NMR spectroscopy and high-resolution mass spectrometry: ^1H NMR spectra were recorded at 400 MHz and ^{13}C NMR spectra were recorded at 100 MHz on a Bruker Avance I spectrometer. Electrospray ionization high-resolution mass spectrometry was performed on an Agilent Technologies mass spectrometer. Red fluorescent cholesteryl Bodipy-ester (cholesteryl 4,4-difluoro-5-(4-methoxyphenyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoate, Fig. 1, right) was purchased from Molecular Probes / Invitrogen (Eugene, OR).

HDL isolation and labeling procedures

Plasma was collected from normolipidemic healthy volunteers. HDL was recovered by serial ultracentrifugation at a density of 1.21 g/ml [24]. The apolipoproteins of HDL were covalently labeled with Alexa 568 (Molecular Probes / Invitrogen) at pH 8.4 according to the manufacturer's instructions. Unbound dye was removed by column chromatography on Sephadex G-25 (Sigma) as described previously [23].

Reconstituted HDL (rHDL) particles were prepared by a modified detergent reconstitution method [25]. Briefly, HDL was delipidated twice with ethanol:diethyl ether (3:2) at -20°C for 2 h, dried under nitrogen, and resuspended in buffer A (150 mmol/l NaCl, 0.01% EDTA, 10 mmol/l Tris/HCl, pH 8.0). Egg phosphatidylcholine (PC, 2.8 mg), cholesterol (67 μg), and cholesteryl oleate (500 μg ; all from Sigma) were dissolved in CHCl_3 :MeOH (2:1), mixed, and the solvents were evaporated. The dried lipid film was resuspended in 200 μl of buffer A. Cholesterol or cholesteryl oleate was replaced by Bodipy-cholesterol, Bodipy-cholesteryl oleate, or cholesteryl Bodipy-ester, respectively. After sodium deoxycholate (50 μl of a 30 mg/ml suspension in buffer A) was added to disperse the lipids, the mixture was stirred at 4°C for 2 h. Delipidated HDL (250 μl of a 4 mg/ml suspension in buffer A) was added, and the mixture was stirred at 4°C overnight. The calculated molar ratio of PC:cholesterol:cholesteryl oleate:protein was 100:48:22:1. Extensive dialysis was performed to separate rHDL from the free dye and detergent. For dual-label experiments, the apoproteins of either rHDL Bodipy-cholesterol or rHDL Bodipy-cholesteryl oleate or rHDL cholesteryl Bodipy-ester were covalently labeled with Alexa 633 as described for Alexa 568 (see above).

X-ray scattering

Small-angle x-ray scattering (SAXS) experiments were performed using a SWAX camera (System 3, Hecus X-ray Systems, Graz, Austria) mounted on a sealed X-ray tube generator from Seifert (Ahrensburg, Germany) that was operated at 50 kV and 40 mA. The X-ray beam size was 0.5 mm $\times$ 3.5 mm (V $\times$ H) and was filtered for CuK_α radiation ($\lambda = 0.1542$ nm) using a Ni-foil and a pulse height discriminator. SAXS patterns were recorded using a linear, one-dimensional, position-sensitive detector (PSD 50, Hecus X-ray Systems, Graz, Austria). Silver stearate was used for detector calibration.

HDL (5.2 mg/ml) and rHDL (2.7 mg/ml) were placed in thin-walled 1-mm reusable quartz glass capillaries, sealed, and measured for 3 h at room temperature. The background corrected data were analyzed in terms of two models. For dilute, non-interacting samples, the scattered intensity is

$$I(q) \propto |F(q)|^2 = P(q) \quad (1)$$

where q is the modulus of the scattering vector ($q = 4 \pi / \lambda \sin \theta$) and $F(q)$ is the particle form factor. HDL was modeled as a spherical particle with an inner hydrophobic core. In this case, it has been shown [26] that

$$F(q) \propto \left\{ \frac{V_{\text{sphere}} [\sin(qr_{\text{sphere}}) - qr_{\text{sphere}} \cos(qr_{\text{sphere}})]}{(qr_{\text{sphere}})^3} + \Delta\rho \frac{V_{\text{core}} [\sin(qr_{\text{core}}) - qr_{\text{core}} \cos(qr_{\text{core}})]}{(qr_{\text{core}})^3} \right\} \quad (2)$$

V_{sphere} and V_{core} are the volumes of the particle and the hydrophobic core, respectively, and r_{sphere} and r_{core} the corresponding radii; $\Delta\rho = \rho_{\text{shell}} - \rho_{\text{core}}$ is the difference of the electron densities of the outer monolayer and the hydrophobic core.

The scattered intensity of the reconstituted HDL particles was modeled as disks as described in [27, 28].

$$P(q) = \int_0^{\pi/2} g^2(q, \alpha) \sin \alpha \, d\alpha \quad (3)$$

where

$$g(q, \alpha) = (\rho_{\text{core}} - \rho_{\text{shell}}) V_{\text{core}} \frac{\sin(x_1)}{x_1} \frac{J_1(y_1)}{y_1} + \rho_{\text{core}} V_{\text{disk}} \frac{\sin(x_2)}{x_2} \frac{J_1(y_2)}{y_2} \quad (4)$$

ρ_{core} and ρ_{shell} are the average electron densities of the hydrophobic core and hydrophilic shell, and V_{core} and V_{disk} are the volumes of the hydrocarbon core and the disk, respectively. J_1 denotes the first order Bessel function and

$$\begin{aligned} x_1 &= \frac{qL}{2} \cos \alpha \\ x_2 &= q(L/2 + t) \cos \alpha \\ y_1 &= qR \sin \alpha \\ y_2 &= q(R + t) \sin \alpha \end{aligned} \quad (5)$$

with L being the thickness of the hydrocarbon core, t the thickness of the hydrophilic layer, and R the disk radius. The form factor is obtained by numerical integration of Eq. (3).

Additionally, we account for heterogeneous HDL and rHDL size distributions by incorporating the Schultz distribution function

$$f(r) = \frac{p^{-2/p^2} \left(\frac{r}{\langle R_0 \rangle} \right)^{(1-p^2)/p^2} \exp \left(-\frac{r}{p^2 \langle R_0 \rangle} \right)}{\langle R_0 \rangle \Gamma(p^{-2})} \quad (6)$$

where Γ is the Gamma function, $p = \sigma / \langle R_0 \rangle$ is the polydispersity index, where σ^2 refers to the variance of R and $\langle R_0 \rangle$ represents the average sphere or disk radius, respectively.

Cell culture

Human hepatocarcinoma (HepG2) cells were cultured according to standard procedures and maintained in minimal essential medium (MEM; Invitrogen, Eugene, OR) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA), 1% nonessential amino acid solution (Sigma), and 1 mmol/l sodium pyruvate (PAA, Linz, Austria). For DAB-photooxidation experiments, the cells were seeded on cover slips in 24-well plates at a density of 5×10^5 cells / well on day 0. On day 1, the cells were washed twice with phosphate-buffered saline (PBS) and re-fed with MEM containing 10% calf lipoprotein deficient serum (cLPDS). On day 2, the cells were incubated with labeled HDL (50 $\mu\text{g/ml}$) diluted in MEM containing 2 mg/ml fatty acid free bovine serum albumin (faf-BSA; PAA)

at 37°C for 3 h. To stimulate esterification cells were kept for 16 h in MEM containing 1 mg/ml BSA and 100 µmol/l sodium oleate.

Sample preparation for DAB photooxidation

To avoid background fluorescence, fixative containing a low concentration of glutaraldehyde (maximum, 0.5%) was used. To achieve low background staining with good fine structural preservation of the cells, 4% formaldehyde/0.5% glutaraldehyde in PBS proved to be optimal. Therefore, the cells incubated with fluorescent lipoproteins were fixed with 4% formaldehyde and 0.5% glutaraldehyde in PBS at 4°C for 45 min and then were washed extensively with PBS. Cells were washed with buffer B (50 mmol/l Tris/HCl, pH 7.4) and pre-incubated with freshly prepared 1 mg/ml 3,3'-DAB (Sigma) in buffer B at room temperature for 30 min. Illumination was done in fresh DAB solutions at 4°C for 20 min with an HBO 100-W high-pressure mercury vapor lamp utilizing a 40× objective (0.60 NA) and a TRITC or FITC filter set for the Alexa 568- and cholesteryl Bodipy-ester dyes or Bodipy-cholesterol and Bodipy-cholesteryl oleate, respectively. As many fluorophores generate singlet oxygen upon illumination, resulting in local oxidation of DAB, we prepared fresh DAB solutions and stored the solutions at 4°C to minimize loss of oxygen. The intensity of irradiation depends on the objective and the singlet oxygen generation of the fluorophores, rather than on the brightness alone.

After photooxidation, the cells were washed with buffer B and post-fixed in 1% osmium-ferrocyanide (a 1:1 mixture of 2% aqueous OsO₄ and 3% K₄Fe(CN)₆·3H₂O; Merck, Darmstadt, Germany) for 15 min, followed by 1% veronal acetate buffered OsO₄ at 4°C for 30 min. After dehydration in a graded series of ethanol (50, 70, 90, 96, 100%), samples were embedded in Epon (Serva, Heidelberg, Germany). Then, 80–100 nm sections were prepared with an UltraCut-UCT ultramicrotome (Leica, Vienna, Austria), transferred to copper grids, stained with uranyl acetate and lead citrate, and investigated with a Zeiss EM900 transmission electron microscope (Zeiss, Oberkochen, Germany) at 50 kV with a wide-angle dual speed CCD camera (Albert Tröndle Dünzelbach, Moorenweis, Germany).

Fluorescence microscopy

Fixed samples were washed with PBS and water and the cover slips were mounted with Fluoprep (Biomérieux, France) on microscopy slides. Images were acquired with a Nikon eclipse TE300 microscope (Nikon, Tokyo, Japan) using a FITC (for Bodipy-cholesterol or Bodipy-cholesteryl oleate) or a TRITC (for Alexa 568 or cholesteryl Bodipy-ester) filter set. For double staining experiments, a Zeiss LSM510 confocal microscopy (Zeiss) was used; for dye excitation 488 nm (for Bodipy-cholesterol or Bodipy-cholesteryl oleate), 543 nm (for cholesteryl Bodipy-ester), and 633 nm lasers (for Alexa 633) were utilized.

Results

Size and shape of the reconstituted HDL particle

To study the transfer of fluorescent lipids from HDL particles to cells, HDL particles were loaded with Bodipy-cholesterol, Bodipy-cholesteryl oleate, or cholesteryl Bodipy-ester (for structures, see Fig. 1). For this, native HDL was delipidated and then reconstituted using deoxycholate (for details, see Methods) [25], yielding reconstituted HDL (rHDL). To compare the size and shape of rHDL with native HDL, we used X-ray small angle scattering analysis. Figure 2 shows the scattered intensities of native HDL (graph I) and rHDL (graph II), exhibiting differences as $I(q)$ decreases as a function of the scattering vector. We were able to fit both patterns with the models described in the Methods section. For native HDL, an average radius of 4.0 ± 0.3 nm, with a thickness of ~ 1.0 nm for the outer hydrophilic layer, was found, which is in agreement with previously published values [29], with a

polydispersity index of $p = 0.13$. The diameter of rHDL disks, determined from the fits, was 7.0 ± 0.3 nm. This value and the observed thickness of the disk of 3.5 ± 0.3 nm are largely consistent to previous reports [30-32]. The polydispersity index obtained from the fit was 0.1. Thus, rHDL particles are self-assembled discoidal particles with a size comparable to native HDL and seem to be promising to follow their uptake and the unloading of lipids to cells.

Uptake of HDL-Alexa 568 and rHDL-Alexa 568 by HepG2 cells

Next we aimed to investigate whether rHDL shows an uptake pattern comparable to that of HDL and thus can be used as an HDL mimetic for ultrastructural analysis. Therefore, the apoproteins of both native and rHDL particles were labeled with Alexa 568. A similar uptake pattern was found with both particles in HepG2 cells after 3 h of incubation (Fig. 3). Fluorescence microscopy showed that HDL was localized in bright fluorescent spherical organelles distributed throughout the cytoplasm and enriched in the perinuclear area for both native and reconstituted HDL (Fig. 3b,c). In both cases, multivesicular bodies (MVBs) were commonly DAB-positive (compare Fig. 3e and Fig. 3f), as analyzed by electron microscopy after DAB photooxidation. In control cells incubated without fluorophores, no DAB precipitate was seen (Fig. 3a,d). These data indicate that the reconstitution method used yielded rHDL particles with an uptake pattern resembling that of native HDL.

Transport of the fluorescent lipid surrogates to lipid droplets

It is well established that excess cellular cholesterol is esterified in the endoplasmic reticulum (ER) to be deposited in lipid droplets [33]. The esterification can be driven by the addition of sodium oleate [33]. This prompted us to test if Bodipy-cholesterol and the two cholesteryl ester surrogates contained in rHDL are deposited in lipid droplets after stimulation with oleate (Fig. 4). Therefore, HepG2 cells were incubated with 100 $\mu\text{mol/l}$ oleate for 16 h to stimulate esterification and simultaneously with rHDL labeled with either Bodipy-cholesterol (Fig. 4a,b), cholesteryl Bodipy-ester (Fig. 4c,d), or Bodipy-cholesteryl oleate (Fig. 4e,f). After supplementation with oleate, cells incubated with rHDL containing Bodipy-cholesterol display spherical organelles that resemble lipid droplets (Fig. 4b), indicating the formation of fluorescent cholesteryl ester and its deposition in lipid droplets. Similarly, the Bodipy-labeled lipid moiety derived from the cells incubated with rHDL containing both fluorescent cholesteryl esters exhibited similar perinuclear organelles after oleate treatment (Fig. 4d,f). Therefore, we conclude that fluorescently labeled cholesteryl esters taken up from HDL were hydrolyzed rapidly by a cholesteryl ester hydrolase, forming free cholesterol and fatty acid regardless of the location of the attached fluorophore in the cholesteryl ester. The resulting Bodipy-cholesterol or Bodipy-fatty acid underwent esterification, accounting for the deposits of cholesteryl ester in lipid droplets seen in Fig. 4d,f. Thus, all three surrogates or rather their hydrolysis products are transported to the ER to become esterified again after stimulation with oleate. These results indicate that the Bodipy moiety, regardless of how it is conjugated to the sterol, did not alter the behavior of the cholesterol and cholesteryl ester or Bodipy-cholesteryl oleate surrogates.

Visualization of the HDL particle and its lipid moiety in HepG2 cells

After having analyzed the fate of the protein components of HDL particles in HepG2 cells using Alexa 568 (see Fig. 3), we next visualized the uptake of the lipid moiety of HDL particles. We used Bodipy-cholesterol, Bodipy-cholesteryl oleate, and cholesteryl Bodipy-ester to follow their transfer from rHDL particles to HepG2 cells by fluorescence microscopy. Additionally, DAB photooxidation was performed to convert fluorophores into electron-dense DAB precipitates enabling the detailed identification of small cellular compartments at high resolution (Fig. 5).

Incubation with protein labeled HDL-Alexa 568 resulted in brightly stained vesicular organelles (Fig. 5a), which were identified as MVBs after DAB photooxidation (Fig. 5c). In contrast, the HDL particles labeled with Bodipy-cholesterol (Fig. 5b) gave a different cellular staining pattern; in addition to the vesicles distributed throughout the cell, the plasma membrane and in a perinuclear network were stained. Diffuse staining was observed, which likely represents non-vesicular transport. After photooxidation, MVBs, but also tubular endosomes, the *trans*-Golgi network, and stacked Golgi cisternae were positive for the DAB reaction product (Fig. 5d). Interestingly, the staining within the Golgi apparatus was generally limited to single *trans* localized cisternae (Fig. 5d, inset). In contrast, the ER and the nuclear envelope were rather infrequently positive for cholesterol.

Incubation of HepG2 cells with rHDL containing fluorescent cholesteryl Bodipy-ester resulted in a vesicular staining pattern (Fig. 5e) comparable to protein-labeled HDL-Alexa 568, but different from Bodipy-cholesterol. The electron microscopic findings again demonstrate MVBs as the main compartment involved in its uptake (Fig. 5g). HDL-derived Bodipy-cholesteryl oleate again displayed a vesicular staining pattern after uptake in HepG2 cells (Fig. 5f). However, in addition to the stained spherical structures, larger compartments, heterogeneous in size and shape, were apparent. After photooxidation, DAB staining was observed again in MVBs (Fig. 5h), and also in lysosomes (Fig. 5i), which may correspond to the large, inhomogenously stained compartments identified by fluorescence microscopy (compare Fig. 5f). The differences in the staining pattern of the two fluorescent cholesteryl esters are likely due to the different fates of the compounds after their hydrolysis: Bodipy-cholesterol released from Bodipy-cholesteryl oleate seems to accumulate in late endosomal/lysosomal vesicles while the fluorescent fatty acid, derived from cholesteryl Bodipy-ester, appears to take another path to the ER.

To directly show if there is significant concomitant uptake of lipid and HDL or if the distribution patterns of HDL and its free cholesterol and cholesteryl ester diverge, we used rHDL particles that were labeled in both the lipid and the apolipoprotein components (Fig. 6). Using rHDL labeled with both Bodipy-cholesterol and Alexa 633, we found colocalization within organelles scattered throughout the HepG2 cells after 3 h of incubation (Fig. 6c). However, cholesterol staining was seen throughout the cell, in the plasma membrane, and also in distinct organelles (Fig. 6a). These data indicate a significant extent of exchange of free cholesterol between the HDL particle and the cells.

In HepG2 cells incubated with HDL labeled with both cholesteryl Bodipy-ester and with Alexa 633, the staining pattern differed remarkably (Fig. 6d-f). Mostly vesicular staining was observed for both the apolipoprotein and the lipid components, and partial colocalization was observed. Similarly, when HDL labeled with both Bodipy-cholesteryl oleate and with Alexa 633 was used, a vesicular staining pattern was seen with partial colocalization mainly in perinuclear areas. Thus, we could clearly follow the fate of both the apoproteins of the HDL particle and its Bodipy-cholesterol, Bodipy-cholesteryl oleate, and cholesteryl Bodipy-ester, indicating that this method is suitable for following lipids derived from the HDL particle intracellularly.

Discussion

In this study, we applied a combination of light and electron microscopy to visualize the lipid cargo of HDL en route to the cell using DAB photooxidation to convert the fluorescent signals in electron-dense precipitates for electron microscopical inspection. We used fluorescent surrogates of free cholesterol and cholesteryl ester, namely Bodipy-cholesterol, Bodipy-cholesteryl oleate, and cholesteryl Bodipy-ester, respectively (Fig. 1). To incorporate labeled lipids into HDL, rHDL particles are generally used [25]. In our study,

particle analyses confirmed the formation of rHDL particles, which are similar in size to native HDL, but discoidal in shape (Fig. 2). The uptake behavior of rHDL was identical to that of native HDL (Fig. 3). Furthermore, HDL-derived Bodipy-cholesterol, Bodipy-cholesteryl oleate, and cholesteryl Bodipy-ester surrogates were transported to the ER to be (re)esterified and deposited in lipid droplets (Fig. 4), which are known to form when excess cholesterol is present [33]. These results show that reconstituted HDL particles labeled with Bodipy-cholesterol, Bodipy-cholesteryl oleate, or cholesteryl Bodipy-ester are an appropriate tool to investigate lipid transfer and subsequent cellular distribution.

rHDL particles are, in fact, important for the delineation of selective cholesteryl ester uptake, one of the major uptake pathways to supply organs such as adrenals and liver with cholesterol needed for steroid and bile acid synthesis, respectively. Glass et al. [34, 35] used spherical particles, reconstituted from rat HDL by partial delipidation and incorporation of radiolabeled cholesteryl ethers, to follow their delivery to liver, adrenal gland, and gonads of rats, demonstrating for the first time this selective cholesteryl ester uptake pathway in which the cholesteryl esters are transferred to the tissues without degradation of the HDL particle. Thus, rHDL, discoidal and spherical, apparently retain their characteristics necessary for recognition by specific receptors such as the scavenger receptor class B type I (SR-BI) and are, therefore, a valid tool to investigate lipid transfer.

The fluorescent probe DiI is often used to label the lipid part of HDL, as DiI integrates easily into the HDL particle via diffusion [36-38]. For example, Krieger et al. demonstrated the importance of SR-BI for selective cholesteryl ester uptake using DiI-labeled HDL [37]. However, DiI does not mimic cholesterol movement per se since it is a fluorophore that resembles phospholipids in structure. As stated above, fluorescent cholesterol derivatives have their limitations, thus hampering the analysis of cholesterol in living cells. Recently, Bodipy-cholesterol, a bright, photostable fluorescent sterol derivative that closely resembles free cholesterol, was synthesized [16]. It was demonstrated that this cholesterol analogue is packed in the membranes and behaves similarly to cholesterol both in tissue culture cells and in cells from patients with diseases in cholesterol metabolism and *in vivo* in zebrafish [17]. Furthermore, trace amounts of this sterol can be detected, thereby making visualization of its movement in living systems possible [17]. Thus, this probe mimics membrane partitioning and trafficking of cholesterol, allowing sterol movement to be monitored by time-lapse imaging [17].

Using DAB photooxidation, we analyzed the vesicles/compartments involved in cholesterol transfer (Fig. 5). A difference was seen in the staining signal on comparing fluorescence and electron microscopy (Fig. 5b vs. Fig. 5d). After unloading, the fluorescence signal of cholesterol derived from HDL is dispersed over all areas of the cells with distinct staining of the plasma membrane and the perinuclear area. Vesicular staining can be seen in the fluorescence microscope (Fig. 5b). The nature of these vesicles was identified using DAB photooxidation: endosomal vesicles, MVBs, the *trans*-Golgi network and stacked Golgi cisternae were heavily stained. In contrast, the cytoplasm, which was found to be stained by fluorescence microscopy, was devoid of structures stained by DAB-reaction products in the electron microscope (Fig. 5d). It is possible that the photooxidation method presented here does not detect fluorophores present at low concentrations in the cytoplasm.

The intracellular staining pattern of cholesteryl ester derived from HDL is strikingly different from that of cholesterol. Most notably, only a vesicular but no diffuse staining pattern can be seen (Fig. 5e, f) with multivesicular bodies as the main DAB-positive compartment visualized at the ultrastructural level (Fig. 5g, h). Almost no signal was apparent in the cytosol (Fig. 5e, f). Our data indicate that cholesterol is distributed via different pathways in HepG2 cells than cholesteryl esters, which seem to be trafficked

within the cell by vesicle-mediated transport. HDL-derived Bodipy-cholesterol was preferentially transported to intracellular compartments, which were shown to exhibit high cholesterol concentrations per se, as identified by Möbius and colleagues, who detected endogenous cholesterol with biotinylated perfringolysin O [7, 8]. We found that especially tubular endosomes and MVBs, and also the *trans* Golgi network and stacked Golgi cisternae, were positive for cholesterol. Although the ER is a central compartment in maintaining cellular cholesterol homeostasis (as the site for cholesterol synthesis and esterification), hardly any Bodipy-cholesterol was found in the ER. However, Bodipy-cholesterol was esterified when oleate was added, indicating that Bodipy-cholesterol is indeed transported to the ER, where it is rapidly esterified (Fig. 4b). This result is consistent with biochemical data, which showed that only 0.5 to 1% of the total cellular cholesterol is localized in the ER [39].

In summary, we have shown that DAB photooxidation is a powerful method to analyze the intracellular transport of HDL-derived lipids by combined light and electron microscopical methods. This methodology enables the analyses of lipid transport at the ultrastructural level and the detailed description of the cellular compartments involved in this process.

Acknowledgments

The authors thank Loredana Ionce, Ulrich Kaindl, Barbara Kornprat, Beatrix Mallinger and Regina Wegscheider for excellent technical assistance. We are grateful to Hildegard Laggner and Werner J. Kovacs for carefully reading the manuscript and scientific discussion. This project was supported by Austrian research funds (FWF) grant P20116 (C.R., C.M.-R., M.P., H.S.) and NIH Grant HL-083187 (R.B.).

References

- [1]. Ikonen E, Jansen M. Cellular sterol trafficking and metabolism: spotlight on structure. *Curr Opin Cell Biol.* 2008; 20:371–7. [PubMed: 18502634]
- [2]. Wüstner D. Fluorescent sterols as tools in membrane biophysics and cell biology. *Chem Phys Lipids.* 2007; 146:1–25. [PubMed: 17241621]
- [3]. McIntosh AL, Atshaves BP, Huang H, Gallegos AM, Kier AB, Schroeder F. Fluorescence techniques using dehydroergosterol to study cholesterol trafficking. *Lipids.* 2008; 43:1185–208. [PubMed: 18536950]
- [4]. Mukherjee S, Zha X, Tabas I, Maxfield FR. Cholesterol distribution in living cells: fluorescence imaging using dehydroergosterol as a fluorescent cholesterol analog. *Biophys J.* 1998; 75:1915–25. [PubMed: 9746532]
- [5]. Wüstner D, Mondal M, Tabas I, Maxfield FR. Direct observation of rapid internalization and intracellular transport of sterol by macrophage foam cells. *Traffic.* 2005; 6:396–412. [PubMed: 15813750]
- [6]. Scheidt HA, Muller P, Herrmann A, Huster D. The potential of fluorescent and spin-labeled steroid analogs to mimic natural cholesterol. *J Biol Chem.* 2003; 278:45563–9. [PubMed: 12947110]
- [7]. Möbius W, Ohno-Iwashita Y, van Donselaar EG, Oorschot VM, Shimada Y, Fujimoto T, Heijnen HF, Geuze HJ, Slot JW. Immunoelectron microscopic localization of cholesterol using biotinylated and non-cytolytic perfringolysin O. *J Histochem Cytochem.* 2002; 50:43–55. [PubMed: 11748293]
- [8]. Möbius W, van Donselaar E, Ohno-Iwashita Y, Shimada Y, Heijnen HF, Slot JW, Geuze HJ. Recycling compartments and the internal vesicles of multivesicular bodies harbor most of the cholesterol found in the endocytic pathway. *Traffic.* 2003; 4:222–31. [PubMed: 12694561]
- [9]. Waheed AA, Shimada Y, Heijnen HF, Nakamura M, Inomata M, Hayashi M, Iwashita S, Slot JW, Ohno-Iwashita Y. Selective binding of perfringolysin O derivative to cholesterol-rich membrane microdomains (rafts). *Proc Natl Acad Sci U S A.* 2001; 98:4926–31. [PubMed: 11309501]

- [10]. Mendis-Handagama SM. Peroxisomes and intracellular cholesterol trafficking in adult rat Leydig cells following Luteinizing hormone stimulation. *Tissue Cell*. 2000; 32:102–6. [PubMed: 10798324]
- [11]. Lange Y, Echevarria F, Steck TL. Movement of zymosterol, a precursor of cholesterol, among three membranes in human fibroblasts. *J Biol Chem*. 1991; 266:21439–43. [PubMed: 1939176]
- [12]. Lange Y, Swaisgood MH, Ramos BV, Steck TL. Plasma membranes contain half the phospholipid and 90% of the cholesterol and sphingomyelin in cultured human fibroblasts. *J Biol Chem*. 1989; 264:3786–93. [PubMed: 2917977]
- [13]. Berdan RC, Shivers RR. Filipin-cholesterol complexes in plasma membranes and cell junctions of *Tenebrio molitor* epidermis. *Tissue Cell*. 1985; 17:177–87. [PubMed: 4012757]
- [14]. Severs NJ. Cholesterol cytochemistry in cell biology and disease. *Subcell Biochem*. 1997; 28:477–505. [PubMed: 9090304]
- [15]. Gimpl G, Gehrig-Burger K. Cholesterol reporter molecules. *Biosci Rep*. 2007; 27:335–58. [PubMed: 17668316]
- [16]. Li Z, Mintzer E, Bittman R. First synthesis of free cholesterol-BODIPY conjugates. *J Org Chem*. 2006; 71:1718–21. [PubMed: 16468832]
- [17]. Hölttä-Vuori M, Uronen RL, Repakova J, Salonen E, Vattulainen I, Panula P, Li Z, Bittman R, Ikonen E. BODIPY-cholesterol: a new tool to visualize sterol trafficking in living cells and organisms. *Traffic*. 2008; 9:1839–49. [PubMed: 18647169]
- [18]. Maranto AR. Neuronal mapping: a photooxidation reaction makes Lucifer yellow useful for electron microscopy. *Science*. 1982; 217:953–5. [PubMed: 7112109]
- [19]. Meisslitzer-Ruppitsch C, Röhl C, Neumüller J, Pavelka M, Ellinger A. Photooxidation technology for correlated light and electron microscopy. *J Microsc*. 2009; 235:322–35. [PubMed: 19754726]
- [20]. Tabas I, Lim S, Xu XX, Maxfield FR. Endocytosed beta-VLDL and LDL are delivered to different intracellular vesicles in mouse peritoneal macrophages. *J Cell Biol*. 1990; 111:929–40. [PubMed: 2391369]
- [21]. Pagano RE, Martin OC, Kang HC, Haugland RP. A novel fluorescent ceramide analogue for studying membrane traffic in animal cells: accumulation at the Golgi apparatus results in altered spectral properties of the sphingolipid precursor. *J Cell Biol*. 1991; 113:1267–79. [PubMed: 2045412]
- [22]. Meisslitzer-Ruppitsch C, Vetterlein M, Stangl H, Maier S, Neumüller J, Freissmuth M, Pavelka M, Ellinger A. Electron microscopic visualization of fluorescent signals in cellular compartments and organelles by means of DAB-photoconversion. *Histochem Cell Biol*. 2008; 130:407–19. [PubMed: 18463889]
- [23]. Röhl C, Pagler TA, Strobl W, Ellinger A, Neumüller J, Pavelka M, Stangl H, Meisslitzer-Ruppitsch C. Characterization of endocytic compartments after holo-high density lipoprotein particle uptake in HepG2 cells. *Histochem Cell Biol*. 2010; 133:261–272. [PubMed: 20039053]
- [24]. Schumaker VN, Puppione DL. Sequential flotation ultracentrifugation. *Methods Enzymol*. 1986; 128:155–70. [PubMed: 3724500]
- [25]. Jonas A. Reconstitution of high-density lipoproteins. *Methods Enzymol*. 1986; 128:553–82. [PubMed: 3724523]
- [26]. Pedersen JS. Analysis of small-angle scattering data from colloid and polymer solutions: modeling and least-square fitting. *Adv. Colloid Interface Sci*. 1997; 70:171–210.
- [27]. Nieh MP, Glinka CJ, Krueger S, Prosser RS, Katsaras J. SANS study of the structural phases of magnetically alignable lanthanide-doped phospholipid mixtures. *Langmuir*. 2001; 17:2629–38.
- [28]. Sevcsik E, Pabst G, Jilek A, Lohner K. How lipids influence the mode of action of membrane-active peptides. *Biochim Biophys Acta*. 2007; 1768:2586–95. [PubMed: 17662236]
- [29]. Laggner P, Müller KW. The structure of serum lipoproteins as analysed by X-ray small-angle scattering. *Q Rev Biophys*. 1978; 11:371–425. [PubMed: 223189]
- [30]. Jonas A, Wald JH, Toohill KL, Krul ES, Kezdy KE. Apolipoprotein A-I structure and lipid properties in homogeneous, reconstituted spherical and discoidal high density lipoproteins. *J Biol Chem*. 1990; 265:22123–9. [PubMed: 2125044]

- [31]. Silva RA, Huang R, Morris J, Fang J, Gracheva EO, Ren G, Kontush A, Jerome WG, Rye KA, Davidson WS. Structure of apolipoprotein A-I in spherical high density lipoproteins of different sizes. *Proc Natl Acad Sci U S A*. 2008; 105:12176–81. [PubMed: 18719128]
- [32]. Petrache HI, Tristram-Nagle S, Nagle JF. Fluid phase structure of EPC and DMPC bilayers. *Chem Phys Lipids*. 1998; 95:83–94. [PubMed: 9807810]
- [33]. Chang TY, Chang CC, Cheng D. Acyl-coenzyme A:cholesterol acyltransferase. *Annu Rev Biochem*. 1997; 66:613–38. [PubMed: 9242919]
- [34]. Glass C, Pittman RC, Civen M, Steinberg D. Uptake of high-density lipoprotein-associated apoprotein A-I and cholesterol esters by 16 tissues of the rat in vivo and by adrenal cells and hepatocytes in vitro. *J Biol Chem*. 1985; 260:744–50. [PubMed: 3918032]
- [35]. Glass C, Pittman RC, Weinstein DB, Steinberg D. Dissociation of tissue uptake of cholesterol ester from that of apoprotein A-I of rat plasma high density lipoprotein: selective delivery of cholesterol ester to liver, adrenal, and gonad. *Proc Natl Acad Sci U S A*. 1983; 80:5435–9. [PubMed: 6412229]
- [36]. Schouten D, Kleinherenbrink-Stins MF, Brouwer A, Knook DL, Kamps JA, Kuiper J, van Berkel TJ. Characterization in vitro of interaction of human apolipoprotein E-free high density lipoprotein with human hepatocytes. *Arteriosclerosis*. 1990; 10:1127–35. [PubMed: 2244860]
- [37]. Acton S, Rigotti A, Landschulz KT, Xu S, Hobbs HH, Krieger M. Identification of scavenger receptor SR-BI as a high density lipoprotein receptor. *Science*. 1996; 271:518–20. [PubMed: 8560269]
- [38]. Gu X, Lawrence R, Krieger M. Dissociation of the high density lipoprotein and low density lipoprotein binding activities of murine scavenger receptor class B type I (mSR-BI) using retrovirus library-based activity dissection. *J Biol Chem*. 2000; 275:9120–30. [PubMed: 10734045]
- [39]. Lange Y, Ye J, Rigney M, Steck TL. Regulation of endoplasmic reticulum cholesterol by plasma membrane cholesterol. *J Lipid Res*. 1999; 40:2264–70. [PubMed: 10588952]

Figure 1. Structures of Bodipy-cholesterol, Bodipy-cholesteryl oleate, and cholesteryl Bodipy-ester.

Figure 2. Scattered intensities of HDL isolated from plasma (I) and rHDL (II). Data have been shifted vertically by a multiplication factor ($\times 100$) for better representation. Solid lines correspond to the fits of either spherical (HDL) or discoid (rHDL) particles.

Figure 3. Reconstituted HDL particles (rHDL) show normal cellular uptake.

HepG2 cells were incubated without (control; a, d), with native HDL-Alexa 568 (b, e), or with rHDL-Alexa 568 (c, f) for 3 h. HDL and rHDL exhibit the same uptake pattern; bright vesicular organelles enriched in the perinuclear area are shown by fluorescence microscopy (b, c). Prominent positive structures are MVVs for both native HDL (e) and rHDL (f), as shown by DAB photooxidation and electron microscopy. Control cells are devoid of the intracellular reaction product (d). Bar: 5 μ m (a-c), 250 nm (d-f).

Figure 4. Effect of oleate supplementation on esterification of fluorescent lipids.

Bodipy-cholesterol (a, b), cholesteryl Bodipy-ester (c, d), and Bodipy-cholesteryl oleate (e, f) derived from rHDL are esterified and deposited in lipid droplets in HepG2 cells. Cells were incubated with (b, d, f) and without (a, c, e) 100 $\mu\text{mol/l}$ sodium oleate (which stimulates esterification) for 16 h together with rHDL. Note that after stimulation with oleate (b, d, f), lipid droplets are loaded with Bodipy-containing lipid, indicating that the modified lipids show their natural behavior. Bar: 5 μm .

Figure 5. Cellular localization of the HDL protein and HDL-derived lipids.

HepG2 cells were incubated with fluorescently labeled lipoproteins for 3 h. HDL was labeled with Alexa 568 at the apolipoprotein moiety to analyze holo-particle uptake (a, c), or with fluorescent free cholesterol (b, d), cholesteryl Bodipy-ester (e, g), and Bodipy-cholesteryl oleate (f, h, i) to visualize HDL-associated lipids and their intracellular transport. Images were acquired by fluorescence microscopy (a, b, e, f) or after DAB photooxidation by electron microscopy (c, d, g, h, i). Cholesterol is transported to MVBs, and also to tubular endosomes, the *trans*-Golgi network, and Golgi cisternae (d, inset). The cholesterol uptake pattern differs substantially from the pattern seen with either HDL labeled in the apoproteins or in the cholesteryl esters. Bar: 5 μ m (a, b, e, f), 250 nm (c, d, g, h, i).

Figure 6. Visualization of dual-labeled rHDL in HepG2 cells.

Cells were incubated for 3 h with the fluorescent dual-labeled rHDL particles. Alexa 633 dye was covalently linked to the protein component of rHDL (b, e, h, red), and the lipid moiety was labeled with either Bodipy-cholesterol (a), cholesteryl Bodipy-ester (d), or Bodipy-cholesteryl oleate (g). For overlay, see panels c, f, and i. Note that there is colocalization of Alexa-HDL labeled with the cholesteryl esters and Alexa-HDL labeled with Bodipy-cholesterol but their pattern of distribution is different. These data demonstrate a difference in the uptake pathway of free cholesterol and cholesteryl ester derived from the HDL particle. Bar: 5 μ m.