Mechanisms of Selective Delivery of Xanthophylls to Retinal Pigment Epithelial Cells by Human Lipoproteins

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FOOTNOTES
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2Abbreviations: AMD, age related macular degeneration; AREDS2, Age-Related Eye Disease Study 2; BCO2, β-carotene 9'-10'-oxygenase; BLT-1, blocking lipid transport 1; Cameo2, high-density lipoprotein receptor-2; CAREDS, Carotenoids in Age-Related Macular Degeneration Study; CD36, lipid transporters cluster determinant 36; GSTP1, glutathione S-transferase; LDLR, low density lipoprotein receptor; LIMP-2, lysosome membrane protein 2; MTBE, methyl-tertiary-butyl-ether; RLBP1, retinaldehyde binding protein 1; RPE, retinal pigment epithelium; RPE65, retinal pigment epithelium specific protein 65 kDa; SCRBI5, scavenger receptor class B member 15; SAA, serum amyloid A; SR-B1, scavenger receptor class B1; StARD, steroidogenic acute regulatory domain.

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Abstract
The xanthophylls, lutein and zeaxanthin, are dietary carotenoids that selectively accumulate in the macula of the eye providing protection against age-related macular degeneration (AMD). To reach the macula, carotenoids cross the retinal pigment epithelium (RPE). Xanthophylls and β-carotene mostly associate with HDL and LDL, respectively. HDL binds to cells via a scavenger receptor class B1 (SR-B1)-dependent mechanism while LDL binds via the LDL receptor (LDLR). Using an in-vitro, human RPE cell model (ARPE-19), we studied the mechanisms of carotenoid uptake into the RPE by evaluating kinetics of cell uptake when delivered in serum or isolated LDL or HDL. For lutein and β-carotene, LDL delivery resulted in the highest rates and extents of uptake. In contrast, HDL was more effective in delivering zeaxanthin and meso-zeaxanthin leading to the highest rates and extents of uptake of all four carotenoids. Inhibitors of SR-B1 suppressed zeaxanthin delivery via HDL. Results show a selective HDL-mediated uptake of zeaxanthin and meso-zeaxanthin via SRB1 and a LDL-mediated uptake of lutein. This demonstrates a plausible mechanism for the selective accumulation of zeaxanthin > lutein and xanthophylls over β-carotene in the retina. We found no evidence of xanthophyll metabolism to apocarotenoids or lutein conversion to meso-zeaxanthin.

Supplementary Key Words
Dietary Lipids, Scavenger Receptors, LDL, HDL, Transport, Carotenoids, Eye, Retina, Lutein, Zeaxanthin
Introduction

Age related macular degeneration (AMD) is an incurable disease in adults 55 years of age and older and is the leading cause of vision loss in this population (1). A 2014 meta-analysis predicts that 196 million people will have AMD by 2020 increasing to 288 million by 2040 (2). AMD occurs due to deterioration of the macula located in the retina of the eye impacting central vision and the ability to see fine detail.

The xanthophylls, lutein and zeaxanthin (Figure 1A and 1B), are dietary carotenoids of interest since they accumulate in the retina of the eye and may provide protection from AMD. Common xanthophylls in the human diet include lutein, zeaxanthin, and β-cryptoxanthin (3) and sources include corn, kale, spinach, eggs, and broccoli (4). Meso-zeaxanthin (Figure 1C), a stereoisomer of zeaxanthin, is present in the macula of the eye but is not a common dietary component.

Of the 700 carotenoids found in nature, only about 25 are found in the diet and human serum (5). The top 6 carotenoids in the human plasma include α-carotene, β-carotene, β-cryptoxanthin, lutein, zeaxanthin, and lycopene (6). Although the concentration of carotenoids in the serum vary widely among individuals, the typical order from highest to lowest is β-carotene > lutein > zeaxanthin (7,8). Xanthophylls comprise about 20% of the carotenoids in the human plasma with a lutein:zeaxanthin ratio between 2:1 and 4:1 (6,9,10). Xanthophylls accumulate in the macula of the retina, imparting the yellow macular pigment color named macula lutea. The functions of xanthophylls in the macula are not fully understood, but they are thought to filter light preventing damage to the macula and might provide protection as antioxidants.

Unlike carotenoid concentrations in the blood, lutein and zeaxanthin represent 80% of carotenoids in the retina while β-carotene is present in only trace amounts (11). In the peripheral macula, lutein dominates over zeaxanthin with a ratio of between 2:1 and 3:1 (9,12). Moving closer to the central macula from the peripheral macula, the ratio changes to predominantly zeaxanthin with a 1:2 ratio of lutein to zeaxanthin. About 50% of the zeaxanthin within the central macula is present as a stereo-isomer of zeaxanthin called meso-zeaxanthin. It is unclear what mechanisms are responsible for the selective accumulation of xanthophylls and particularly zeaxanthin in the retina.
Meso-zeaxanthin is not found in detectable amounts in the blood, liver, or in the typical human diet to account for the concentrations found in the macula (13). It is hypothesized that lutein is converted to meso-zeaxanthin either enzymatically or induced by light (14,15). Thus, primates given a xanthophyll-free diet from birth followed by a lutein supplement showed the presence of meso-zeaxanthin in the retina while those provided no xanthophylls or with just zeaxanthin alone did not have meso-zeaxanthin in the retina (16). However, no studies have been able to show how this conversion occurs in the macula. Even so, the discovery of meso-zeaxanthin as an additional macular pigment xanthophyll has prompted supplement companies to promote products containing lutein, zeaxanthin, and meso-zeaxanthin for the prevention of AMD. When supplemented, meso-zeaxanthin is found in the human serum (17), leading researchers to investigate whether its supplementation could increase macular pigment and thus improve the outcome of retinal diseases like AMD. Human studies measuring serum concentration and macular pigment optical density have shown that taking a supplement predominantly composed of meso-zeaxanthin and small amounts of lutein and zeaxanthin results in the presence of all three xanthophylls in human serum and an increased macular optical pigment density compared to those un-supplemented (18). Since meso-zeaxanthin is found in the blood and may increase macular pigment, it is worth investigating the mechanisms of its retinal uptake.

The RPE is similar to the blood-brain barrier for the retina in that it serves as a cellular and metabolic interface between the retina and the blood supply from the choroid (19). RPE cells display polarity with a basolateral side of tight junctions creating a barrier to the choriocapillaris and an apical side where villi extend and perform phagocytosis on photoreceptors of the retina. This unique position of the RPE allows it to provide nutrients to the photoreceptor cells while also eliminating waste from the retina. Differentiated ARPE-19 cells are often used as a model for the study of retinal metabolism since they show structural and functional properties similar to the human RPE (20). ARPE-19 cells express lipoprotein receptors necessary for studying lipoprotein delivery of carotenoids including SR-B1, SR-B2, LDLR, CD-36, and ABCA1 (21-27). Based on analysis of the expression of lipoprotein transporters and
receptors in the retina and two different RPE-cell lines, including ARPE-19 cells, it was proposed that circulating LDL and HDL enter the basolateral side of the RPE via LDLR and the SR-B1 (27).

Dietary carotenoids are incorporated into lipoproteins for distribution to various tissues in the human body. Previous work in our laboratory (28,29) demonstrated that xanthophylls associated mostly with HDL, while β-cryptoxanthin, lycopene, and β-carotene predominantly associated with LDL in human serum. We also showed that zeaxanthin delivered by detergent micelles to ARPE-19 cells is preferentially taken up via an SR-B1-dependent mechanism compared to β-carotene (30). The current work was designed to extend those studies and to investigate in detail the uptake and metabolism of lutein, zeaxanthin, meso-zeaxanthin, and β-carotene delivered in their physiologically relevant transport vehicles. Using differentiated ARPE-19 cells showing structural and functional properties similar to human RPE cells (20), we used human serum and the lipoproteins LDL and HDL. We evaluated the kinetics of uptake, the possible interactions of the carotenoids, and the effects of the presence of other lipoproteins and specific inhibition of SR-B1. We demonstrate that xanthophylls, lutein and zeaxanthin, transported in both HDL and LDL show quite different uptake kinetics. Next, we show that meso-zeaxanthin is very similar to zeaxanthin in its uptake. Our experimental evidence provides strong support for HDL-dependent selective uptake of zeaxanthin and meso-zeaxanthin via SR-B1 in contrast to an LDL-mediated uptake of lutein and β-carotene. Finally, we found no evidence of xanthophyll conversion to meso-zeaxanthin or lutein conversion to apocarotenoids providing evidence that RPE cells do not extensively metabolize xanthophylls.
Materials and Methods

Materials

All-trans-lutein and all-trans-zeaxanthin (≥98% assay (UV) purity) were purchased from Indofine Chemical Company, Inc. (Hillsborough, NJ). All-trans-β-carotene and solvents used for HPLC were purchased from Sigma Alderich (Saint Louis, MO). Meso-zeaxanthin was a gift from DSM Nutritional Products (Heerlen, Netherlands). Recombinant human serum amyloid A (human SAA1 α except for the presence of an N-terminal methionine and substitution of asparagine for aspartic acid at position 60 and arginine for histidine at position 71) was purchased from PeproTech (Rockyhill, NJ). Human and bovine serum albumins were purchased from Thermo Scientific. Block lipid transport 1 (BLT-1) (≥98% assay HPLC purity) and dimethyl sulfoxide (DMSO) were purchased from Sigma Alderich.

Cell Culture

Human retinal pigment epithelial cells, ARPE-19 cells (ATCC®-CRL-2302™), were purchased from the American Type Culture Collection (Rockville, MD). ARPE-19 cells were maintained in Ham's F12 Media: DMEM (1:1) (Dulbecco's modified Eagle's medium) with 10% fetal bovine serum (FBS) (Gibco, Life Technologies, Inc.) as monolayers at 37°C with 5% CO₂ in T-75 flasks. Cultures of ARPE-19 cells were seeded at 1.5 X 10⁵ cells/cm² with Ham's F12 Media: DMEM (1:1) with 10% FBS for experiments on 6-well flat bottom plates. Cells were plated and allowed to become confluent after 7 days. The cell medium was changed 2-3 times per week. Cells were used after differentiation for 6-8 weeks. Pilot experiments confirmed that maximal xanthophyll cell uptake occurs at this point of differentiation (results not shown).

Isolation of Lipoproteins

VLDL, LDL and HDL were isolated from human serum (type AB, sterile, Valley Biomedical, Winchester, VA) using a method previously developed (31). Briefly, chylomicrons were first removed by centrifugation at 100,000 g for 10 minutes in a Beckman Coulter Optima L-90K Ultracentrifuge. Human
serum was then mixed with OptiPrep™ (Sigma Alderich) (4:1 v/v, 12% iodixanol final concentration) and 3.5 mls was transferred to an OptiSeal™ tube (Beckman Coulter). The remaining tube was filled with Phosphate-Buffered Saline (PBS) (Gibco, Life Technologies). The tube was capped and centrifuged in a Beckman Coulter Optima™ TLX Ultracentrifuge at 350,000 g for 2.5 hours at 16°C. Lipoprotein fractions were removed by tube puncture using a syringe. The syringe was inserted into the tube just below the lipoprotein band starting with VLDL at the top followed by LDL and then HDL at the bottom. The volumes were recorded and collected into separate vials. Lipoprotein fractions were confirmed using agarose gel electrophoresis and staining with Sudan Black. Protein amounts in human serum and lipoproteins were measured using the Modified Lowry Method (Thermo Scientific Pierce Modified Lowry Method kit). Collected lipoproteins were used immediately following isolation.

**Carotenoid Enrichment of Human Serum and Lipoproteins**

Whole human serum or lipoproteins isolated by centrifugation were enriched with carotenoids using a procedure previously reported (29). This method was previously shown to successfully enrich the lipoprotein with the intended carotenoid without influencing lipoprotein integrity or redistributing carotenoids among lipoproteins in whole serum when incubated in vitro (28). Carotenoids were added to human serum or lipoproteins dissolved in ethanol (zeaxanthin, *meso*-zeaxanthin, and lutein) or tetrahydrofuran (β-carotene) (<2% final volume) so that when diluted in serum-free medium they would meet the desired concentrations. The solution was mixed and incubated under nitrogen at 4°C in the dark on a mixer overnight for 24 hours. Aliquots of the carotenoid-enriched human serum or lipoproteins were removed for analysis to confirm the initial concentration of carotenoids added to cells prior to experiments.

**Addition of Carotenoids to Differentiated ARPE-19 Cells**

After 6-8 weeks of cell differentiation, cell medium was removed and cells were rinsed 3 times with PBS. For cell uptake experiments, carotenoid-enriched human serum or isolated lipoproteins were added to
serum-free cell medium (10% final volume unless otherwise described) to meet the desired carotenoid concentration and added to cells. For BLT-1 and SAA experiments, HDL protein was first measured using Lowry Assay and carotenoids were added to 10 μg HDL protein/ml to reach the desired final concentration in serum-free medium.

**Carotenoid Extraction**

Extraction of cells and medium was performed as previously described with slight modifications (32,33). For human serum or lipoproteins, one volume was treated with 3 volumes 2-propanol-dichloromethane (2:1, v/v) and vortexed. All samples were kept on ice under yellow light to prevent oxidation during extraction. The mixture was centrifuged for 3 minutes at 2,000 RPM. The top layer was removed and dried under nitrogen. The resulting residue was re-suspended in 200 μl of mobile phase, filtered through a 0.22 μm pore sized filter and injected on to the HPLC. For carotenoid extraction from cells, cell medium was removed and cells were rinsed with 2 mls of ice-cold PBS followed by the addition of 1 ml of 2-propanol-dichloromethane (2:1) for 30 minutes. This was performed 3 times at room temperature. Extracts were then collected, dried under nitrogen, re-suspended in mobile phase, filtered through a 0.22 μM pore sized filter and injected on to the HPLC.

**Method 1: HPLC Analysis of Carotenoids**

Lutein, zeaxanthin, and β-carotene were analyzed using an Agilent Technologies 1200 Series Diode Array and Multiple Wavelength Detector HPLC system (Santa Clara, CA) using a method previously described (30,34). A column C30 Type Carotenoid, 4.6 X 250 mm, 3 μm (YMC, Inc., Milford, MA) was used with methanol: methyl-tertiary-butyl-ether (MTBE) (90:10, v/v) at a flow rate of 0.9 ml/min as mobile phase. When only β-carotene was being measured, the same column was used but with a gradient of 75:25, v/v methanol: MTBE and a flow rate of 1.4 ml/min. Carotenoids are monitored at 450 nm and quantified using external standard curves established for each carotenoid tested.
Method 2: Chiral Column Separation of Xanthophylls

Separation and identification of meso-zeaxanthin from zeaxanthin was analyzed using the same HPLC system described above except using a chiral column (ChiralPak AD, 25 cm length X 4.6 mm ID, Chiral Technologies, Exton, PA) and a method previously described but with slight modifications (13). A three-step gradient was used starting with a mobile phase consisting of 94.5% hexanes and 5.5% 2-propanol for 40 minutes. From 40-50 minutes, 2-propanol was linearly increased to 15% while hexane was reduced to 85 percent. From 50-55 minutes the gradient of hexane and 2-propanol was changed to a 50:50 mixture and maintained for 15 minutes (70 minutes into the total run-time). At 70 minutes, the gradient was re-equilibrated to the initial gradient of 94.5% hexanes and 5% 2-propanol from 70-80 minutes. The flow rate during the run was 0.7 mls/minute and monitored at 453 nm.

Inhibition of SR-B1

BLT-1 is a chemical inhibitor of lipid transport via the SR-B1 pathway (35). Differentiated ARPE-19 cells were pretreated with equal volumes (0.1% final volume) of either DMSO or BLT-1 dissolved in DMSO (10 µM) for 1 hour in serum-free cell medium. This concentration of BLT-1 has been shown to be effective in inhibition of lipid transport from HDL to SR-B1 (36,37). After removal of cell medium, the cells received the previous treatment in addition to 0.1 µM of zeaxanthin- or lutein- (final concentration when added to cell medium) enriched HDL or LDL (~10 ug protein/ml final volume), respectively, in serum-free cell medium for 3 hours at 37°C. After the incubation, zeaxanthin or lutein was extracted from cells using the method previously prescribed. Lipid-free serum amyloid A (SAA) is an inhibitor of SR-B1-dependent binding and selective cholesterol uptake from HDL (38). SAA or BSA (control) were added to differentiated cells at a concentration of 10 ug/ml along with 0.1 µM of zeaxanthin- or lutein-enriched HDL or LDL (~10 ug/ml), respectively, in serum free medium for 3 hours at 37°C. The amounts of SAA and HDL added in this experiment have previously shown a significant decrease in SR-B1-specific HDL binding. After incubation, zeaxanthin or lutein was extracted from cells as described previously above.
Statistical Analysis

Statistical analyses were conducted using R Data Analysis Software. Values are listed as means ± the standard deviation (SD). Results were analyzed using a mixed-effects ANOVA model. Where appropriate, this was followed by a multiple comparison of means using Tukey contrasts. P values <0.05 were considered significant.
Results

Lipoprotein Separation and Carotenoid Distribution

After centrifugation of human serum and separation and removal of lipoprotein fractions, the fractions were analyzed on agarose gel with Sudan black staining. Figure 2 shows the presence of only LDL and HDL staining in lanes 1 and 2, respectively, and the presence of all lipoproteins in whole serum in lane 3. After removal of lipoprotein fractions, carotenoids (β-carotene, lutein, and zeaxanthin) were extracted as described in the methods section and analyzed using HPLC. Each carotenoid was quantified and compared to the total amount of that carotenoid present in whole serum (Figure 3). β-carotene mostly associated with the LDL fraction (64 ± 0.4%) followed by HDL (25 ± 2%) and VLDL (10 ± 1%). Lutein and zeaxanthin mostly associated with HDL (54 ± 9% and 51 ± 14%) followed by LDL (36 ± 4% and 40 ± 10%) and VLDL (10 ± 5% and 8 ± 3%). These data are agreement with other studies showing similar carotenoid distributions among lipoproteins (28,29,39).

Carotenoid Uptake from Whole Serum and Isolated Lipoproteins

We first studied the uptake of β-carotene, lutein, meso-zeaxanthin, and zeaxanthin from human serum enriched separately with each of the carotenoids (Figure 4). Carotenoid uptake is expressed as a percentage of the initial amount added to cells. Zeaxanthin uptake was highest in ARPE-19 cells (69 ± 20%) followed by lutein (20 ± 3%) and β-carotene (3 ± 0.01%) after 24 hours. Meso-zeaxanthin uptake was very similar to zeaxanthin uptake (62 ± 2%). After establishing the kinetics of carotenoid uptake in whole serum, LDL and HDL were isolated and enriched with carotenoids to compare their delivery to cells by the isolated lipoproteins (Figure 5). Strikingly, HDL delivered zeaxanthin (5A) and meso-zeaxanthin (5B) most efficiently at all 3 time points with a greater than 50% cell uptake at 24 hours and a statistically higher uptake than from LDL at all time points. In marked contrast and surprisingly, lutein (5C) was more efficiently delivered from LDL than HDL similar to β-carotene.
We next studied the concentration dependence of the initial rate of cell uptake of lipoprotein-delivered carotenoids. After separation and enrichment of lipoproteins with 1, 10, 20, 30, and 40 µM of zeaxanthin, meso-zeaxanthin, or lutein, we measured cell delivery after 3 hours. As shown in figure 6, the initial rate of uptake of all three compounds was linear over the concentration range used. The results confirm that HDL more efficiently delivers zeaxanthin (6A) and meso-zeaxanthin (6B) while lutein (6C) is more efficiently delivered by LDL.

Influence of Other Carotenoids on Cellular Uptake

We next asked whether the presence of increasing amounts of another carotenoid would inhibit uptake of either zeaxanthin or lutein. Aliquots of human serum (5% (v/v) in serum-free medium) were enriched with 1 µM of zeaxanthin or lutein (final concentration after mixing with cell medium) and added to cells along with increasing amounts of 0, 1, 3, or 5 µM of β-carotene-, lutein-, or zeaxanthin- enriched whole serum (5% (v/v) in serum-free medium). The amount of whole serum added to cells was equal in all treatments (i.e. 10% v/v) while only the amount or presence/absence of the carotenoid added varied. Cells were incubated with the appropriate carotenoid treatment for 3 hours before cells were extracted. Compared to the control treatment, the amount of zeaxanthin taken up remained unchanged when increasing amounts of β-carotene and lutein were present (Figure 7A) (P > 0.05). A small but significant increase (P < 0.05) of 9% of lutein taken up occurred in the presence of 5 µM of zeaxanthin (Figure 6B) likely reflecting the presence of a small amount of lutein in the added zeaxanthin. More strikingly, the presence of increasing amounts of β-carotene resulted in an 8% (P < 0.05) and 41% (P < 0.001) reduction in delivery of lutein to cells at 3 µM and 5 µM of β-carotene compared to baseline, respectively (Figure 7B). In summary, zeaxanthin uptake to cells remained unchanged with increasing amounts of β-carotene and lutein, while lutein cell uptake decreased markedly with increasing amounts of β-carotene.
Impact of Excess Lipoprotein on Carotenoid Uptake

Since we found that LDL most efficiently delivers lutein while HDL most efficiently delivers zeaxanthin to ARPE-19 cells, the goal of the next experiments was to determine if increasing amounts of LDL or HDL devoid of carotenoid would affect the cell delivery of 1 µM lutein-LDL or zeaxanthin-HDL respectively. Excess unenriched LDL resulted in an overall decline in lutein-enriched LDL cell delivery (Figure 8A). The addition of 1X, 3X, 5X, and 10X unenriched LDL resulted in a 5, 10, 15, and 27% decline in lutein-enriched LDL cell delivery compared to the control treatment. Adding increasing amounts of unenriched HDL had a marked impact on zeaxanthin-enriched cell delivery resulting in a 5, 2, 28, and 87% decline in zeaxanthin-enriched HDL cell delivery compared to the control treatment (Figure 8B). It should be pointed out that these effects are not merely due to the “dilution” effect since we have previously shown that xanthophylls do not exchange among lipoproteins when incubated in vitro (28).

Effect of Blocking SR-B1 on the Uptake of Zeaxanthin from HDL or Lutein from LDL

A chemical inhibitor, BLT-1, and SAA, a protein ligand of SR-B1 were used to block HDL-dependent uptake of zeaxanthin into ARPE-19 cells (Figure 9). After pre-incubation with 10 uM BLT-1 in serum-free medium for 1 hour followed by co-incubation with 10 uM of BLT-1 and 0.1 uM zeaxanthin-enriched HDL cell medium for 3 hours, BLT-1 decreased zeaxanthin uptake by 52% compared to the control. Similarly, after exposing cells to 10 ug/ml of SAA and 0.1 uM of zeaxanthin-enriched HDL cell medium for 3 hours, zeaxanthin cell delivery was decreased by 49% compared to the control treatment. When we repeated the same experiments using lutein-enriched LDL (Figure 9), we saw no significant difference in lutein uptake when BLT-1 and SAA were used to block SR-B1. These results suggest that zeaxanthin entry into ARPE-19 cells from HDL is largely SRB1-dependent while lutein entry from LDL is not.
Carotenoids are not Extensively Metabolized by ARPE-19 cells

β-carotene 9'-10’-oxygenase (BCO2) cleaves non pro-vitamin A carotenoids, lutein and zeaxanthin, to create 3-hydroxy-apo-10’-carotenals (40). BCO2 cleaves the asymmetric lutein molecule to yield 3-hydroxy-β-apo-10’-carotenal and 3-hydroxy-α-apo-10’-carotenal. The cleavage of the symmetric zeaxanthin molecule by BCO2 yields 3-hydroxy-β-apo-10’-carotenal. Previous research in our lab using the same method described in “Materials and Methods” to detect 3-hydroxy-apo-10’-carotenals (40), shows that these would appear in our chromatograms with retention times between 4-5 minutes. To determine if ARPE-19 cells formed these 3-hydroxy-apo-10’-carotenals, we looked carefully at the uptake and recovery of xanthophylls.

HPLC chromatograms (Method 1) of lutein, zeaxanthin, and meso-zeaxanthin standards are shown in Figure 10A and 10B. Differentiated ARPE-19 cells incubated with lutein-enriched human serum for 24 hours had a HPLC profile of 93 ± 2% all-trans-lutein, 4 ± 1% all-trans-zeaxanthin, and 3 ± 1% of unidentified peaks (Figure 10C). Cell medium after 24 hours of incubation with lutein-enriched human serum included 91 ± 4% all-trans-lutein, 5 ± 1% all-trans-zeaxanthin, and 4 ± 3% unidentified peaks. The HPLC profile of the initial lutein-enriched human serum prior to cell addition contained 95 ± 1% all-trans-lutein, and 5 ± 1% all-trans-zeaxanthin. Zeaxanthin-enriched human serum prior to cell addition contained 96 ± 2% all-trans-zeaxanthin and 4 ± 2% all-trans-lutein. After 24 hours of incubation with zeaxanthin-enriched human serum, differentiated ARPE-19 cells contained 97 ± 1% all-trans-zeaxanthin, 1 ± 0.5% all-trans-lutein, and 2 ± 1% unidentified peaks (Figure 10D). Cell medium from the 24-hour incubation contained 92 ± 3% all-trans-zeaxanthin, 4 ± 1% all-trans-lutein, and 4 ± 2% other peaks.

Lutein and zeaxanthin are highly conserved after 24 hours in cells and medium with unidentified peaks occurring more in cell culture medium than in cells. In no case were there any detectable metabolites at the retention times of the 3-hydroxy-apo-10’-carotenals (10C-10E). Rather, unidentified peaks are likely isomers of the xanthophylls identified in previous studies using the same HPLC conditions (30,34). HPLC chromatogram profiles are similar to a study using micelles to deliver lutein and zeaxanthin to
differentiated ARPE-19 cells (30). Thus, xanthophylls are not metabolized to 3-hydroxy-apo-10'-
carotenals in ARPE-19 cells. Furthermore, western blot analysis of these cells did not detect any BCO2
protein (data not shown). The antibody did detect BCO2 protein in other human cell lines.

It has been hypothesized that \textit{meso}-zeaxanthin is formed from lutein in the macula of the retina. To
determine if \textit{meso}-zeaxanthin is formed from lutein in the RPE, we incubated ARPE-19 cells for 24 hours
with 10 µM lutein-enriched human serum and measured xanthophylls using the chiral column method 2.
The chiral column is able to separate \textit{meso}-zeaxanthin, zeaxanthin, and lutein as shown in the standard
mixture in Figure 11A. After 24 hours of receiving lutein-enriched human serum, the cell extract
contained 95 ± 0.2% all-trans lutein, 3 ± 0.1% all-trans zeaxanthin, and 2 ± 0.1% other peaks. Cell
medium contained 94 ± 0.1% all-trans lutein, 1 ± 0.1% all-trans zeaxanthin, and 5 ± 1% other peaks.
\textit{Meso}-zeaxanthin was not detected in cell extracts (Figure 11B) or cell medium (not shown) after 24
hours. Similarly, we incubated cells with 10 µM of zeaxanthin- or \textit{meso}-zeaxanthin-enriched whole serum
for 24 hours and measured the uptake of xanthophylls using chiral method 2 to look for any conversion to
the other xanthophylls. After incubation with zeaxanthin, cell extracts contained 97 ± 0.2% all trans
zeaxanthin, 1 ± 0.1% all trans lutein, and 2 ± 0.1% other peaks (Figure 11C) while cell medium contained
93 ± 1% all trans zeaxanthin, 0.4 ± 0.2% all trans lutein, and 7 ± 1% other peaks. Incubation with \textit{meso}-
zeaxanthin resulted in cell extracts containing 97 ± 0.5% \textit{meso}-zeaxanthin, 1 ± 0.01 all trans zeaxanthin,
and 1 ± 0.7% other peaks (Figure 11D) and cell medium containing 94 ± 1% \textit{meso}-zeaxanthin, 0.5 ± 1%
all trans zeaxanthin, and 5 ± 2% other peaks.
Discussion

Xanthophylls preferentially accumulate in the macula of the eye despite higher levels of other carotenoids in the blood, indicating a selective mechanism of delivery in the retina. Human studies have shown a correlation between increased serum xanthophyll concentrations and increased macular pigment density (41). Xanthophylls are transported mostly in HDL while carotenes such as β-carotene are transported mostly in LDL. The RPE is a highly selective transfer point from the blood vessels of the choroid where lipoprotein-bound xanthophylls must cross to reach the photoreceptors of the macular retina. Using differentiated ARPE-19 cells as a model for the RPE, we previously demonstrated that zeaxanthin delivered by detergent micelles is preferentially taken up compared to β-carotene via SR-B1 (30). We now show that despite both lutein and zeaxanthin being mostly associated with HDL in serum, zeaxanthin is much more efficiently delivered to ARPE-19 cells via HDL while lutein is more efficiently delivered via LDL. Thus, there is a selective uptake of zeaxanthin over lutein even though both are transported in HDL. Furthermore, our results show that zeaxanthin delivered by HDL occurs in an SR-B1 dependent process while lutein cell uptake from LDL may involve the LDL receptor. Given the similarities to zeaxanthin in structure and cell delivery, meso-zeaxanthin is also likely taken up from HDL in a SR-B1-dependent process in ARPE-19 cells.

SR-B1 is a transmembrane glycoprotein homodimer embedded in the plasma membrane containing two N- and C-terminal transmembrane domains and a central extracellular domain clustered in caveolae-like domains (42). SR-B1 mediates selective lipid uptake of HDL cholesterol to the liver and other steroidogenic tissues through facilitated diffusion (43). The exact mechanism of selective lipid uptake of cholesterol from HDL via SR-B1 is not well understood, but differs from the LDL receptor pathway where LDL binds to the LDL receptor forming a clathrin-coated pit and the entire LDL particle is taken up into the cell. Instead, SR-B1-mediated selective cholesterol uptake occurs in a two-step process where the HDL particle binds to the extra-cellular domain where selective delivery of cholesterol into the cell occurs without internalization of the HDL particle (44). It was hypothesized that a lipophilic channel
forms between the HDL particle and the plasma membrane localized SR-B1 where selective uptake of cholesterol esters occurs (45). This is supported by a recent publication identifying the crystal structure of the extracellular domain of LIMP-2 (lysosome membrane protein 2) and using this as a model for SR-B1 (46). LIMP-2 belongs to the CD36 superfamily of scavenger-receptor proteins along with SR-B1 and CD-36. In this model, they show a large, predominantly hydrophobic cavity running through the molecule where cholesterol esters are delivered from bound HDL through the plasma membrane.

Several studies report SR-B1 as the transfer protein for carotenoids in intestinal cell delivery (36,37,47,48) as well as the RPE (30). Genetic evidence links variants in several genes including SR-B1 to AMD risk in the Carotenoids in Age-Related Macular Degeneration Study (CAREDS) and these variants were related to levels of lutein and zeaxanthin in human serum and the macula (44). However, given the role of SR-B1 in intestinal absorption of carotenoids and its possible role in other cell types, it is difficult to determine whether these effects are due to variants in SR-B1 at the intestinal level or SR-B1 related uptake in the retina. Using ARPE-19 cells as a model for the RPE, we were able to directly compare delivery of xanthopylls in LDL or HDL to ARPE-19 cells. After 24 hours, zeaxanthin delivery in HDL is highest (66%) while lutein delivery from HDL is only 13% and is higher when lutein is delivered in LDL (33%). We hypothesized that HDL-dependent uptake of zeaxanthin occurs via SR-B1 while LDL-dependent uptake of lutein may occur via the LDL receptor. Indeed, ARPE-19 cells express SR-B1 and LDLR and it was proposed that lipoproteins likely enter the RPE through these lipoprotein receptors (27). Adding increasing amounts of unenriched LDL to lutein-enriched LDL creates competition for entry into the cell and decreased lutein uptake by up to 27 percent. Likewise, adding increasing amounts of unenriched HDL decreased zeaxanthin-enriched HDL uptake by 87 percent supporting competition via SR-B1, an HDL-specific receptor.

To further support SR-B1 as the protein transporter for zeaxanthin, we used BLT-1, an HDL-specific inhibitor of SR-B1 selective lipid uptake (35). It has been effectively used to inhibit carotenoid uptake by SR-B1 (36,37,49) in intestinal cells and it does not inhibit other protein transporters (50). Interestingly,
it’s been found that BLT-1 inhibits cholesterol uptake by SR-B1 in a Cys384-dependent manner (51). BLT-1 attaches to Cys384 which is located in the lumen of the SR-B1 tunnel and blocks cholesterol transport. This is evidenced by the fact that when Cys384 is converted to serine, the effect of BLT-1 on cholesterol transport is lost. Thus it seems that Cys384 contributes to the ability of SR-B1 to mediate selective uptake which is inhibited by BLT-1. It is interesting to note that CD36 does not have the same Cys384 equivalent to SR-B1’s, yet can bind to HDL but is unable to mediate efficient lipid uptake. In our study, BLT-1 inhibited HDL-delivered zeaxanthin cell uptake into ARPE-19 cells by 52 percent while no significant effect was seen with LDL-delivered lutein. Thus, it seems that zeaxanthin may be transported from HDL through the SR-B1 tunnel by a mechanism similar to cholesterol esters. This is consistent with results of another study showing that an SR-B1 antibody reduced zeaxanthin uptake in ARPE-19 cells by 58% while an antibody for CD-36 had no effect (30). Lipid-free SAA, an acute inflammatory protein, was used as an inhibitor of SR-B1 due to its ability to compete with HDL for binding to SR-B1. Zeaxanthin uptake was significantly decreased by 49% when lipid-free SAA was added further supporting SR-B1 as the zeaxanthin transport protein. Lipid-free SAA had no significant effect on cell uptake of LDL-delivered lutein.

Surprisingly, our results demonstrated that LDL was more efficient in delivering lutein to ARPE-19 cells, despite its greater extent of association with HDL in serum. One explanation could be that SR-B1 contains a zeaxanthin-specific transfer factor within the SR-B1 tunnel that discriminates the chemical structure of zeaxanthin, so not only does it recognize HDL for binding and cholesterol transfer, but zeaxanthin as well. A similar function for SR-B1 was characterized in Drosophila Melanogaster where disruption of the NinaD gene codes for an SR-B1 protein that when disrupted causes blindness (52). These authors further identified 2 isoforms of the NinaD gene located in different subcellular compartments where NinaD-1 acts on the plasma membrane to specifically mediate the uptake and transfer of zeaxanthin from micelles (53). Furthermore, NinaD-1 uptake was preferential for zeaxanthin compared to β-carotene. The function of NinaD-11, which is localized to intracellular membranes, is yet
to be determined. Another example is carotenoid delivery by lipophorin, a lipid transporter similar to HDL in humans that occurs in the silkworm, *Bombyx mori* (54). Two genes encode for a high-density lipoprotein receptor-2 (Cameo2) and scavenger receptor class B member 15 (SCRB15) demonstrating selective affinity for xanthophylls and carotenoids respectively. Similar to xanthophylls and HDL, carotenoids are carried by lipophorin and occur as a complex mixture in the lipoprotein yet Cameo2 and SCRB15 demonstrate selective delivery of specific carotenoids. Likewise, while HDL may transport both lutein and zeaxanthin, cell uptake to the RPE may occur selectively and specifically by SR-B1 for zeaxanthin. Although not typical in the human diet, *meso*-zeaxanthin, being a stereoisomer to zeaxanthin, is similar in structure and when consumed in the diet, may be recognized by SR-B1 and taken up into the RPE like zeaxanthin.

Our results show a 41% decline in lutein cell uptake in the presence of 5-fold molar excess of β-carotene when delivered in whole serum. This is indicative of competition occurring between lutein and β-carotene during uptake by the RPE. Although lutein is found mostly in HDL, LDL delivers a higher amount to the RPE. β-carotene mainly associates with LDL in the blood; therefore, any excess β-carotene may somehow inhibit LDL-delivered lutein cell uptake. If translated to retinal uptake, a large amount of β-carotene present in the blood compared to lutein could reduce the amount taken up by the RPE to the retina thus reducing macular pigment and increasing the risk of AMD. This was suggested in the Age-Related Eye Disease Study 2 (AREDS2) randomized clinical trial (55). Oral supplementation of the original AREDS formulation (vitamins C and E, β-carotene, and zinc) reduced the risk of progression to advanced AMD. Adding lutein and zeaxanthin to the supplement did not further reduce the risk of progression to advanced AMD. However, lutein serum levels were lower in those receiving the original AREDS formulation including lutein and zeaxanthin along with β-carotene compared to those with the lutein and zeaxanthin added and without β-carotene. Post-hoc analyses showed that receiving the AREDS supplement without β-carotene but including lutein and zeaxanthin was even more impressive in reducing progression to advanced AMD compared to those receiving the original AREDS supplement with β-
carotene. Although further studies are needed, the results suggest an inhibitory effect on the uptake of lutein by β-carotene. Together, these results suggest that the AREDS formulation containing lutein and zeaxanthin without β-carotene may maximize the delivery of xanthophylls to the retina.

Meso-zeaxanthin is present as approximately half the zeaxanthin found in the central macula of the retina, but is not present in the peripheral regions of the macula where lutein dominates. Meso-zeaxanthin is naturally found in shrimp carapace, fish skin, and turtle fat which are not common foods consumed in the human diet (56-58). As such, meso-zeaxanthin is not found in detectable amounts in human blood. Meso-zeaxanthin in the macula is likely converted from lutein as predicted by chemistry (59). Support of this was found when monkeys fed a lutein-only diet accumulated meso-zeaxanthin while those fed zeaxanthin only did not (16). Similar results were found in leghorn chicks fed a xanthophyll-free diet compared to a control diet containing lutein and zeaxanthin (60). Furthermore, meso-zeaxanthin was produced in the retina of leghorn chickens in a developmentally regulated manner (15). However, the exact mechanism for this conversion in the retina is unknown. We were unable to detect any conversion to meso-zeaxanthin when ARPE-19 cells were incubated for 24 hours with 10 μM lutein or zeaxanthin. Our results suggest that if meso-zeaxanthin is converted from lutein in the macula of the eye, this conversion does not occur robustly in the RPE or at least in our cell model. It’s possible that lutein and zeaxanthin are transported across the RPE to the retinal cells where conversion of lutein to meso-zeaxanthin may occur. Binding proteins, StARD3 (steroidogenic acute regulatory domain) and GSTP1 (glutathione S-transferase), were identified as having a high affinity for lutein and zeaxanthin in the central macula, respectively (59,61). GSTP1 was found to also have a high affinity for not only zeaxanthin, but meso-zeaxanthin, so it’s possible that these proteins are involved in the process of converting lutein to meso-zeaxanthin within the macula.

Immunohistochemical analysis has detected the presence of BCO2 in the human RPE (62). However, the presence of BCO2 in the RPE does not explain why xanthophylls seemingly accumulate in the retina without BCO2 conversion to its metabolites. One explanation is that unlike mouse and chicken BCO2
enzymes, human BCO2 enzyme is inactive in the retina (63) which explains why only primates accumulate xanthophylls. An amino acid insertion was discovered near the substrate binding tunnel of human BCO2 that when inserted into the mouse BCO2 enzyme leads to its inactivation. Additionally, zeaxanthin accumulated in the retinas of BCO2 knockout mice. However, another group finding a similar result of accumulation of zeaxanthin in the retinas of BCO2 knockout mice offers an alternative explanation. They propose that human retinal BCO2 resides in a different cellular compartment of the retina than the xanthophylls (64,65). We were unable to detect any 3-hydroxy-apo-10’-carotenals in cell extracts after incubation with lutein or zeaxanthin nor did we detect the presence of BCO2 protein.

In summary our results suggest that zeaxanthin is taken up by RPE cells via a mechanism of selective uptake from HDL similar to that of cholesterol ester by way of transfer through the lipophilic channel of SR-B1. Meso-zeaxanthin likely follows a similar pathway given its similar structure and kinetics of uptake compared to zeaxanthin. We provide strong evidence that lutein is transported into the RPE by a different mechanism, perhaps involving the LDL receptor. This would also explain the inhibitory interaction between lutein and β-carotene, as both are taken up into the RPE mainly from LDL. Finally, xanthophylls are transported intact into the RPE and we did not detect any measurable conversion to meso-zeaxanthin or to other metabolites. Thus the RPE acts as a transporter of xanthophylls from the choroid to the retina where conversion of lutein to meso-zeaxanthin may occur in the macula mediated by other binding proteins and/or enzymes.

These results suggest that lutein and zeaxanthin are selectively taken up by different mechanisms and that this leads to markedly preferential uptake of zeaxanthin. The exact molecular mechanism by which zeaxanthin and meso-zeaxanthin are taken up preferentially from HDL that also contains lutein remains to be resolved. Lutein and β-carotene appear to interact for entry into the retina via LDL possibly due to competition for the LDLR. Thus nutritional treatment of AMD should focus on supplementation of xanthophylls without β-carotene. Future studies are needed to confirm that lutein uptake occurs via LDLR in the RPE and to explore this mechanism in detail. The preferential uptake of zeaxanthin compared to
lutein and the possible conversion of lutein to meso-zeaxanthin in the macula of the eye may indicate that focus should be placed on consuming higher amounts zeaxanthin. For example, the AREDS2 supplements 10 mg of lutein and 2 mg of zeaxanthin (55). Given the preferential uptake of zeaxanthin compared to lutein in the RPE and its lower amount in the blood compared to lutein, it remains to be seen if supplementing a higher amount of zeaxanthin compared to lutein would be more beneficial for lowering the risk of AMD.
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References


Figure 1. Macular Xanthophylls

Structure of xanthophylls which selectively accumulate in the macula of the eye: Lutein (A), zeaxanthin (B), and meso-zeaxanthin (C). Zeaxanthin and meso-zeaxanthin are stereo-isomers while lutein differs in the placement of a double bond.
Figure 2. Agarose Gel Confirmation of Lipoproteins

After isolation by ultracentrifugation, lipoprotein fractions were confirmed using agarose gels and staining with Sudan Black. Lanes 1 and 2 indicate a single band for LDL and HDL fractions, respectively. Lane 3 contains whole serum and stains for VLDL, LDL, and HDL. There is a clear separation between LDL and HDL in whole serum and a small amount of VLDL migrates in front of LDL.
Figure 3. Carotenoid Distribution among Lipoproteins

Lipoprotein fractions from human serum were separated and endogenous levels of β-carotene, lutein, and zeaxanthin were measured in each lipoprotein fraction. Carotenoid amounts in each lipoprotein fraction are listed as a percentage of the total amount recovered in all lipoprotein fractions. Total recovery from lipoprotein fractions from the initial amount measured in whole serum was as follows: 110 ± 26 % β-carotene, 107 ± 30% lutein, and 113 ± 34% zeaxanthin. Data represent means ± SD of triplicate separations of lipoprotein fractions.
Figure 4. ARPE-10 Carotenoid Uptake: Delivery in Whole Serum

Kinetics of ARPE-19 cell uptake of 1 µM of β-carotene, lutein, zeaxanthin, and meso-zeaxanthin delivered in whole serum. Cells were grown to confluency in 6-well plates (3-4 wells/treatment) and allowed to differentiate for 6 weeks. Carotenoid amounts are represented as a percentage of the amount of that carotenoid measured in whole serum prior to addition to cells. Carotenoids were measured using HPLC. After 24 hours, we recovered 92 ± 10% β-carotene, 92 ± 14% lutein, 103 ± 30% zeaxanthin, and 89 ± 3% meso-zeaxanthin in cells and medium. Data are means ± SD of 2-3 independent experiments.
Figure 5. Kinetics of Carotenoid Uptake of LDL and HDL

ARPE-cell uptake of 1 μM of (A) zeaxanthin, (B) meso-zeaxanthin, (C) lutein and (D) β-carotene delivered in either LDL or HDL was measured at 0, 4, 6, and 24 hours. Cells were grown to confluency in 6-well plates (3-4 wells/treatment) and allowed to differentiate for 6 weeks. LDL and HDL were separated and enriched with 1 μM of the indicated carotenoid, added to cells, and uptake was measured at
the indicated time points. Total zeaxanthin, *meso*-zeaxanthin, lutein, and β-carotene recovered from cells and medium delivered by: LDL (82 ± 4%, and 72 ± 9%, 98 ± 19%, and 88 ± 11%) and HDL (103 ± 16%, 86 ± 5%, 97 ± 8%, and 92 ± 4%) respectively. Data are means ± SD of two or more independent experiments. * P < 0.05, LDL vs. HDL at the time indicated.
Figure 6. Concentration-Dependent Carotenoid Uptake by LDL or HDL

Different concentrations of (A) zeaxanthin, (B) meso-zeaxanthin, or (C) lutein were delivered in LDL or HDL and the amount of xanthophyll taken up by the cells was measured after 3 hours. Cells were grown to confluency in 6-well plates (2 wells/treatment) and allowed to differentiate for 6 weeks. LDL and HDL were separated and enriched with 1, 5, 10, 20, 30, and 40 µM of the indicated carotenoid, added to cells,
and cell uptake of xanthophylls was measured by HPLC after 3 hours. Data are means ± SD of 3 independent experiments.
Figure 7. Interactions of Carotenoids during Cell Uptake

Impact of increasing concentrations (1, 3, 5 µM) of lutein or β-carotene on cell uptake of 1 µM zeaxanthin (A) and impact of increasing concentrations (1, 3, 5 µM) of zeaxanthin or β-carotene on cell uptake of 1 µM lutein (B). Xanthophyll cell uptake is represented as a percentage of the control without the added carotenoid ((1 µM zeaxanthin (A) or 1 µM lutein (B) only)). Whole serum was separately enriched with the given amounts of carotenoids and added at the same time. Total amount of whole serum added to each treatment was the same. Cells were analyzed for carotenoid content after 4 hours using HPLC. Data are means ± SD of 3 independent experiments. * P < 0.05 compared with the control treatment.
Figure 8. Impact of Excess Lipoprotein on Carotenoid Uptake

ARPE-19 cells received 50 ul of lutein-enriched LDL (A) or zeaxanthin-enriched HDL (B) along with the following treatments: 0X (control), 1X, 3X, 5X, or 10X of un-enriched LDL (LDL-lutein treatment) (A) or HDL (HDL-zeaxanthin treatment) (B). Cellular carotenoid content was measured after 3 hours and
compared to the control (lutein-enriched LDL without excess LDL or zeaxanthin-enriched HDL without excess HDL). Data are means ± SD >3 independent experiments. * P < 0.05 compared with the control treatment.
Figure 9. Effect of BLT-1 and SAA on Zeaxanthin or Lutein Uptake

ARPE-19 cells were pre-incubated for 1 hour with 10 μM of BLT-1 or control (DMSO). After pre-incubation with BLT-1, medium was removed from cells and 0.1 μM of zeaxanthin-enriched HDL or lutein-enriched LDL was added to ARPE-19 cells along with 10 μM of BLT-1 or control. After 3 hours, cells were analyzed for zeaxanthin or lutein content using HPLC. SAA or control (bovine serum albumin) was added to ARPE-19 cells at a concentration of 10 μg/ml at the same time as 0.1 μM of zeaxanthin-enriched HDL or lutein-enriched LDL. After 3 hours of incubation, zeaxanthin or lutein content was measured in the cells using HPLC. The amount of zeaxanthin or lutein in the cells treated with SAA was compared with the control. Data are means ± SD for 3 independent experiments. * P < 0.05 compared with the control treatment.
Standards

Meso-zeaxanthin Standard

24-Hour Cell Extract – Lutein Treated

24-Hour Cell Extract – Zeaxanthin Treated

24-Hour Cell Extract – meso-Zeaxanthin Treated
Figure 10. Xanthophylls are not Extensively Metabolized to Apocarotenoids by ARPE-19 Cells

HPLC chromatograms of a mixture of lutein and zeaxanthin standards (A), *meso*-zeaxanthin standard (B), 24-hour cell extract of lutein-treated cells (C), 24-hour cell extract of zeaxanthin-treated cells (D), and 24-hour cell extract of *meso*-zeaxanthin-treated cells (E). Under these conditions, 3-hydroxy-apo-10’-carotenals, the products of BCO2 catalytic conversion of lutein and zeaxanthin, would be seen between retention times 4 and 5 minutes. A column C30 Type Carotenoid, 4.6 X 250 mm, 3 μm (YMC, Inc., Milford, MA) was used with methanol-MTBE (90:10, v/v) at a flow rate of 0.9 ml/min as mobile phase. mAU = absorbance units, min = minutes.
Figure 11. Lutein is not Converted to Meso-Zeaxanthin by ARPE-19 Cells

HPLC chromatogram (A) showing the separation of *meso*-zeaxanthin, zeaxanthin, and lutein in a mixture of standards. HPLC chromatogram of cell extracts after 24 hours of incubation with 10 µM of lutein (B), zeaxanthin (C), or *meso*-zeaxanthin (D) in whole serum. A Chiralpak AD, 25 cm length X 4.6 mm ID column was used with a 3-step gradient as described in the “Materials and Methods” section. mAU = absorbance units, min = minutes