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Mechanism for the Selective Uptake of Macular Carotenoids Mediated by the HDL Cholesterol Receptor SR-BI

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Abstract

The macular carotenoids lutein and zeaxanthin are taken up from the bloodstream into the human retina through a selective process, for which the HDL cholesterol receptor scavenger receptor BI (SR-BI) in the cells of retinal pigment epithelium (RPE) is thought to be a key mediator. However, the mechanism of SR-BI-mediated selective uptake of macular carotenoids is still not fully understood. Here, we investigate possible mechanisms using biological assays and cultured HEK293 cells, a cell line without endogenous SR-BI expression. Binding affinities between SR-BI and various carotenoids were measured by surface plasmon resonance (SPR) spectroscopy, which shows that SR-BI cannot bind lutein or zeaxanthin specifically. Overexpression of SR-BI in HEK293 cells results in more lutein and zeaxanthin taken up than β-carotene, and this effect can be eliminated by an SR-BI mutant (C384Y) whose cholesterol uptake tunnel is blocked. Next, we determined the effects of HDL and hepatic lipase (LIPC), SR-BI's partners in HDL cholesterol transport, on SR-BI-mediated carotenoid uptake. HDL addition dramatically reduced lutein, zeaxanthin, and β-carotene in HEK293 cells expressing SR-BI, but the cellular lutein and zeaxanthin are higher than β-carotene. LIPC addition increases the uptake of all three carotenoids in HDL-treated cells, and promotes the transport of lutein and zeaxanthin better than β-carotene.

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Our results suggest that SR-BI and its HDL cholesterol partner HDL and LIPC may be involved in the selective uptake of macular carotenoids.

Keywords

SR-BI; HDL; LIPC; Macular pigment; Lutein; Zeaxanthin; Transport; Uptake

1. Introduction:

The dietary carotenoids lutein and zeaxanthin accumulate in the human macular region at a very high concentration, forming a visible yellow spot on the retina (Bernstein et al., 1998; Bone et al., 1988; Li et al., 2020; Wald, 1945). These macular carotenoids are well-known antioxidants that also absorb phototoxic blue light. Therefore, as soon as they were identified in the human macula, they were thought to protect the retina from oxidative stress-induced degeneration, especially blue light-mediated oxidative damage (Bernstein et al., 2016; Krinsky et al., 2003; Li et al., 2010; Whitehead et al., 2006). In fact, many clinical studies have demonstrated that supplementation with carotenoids can reduce the risk of age-related macular degeneration (AMD), a common cause of blindness in modern countries (Age-Related Eye Disease Study 2 Research, 2013; Age-Related Eye Disease Study Research, 2001; Bhosale et al., 2007; Landrum et al., 1997; Nolan et al., 2007; Seddon et al., 2001). However, human beings and animals cannot synthesize carotenoids and have to obtain them from the diet, making it important to understand the mechanism of carotenoid transport from the gut to the human eye (Zimmer and Hammond, 2007).

The uptake of macular carotenoids from the diet to the retina is a selective process likely to be controlled by HDL-related proteins. There are around 50 carotenoids in the human diet, of which ~20 can be taken up into the bloodstream, while only lutein and zeaxanthin can eventually reach the retina (Bernstein et al., 2016; Harrison, 2019; Loane et al., 2008). Although lutein and zeaxanthin have been detected in both LDL and HDL in serum, genome-wide association studies (GWAS) reveal that it is not the genetic variants of LDL-related proteins but rather HDL-related proteins, such as HDL-cholesterol receptors scavenger receptor BI (SR-BI), ABCA1, LIPC, LCAT, CETP, and Apo A1, that are significantly associated with the levels of carotenoids in the human retina (Barua and Furr, 1992; Johnson et al., 2000; Khachik et al., 1997; Meyers et al., 2013; Meyers et al., 2014; Montezuma et al., 2007; SanGiovanni and Neuringer, 2012). We recently found that disruption of the HDL lipoprotein in *Bco2* knockout mice resulted in an increase in hepatic carotenoids and a decrease in serum and retinal carotenoids, suggesting that HDL is the primary transporter for macular carotenoids from the liver to the eye (Li et al., 2022). Therefore, the selective uptake of macular carotenoids is likely controlled by HDL-related proteins.

Multiple lines of evidence suggest that the HDL cholesterol receptor SR-BI is a major mediator that controls the uptake of macular carotenoids. A blind *Drosophila* mutant whose dysfunctional *ninaD* gene encoding an SR-BI-like protein leads to a 90% reduction of lutein and zeaxanthin in the fly head (Voolstra et al., 2006), In contrast, upregulation of

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SR-BI in the intestines by depleting its repressor ISX results in more retinal accumulation of lutein and zeaxanthin in *Bco2*$^{-/-}$ mice (Lobo et al., 2010; Seino et al., 2008). SR-BI has been detected in the RPE of both primates and rodents, which implies that the SR-BI may mediate the transport of lutein and zeaxanthin from the choroidal bloodstream to the retina, and it is likely involved in the selective uptake of these carotenoids, too (Duncan et al., 2009; Provost et al., 2003; Shyam et al., 2017b; Tserentsoodol et al., 2006). Inhibition of activity and expression of SR-BI in ARPE19 cells by treatments with anti-SR-BI antibody and small interfering RNA (siRNA) against SR-BI reduced more cellular lutein and zeaxanthin than β-carotene (During et al., 2008). However, the mechanisms underlying the SR-BI-mediated selective uptake of macular carotenoids are still not established.

Several mechanisms have been proposed for the selective uptake of macular carotenoids mediated by SR-BI. Based on whether or not owning oxygen, carotenoids are classified into xanthophylls and carotenes, respectively (Khachik et al., 1992; Khachik et al., 2002). The macular carotenoids lutein and zeaxanthin are the two major xanthophylls in the serum, along with β-carotene, α-carotene, cryptoxanthin, lycopene, and trace amounts of other carotenoids (Barua and Furr, 1992; Johnson et al., 2000; Khachik et al., 1992). Due to the presence of an extra hydroxyl group on the ring at each side of the molecule, lutein and zeaxanthin are more hydrophilic than carotenes. Hence, the stronger binding affinity between SR-BI protein and lutein and zeaxanthin might be responsible for the selectivity of uptake. Meanwhile, SR-BI possesses a hydrophobic cholesterol tunnel (Neculai et al., 2013), which can also contribute to the preferential selection of xanthophylls. In addition, SR-BI's partners in HDL cholesterol transport, such as HDL and LIPC, may also facilitate the selective uptake of macular carotenoids, just like in the selective uptake of cholesterol ester in the liver (Fliesler, 2015; Kontush, 2020). Indeed, recent research has shown that HDL promotes zeaxanthin uptake via SR-BI in cultured human RPE cells more than lutein (Shyam et al., 2017b; Thomas and Harrison, 2016).

In this manuscript, we investigate how SR-BI mediates the selective uptake of macular carotenoids and whether HDL and LIPC, partners of SR-BI in esterified cholesterol uptake, may facilitate this process by using surface plasmon resonance (SPR) spectroscopy, mutagenesis, and biological assays in cultured HEK293 cells.

2. Methods and Materials

2.1 Materials.

Sodium taurocholate hydrate, butylated hydroxytoluene (BHT), dimethyl sulfoxide (DMSO), methanol, polyvinylpyrrolidone, and tetrahydrofuran (THF) were from Sigma Chemicals (Saint Louis, MO, USA). Ethyl acetate, dichloromethane, N, N-diisopropylethylamine (DIPEA), and hexane were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Lutein was provided by Kemin Health (Des Moines, IA, USA). Zeaxanthin and *meso*-zeaxanthin were gifts from DSM Nutritional Products, Ltd. (Kaiseraugst, Switzerland). β-Carotene and astaxanthin were obtained from Sigma Chemicals (Saint Louis, MO, USA).

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Human high-density lipoprotein (HDL; #361-10) and low-density lipoprotein (LDL; #360-10) were purchased from LEE Biosolutions (Maryland Heights, MO, USA). Recombinant human hepatic lipase (LIPC) was from OriGene Technologies, Inc. (#TP315870; Rockville, MD, USA). SR-BI antibody was from LifeSpan BioSciences, Inc. (# LS-C2880/145100; Seattle, WA, USA), and actin antibody was from Sigma-Aldrich (#A2066; Saint Louis, MO, USA). Goat anti-rabbit IgG conjugated to HRP was from Alpha Diagnostic International (# 20320; San Antonio, TX, USA). Recombinant human SR-BI (#11069-H08H), ApoE (#10817-H30E-B), and ApoD (# 11297-H08H) were from Sino Biological Inc. (Beijing, China), while ApoM (#MBS143944) and ApoAII (#MBS318206) came from MyBioSource, Inc. (San Diego, CA, USA). Human glutathione-S transferase P1 (GSTP1) proteinwas expressed in *E.coli* and purified as reported in our laboratory (Li et al., 2014).

Human embryonic kidney HEK293 cells were purchased from ATCC (#CRL-11268™; Manassas, VA, USA). Dulbecco's Modified Eagle Medium (DMEM; #11965-092), fetal bovine serum (FBS; #10439-024), penicillin/streptomycin (#15070-063), and trypsin-EDTA (#25300-063) were all obtained from Thermo Fisher Scientific (Waltham, MA, USA).

2.2 SPR binding assays.

Surface plasmon resonance (SPR) spectroscopy analyses were conducted using a fully automated SensiQ Pioneer optical biosensor equipped with carboxyl-modified sensor chips (Pall ForteBio, Fremont, CA, USA). Using this platform, a protein surface and an unmodified reference surface were prepared for simultaneous analyses. Proteins were immobilized using a standard amine coupling method. Carotenoids were prepared in 100% DMSO and then diluted to a 5% working dilution (1–100 μM) in running buffer (10 mM phosphate-buffered saline, 0.1% polyvinylpyrrolidone, and 5% DMSO, pH 7.4), and injected using the Onestep® (Taylor dispersion) injection method. A concentration gradient was created by slowly diffusing the analyte in the running buffer. Buffer blanks were injected for doublereferencing purposes. Each carotenoid concentration was injected three times, and assays were performed at 25°C. Data were collected at 10 Hz. For affinity determination, SPR response data (sensorgrams) were zeroed at the beginning of each injection and double-referenced. Equilibrium binding constants were determined by using Qdat® analysis software (Pall ForteBio, Fremont, CA, USA).

2.3 Cell culture.

HEK293 cells were cultured in DMEM containing 10% FBS and a 1% penicillin/streptomycin antibiotic mixture at 37 °C with 5% CO_2 in 75 cm^2 tissue culture flasks (#25–209; Genesee Scientific)) in a NAPCO 8000 Direct-Heat CO_2 incubator from Thermo Fisher Scientific (Waltham, MA, USA). Cells at passages 8 to 10 were cultured to 80% confluency and utilized in the following transient transfection and carotenoid assays.

2.4 Plasmid preparation and transient transfection.

Expression vectors of wild-type SR-BI (WT-SR-BI), C384Y-mutant SR-BI (MT-SR-BI) with a red mCherry tag, and the backbone (control) vector were provided by Dr. Sergio Grinstein of the Department of Biochemistry, University of Toronto (Toronto, Ontario,

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Canada). All of the vectors were transfected into DH5α cells for amplification and extracted using a QIAGEN Plasmid Midi Kit (#12143; Germantown, MD, USA). The sequence of each expression vector was confirmed by DNA sequencing at the DNA Sequencing Core Facility of the University of Utah. Upon reaching 80% confluency, HEK293 cells in each flask were transfected with 10 μg plasmid using a jetPRIME® transfection reagent kit (Poly Transfection, Illkirch, France). Transfection efficiencies were determined by checking the expression of the mCherry tag using live-cell fluorescence microscopy (Zeiss Axiovert 200; Carl Zeiss Microimaging, Maple Grove, MN, USA). Carotenoid assays were conducted after 24h of transfection.

2.5 Carotenoid Assays.

To conduct carotenoid uptake assays, we first prepared soluble carotenoids. To make the final concentration of each of the three carotenoids as 4 μM, 400 μL of 100 μM lutein, 400 μL of 100 μM zeaxanthin in methanol, and 400 μL of 100 μM β-carotene in hexane with 30 μL of Tween-40 and 200 μL of acetone were put into a 2 mL centrifuge tube and dried down using argon gas. We then added 300 μL of DMEM cell culture media to the centrifuge tube, vortexed it for 20s, and placed it on a shaker incubating at 4°C for 20 mins. This solution of mixed carotenoids was used in the following assays.

To determine the effect of SR-BI on the uptake of macular carotenoids, the HEK293 cells transfected with WT-SR-BI, MT-SR-BI, and control vectors were cultured for 24h. These cells were then washed three times with PBS after removing the old culture medium. Next, the solution of the mixed carotenoids was added to the cell culture flask to make the final concentration of each carotenoid 4 μM in 10 mL of complete cell culture medium. These cells were cultured for another 24h, washed three times with PBS containing 5% sodium taurocholate hydrate to remove the extracellular carotenoids, and then collected by centrifugation at 900 × *g* for 5 min. The cell pellet was weighed and ready for carotenoid extraction.

To test the effects of HDL on carotenoid uptake, HEK293 cells transfected with WT-SR-BI vector were cultured in DMEM containing 10% lipoprotein–depleted FBS (#BS041101, Kalen Biomedical, LLC, Montgomery Village, MD) and 4 μM mixed carotenoids with or without 350 μg/mL of human HDL lipoproteins (#361-10, Lee Biosolutions, Maryland Heights, MO). After 24h incubation, the cells were collected as above. The HDL protein may have some endogenous carotenoids, but the content is too low to have influence as reported (Harrison, 2019).

To determine the effect of disrupted HDL on carotenoid uptake, the sera from wild-type C57BL/6 (*ApoA1*$^{+/+}$) mice and *ApoA1* knockout (*ApoA1*$^{-/-}$) mice were prepared as before (Li et al., 2014). Briefly, mouse blood was collected by cardiac puncture in BD Microtainer SST Tubes (#365978, Franklin Lakes, NJ, USA), then centrifuged at 1000 × *g* for 5 min for separation of serum. Next, HEK293 cells transfected with WT-SR-BI vector were cultured in DMEM containing 4 μM mixed carotenoids and 1mL serum from *ApoA1*$^{+/+}$ or *ApoA1*$^{-/-}$mice. After 24h incubation, all cells were collected as above.

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Finally, to test the effect of LIPC on carotenoid uptake, HEK293 cells transfected with WT-SR-BI vector were cultured in DMEM containing 10% lipoprotein–depleted FBS, 4 μM mixed carotenoids, and 350 μg/mL HDL with or without 0.5 μg/mL human LIPC protein for 24h. Then, cells were collected for carotenoid analysis.

2.6 Carotenoid extraction and analysis by HPLC.

One mL of THF containing 0.1% BHT was added to each tube with cell pellets and then sonicated for 10s. Next, after brief vortexing, the samples were sonicated in a water bath at 5–10 °C for 30 mins and centrifuged at 1000 × *g* for 5 mins. The organic solvent layer was taken out and dried under nitrogen gas. The above steps were repeated two more times. All of the organic solvent layers with carotenoids were combined and dried down. We then added 0.5 mL hexane, 0.5 mL methanol containing 0.1% BHT, and 0.2 mL H_2O, and then sonicated again in a water bath at 5–10 °C for 30 mins, followed by centrifugation at 1000 × *g* for 5 mins. The upper hexane layer with carotenoids was collected and dried down for HPLC analysis.

The carotenoid samples were dissolved in solvents composed of 95% methanol and 5% *t*-butyl methyl ether and then analyzed on an Agilent 1260 series HPLC system equipped with a quaternary pump, temperature-regulated autosampler, and PDA detector (Agilent Technologies, Santa Clara, CA, USA). A C30 column (YMC, Kyoto, Japan; 30 cm length × 4.6 mm i.d.) was used with methanol and *t*-butyl methyl ether in a linear gradient (% methanol at min: 95 at 0; 70 at 20; 40 at 30; 5 at 40; 95 at 45; 95 at 50) with a flow rate set to 1.0 mL/min. Carotenoids were monitored at 445 nm, identified by PDA spectra and co-elution with standards, and quantified using ChemStation software.

2.7 Western blot.

Pellets of HEK293 cells with and without SR-BI expression were homogenized in PBS buffer using a Branson 102C Digital Sonifier (Danbury, CT, USA). 30 μL of ~1 mg/mL protein per sample was loaded and separated on a 4–15% Bio-Rad precast polyacrylamide gel (#456-1084), and then transferred onto a 0.45 μm nitrocellulose membrane as before (Li et al., 2011). The blotted membrane was incubated with an anti-SR-BI primary antibody (1:1000) overnight at 4°C, then with a secondary antibody (1:2000) for 2 h at room temperature. A parallel experiment was conducted to test the expression of housekeeping protein β-actin under similar conditions. The membranes were developed using an ECL plus Western blot detection kit from GE Healthcare (Lafayette, CO, USA), and imaged on an iBright 750 digital imager from Thermo Fisher Scientific (Waltham, MA, USA).

2.8 Statistical analysis.

Statistical analyses of results were carried out using GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA). The bars on the graphs represent means with ± SD. Two-tailed $P < 0.05$ and $P < 0.001$ were considered significant and highly significant, respectively.

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3. Results:

3.1. The binding affinity between human SR-BI protein and various carotenoids.

The selective uptake of macular carotenoids may be due to high-affinity binding interactions between SR-BI and lutein and zeaxanthin. To investigate whether SR-BI can bind xanthophylls stronger than carotenes, we investigated the binding affinities between the recombinant human SR-BI protein and lutein, zeaxanthin, *meso*-zeaxanthin, β-carotene, and astaxanthin using surface plasmon resonance (SPR) spectroscopy. The zeaxanthin-binding protein GSTP1 and interphotoreceptor retinoid-binding protein (IRBP), which is a retinoid and carotenoid transporter between retina and RPE (Bhosale et al., 2004; Vachali et al., 2013), were used as controls. Smaller K_D values represent stronger binding affinities. Table 1 demonstrates that the binding affinities between human SR-BI and xanthophyll carotenoids have no significant difference with β-carotene. . These binding affinities are similar to IRBP butmuch weaker than that between GSTP1 and zeaxanthin. This suggests that SR-BI may not be able to selectively capture lutein and zeaxanthin.

3.2 The influence of a cholesterol-tunnel-blocked SR-BI mutant on the uptake of macular carotenoids.

Next, we investigated if the hydrophobic cholesterol tunnel in SR-BI is responsible for the selective uptake of macular carotenoids in cell culture assays. To eliminate the effects of endogenous SR-BI on the uptake of carotenoids, we selected the HEK293 cell line because it reportedly expresses no SR-BI. Immunocytochemistry (ICC) and western blot analysis confirmed no SR-BI expression in the control HEK293 cells, while there was strong expression in the HEK293 cells transfected with the SR-BI expression vector (Figures 1 A and B).

Substitution of SR-BI's cysteine (C) residue positioned at 384 in the cholesterol tunnel with a tyrosine (Y) residue can dramatically block the uptake of esterified cholesterol ((Neculai et al., 2013; Yu et al., 2012). Therefore, this SR-BI mutant (C384Y) was used to investigate the effects of the hydrophobic cholesterol tunnel on SR-BI-mediated uptake of macular carotenoids. After culturing to ~80% confluency, HEK293 cells were transfected with the expression vectors of wild-type SR-BI (WT-SR-BI), SRBI-C384Y mutant (MT-SR-BI), or empty vector (control) for 24 hours. These cells were then treated with 4 μM lutein, zeaxanthin, and β-carotene dissolved in 0.05% Tween-40 for 24 hours and harvested to measure the cellular carotenoids by HPLC. Figure 1C demonstrates that WT-SR-BI expression increased the uptake of all three carotenoids, whereas the SR-BI mutant offset its effects. In the control cells, lutein and zeaxanthin were about 10 times higher than β-carotene. It also shows that WT-SR-BI caused net increases of lutein, zeaxanthin, and β-carotene at about 2 ng, 2.6 ng, and 0.5 ng per gram of wet cells, respectively.

3.3 Effects of HDL on SR-BI-mediated selective uptake of macular carotenoids.

To determine if HDL facilitates SR-BI mediated selective uptake of macular carotenoids, we investigated the uptakes of lutein, zeaxanthin, and β-carotene in HEK293 cells expressing SR-BI with and without HDL addition. Unexpectedly, we found that HDL addition significantly reduced the cellular contents of lutein, zeaxanthin, and β-carotene to around

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13%, 14%, and 3% of the control, respectively (Figure 2). This implies that HDL probably holds the carotenoids too tightly to release them readily to SR-BI. If true, disruption of HDL by depletion of its key apolipoprotein ApoA1 may result in more carotenoids taken up by SR-BI.

To test this hypothesis, we further investigated the effects of sera of *ApoA1* knockout (*ApoA1*$^{-/-}$) mice and C57BL/6 wild-type (*ApoA1*$^{+/+}$) mice on carotenoid uptake in HEK293 cells expressing SR-BI. As shown in Figure 3, in comparison with the control, the addition of *ApoA1*$^{-/-}$ serum led to a 1.72 times increase in zeaxanthin and a 2.91 times increase in β-carotene, whereas no significant change occurred in lutein. This indicates that disruption of HDL may result in more carotenoids taken up by cells. On the other hand, we can see that the effects of ApoA1 deletion on carotenoid uptake vary among carotenoids. It affected β-carotene>zeaxanthin> lutein, following the binding affinities between ApoA1 and these carotenoids.

It appears that ApoA1 likely plays a role in the selective uptake of macular carotenoids. Therefore, we further measured the binding affinities between other apolipoproteins of HDL and various carotenoids by SPR. Table 2 exhibits the binding affinities between ApoAI, ApoAII, ApoE, ApoD, and β-carotene>> zeaxanthin ≈*meso*-zeaxanthin> lutein. The binding affinity between ApoM and β-carotene is also the strongest, but it binds lutein and *meso*-zeaxanthin tighter than zeaxanthin. These data suggest that HDL can facilitate the SR-BI-mediated selective uptake of macular carotenoids through the selectivity of its apolipoproteins, while HDL addition may not be able to promote the uptake of carotenoids.,

3.4 Effects of LIPC on SR-BI-mediated selective uptake of macular carotenoids.

LIPC is one of the three essential proteins involved in the selective uptake of esterified cholesterol (Holmes et al., 2011; Santamarina-Fojo et al., 2004). It functions as a bridge ligand between HDL and SR-BI to facilitate selective uptake of esterified cholesterol. To gain more complete insights into the SR-BI-mediated selective uptake of macular carotenoids, we further evaluated the effects of LIPC on the uptake of carotenoids. HEK293 cells expressing SR-BI were incubated with carotenoids and HDL with and without LIPC addition for 24 hours, and they were then harvested for carotenoid analysis by HPLC. Figure 4 demonstrates that LIPC addition increased uptake of lutein by 18%, zeaxanthin by 20%, and β-carotene by 11% in HEK293 cells relative to the control. This suggests that LIPC may also facilitate the selective uptake of macular carotenoids mediated by SR-BI.

4. Discussion:

In this work, we systematically investigated the mechanism of the SR-BI-mediated selective uptake of macular carotenoids that may occur in human RPE. We found that the hydrophobic cholesterol tunnel of SR-BI is responsible for the selective uptake of lutein and zeaxanthin, and SR-BI's partners in the esterified cholesterol uptake, HDL and LIPC, may also facilitate this process.

Although biological and genetic evidence has demonstrated that SR-BI may mediate the selective uptake of lutein and zeaxanthin (During et al., 2008; McKay et al., 2013; Meyers

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et al., 2013), the mechanisms by which SR-BI controls the selective uptake of lutein and zeaxanthin remain unelucidated. Our results suggest that the selective uptake of lutein and zeaxanthin is unlikely to be controlled by specific capture by SR-BI but rather by its cholesterol transport tunnel. Our SPR data in Table 1 show that SR-BI cannot preferentially bind lutein or zeaxanthin over β-carotene, which rules out the possibility that SR-BI could specifically capture them. Another possible mechanism is that the hydrophobic cholesterol tunnel in SR-BI might create selectivity when carotenoids pass through it. After all, lutein and zeaxanthin are more hydrophilic than β-carotene. Taking advantage of a known cholesterol-tunnel mutant of SR-BI, C384Y, which can block cholesterol transport, we found that this SR-BI mutant impaired the uptake of lutein and zeaxanthin more than β-carotene (Figure 1C), suggesting that lutein and zeaxanthin could pass through this hydrophobic tunnel more easily. This evidence supports our hypothesis that SR-BI's cholesterol tunnel controls the selective uptake of macular carotenoids. To our knowledge, this is the first attempt to explore the molecular mechanism of SR-BI-mediated carotenoid uptake by mutagenesis experiments.

SR-BI's partner in the uptake of esterified cholesterol, HDL, may also facilitate the selective uptake of lutein and zeaxanthin. Indeed, it has been reported that HDL is able to selectively deliver zeaxanthin into cells with SR-BI expression, and more zeaxanthin is accumulated in cells after incubation with more carotenoids or for a longer time (Shyam et al., 2017b; Thomas and Harrison, 2016). We found that approximately a 4 times higher percentage of zeaxanthin and lutein were detected in cells treated with HDL than β-carotene (Figure 2), suggesting that HDL can facilitate the selective uptake of xanthophylls. Unexpectedly, we found that HDL addition dramatically reduced all three carotenoids relative to the cells treated with no HDL. To explain this result, we incubated the HEK293 cells expressing SR-BI with sera from WT mice and mice deficient in ApoA1, the key HDL apolipoprotein. Zeaxanthin and β-carotene were increased 2 to 3 times in cells treated with *ApoA1*$^{-/-}$ serum than WT (ApoA$^{+/+}$) serum (Figure 3), showing that HDL disruption resulted in more carotenoid uptake. It revealed that HDL probably binds carotenoids too tightly to easily hand them over to SR-BI, thereby reducing the uptake of carotenoids. Additionally, we saw that the impacts of ApoA1 deletion varied among three carotenoids, suggesting ApoA1 may also be involved in the selective uptake of lutein and zeaxanthin. We measured the binding affinities between human ApoA1 and carotenoids and found that ApoA1 tightly binds β-carotene>> zeaxanthin>lutein. A similar tendency existed among HDL's other apolipoproteins, such as ApoAII, ApoE, ApoD, and ApoM. Our SPR data suggest that the apolipoprotein of HDL could contribute to the selective uptake of xanthophylls, too.

LIPC can function as not only a lipase but also a ligand that facilitates the selective uptake of esterified cholesterol from HDL to SR-BI (Santamarina-Fojo et al., 2004). Our results demonstrate that LIPC can also promote SR-BI-mediated carotenoid uptake, slightly favoring the uptake of xanthophylls (Figure 4). Therefore, both HDL and LIPC appear involved in the uptake of macular carotenoids mediated by SR-BI.

The mechanism of SR-BI-mediated carotenoid uptake proposed in this manuscript is more suitable for understanding the selective uptake of lutein and zeaxanthin from the choroidal bloodstream to the RPE. SR-BI also is present in the intestines and is known to be

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an essential transporter of carotenoids (Seino et al., 2008); however, the mechanism of carotenoid uptake mediated by intestinal SR-BI may vary because the intestinal SR-BI has to take up carotenoids from the food matrix instead of the bloodstream. Our mutagenesis data reveal that SR-BI mainly controls the selectivity between xanthophylls and carotenes but not that between lutein and zeaxanthin. This discrepancy between the uptake of lutein and zeaxanthin might be ascribed to the lipoproteins, as we and others have reported that HDL prefers to transport zeaxanthin over lutein (Shyam et al., 2017b; Thomas and Harrison, 2016). There are a couple of other limitations, too. HEK293 cell is a human kidney cell line, whose properties may be differ from the retinal cells. It is unknown whether or not there are receptors other than SR-BI controlling carotenoid transport in the RPE cells; however, our results show that the HEK 293 cell line itself, which has no endogenous SR-BI, preferentially takes up xanthophylls over β-carotene (the control in Figure 1C), indicating there probably are other receptors. In addition, in the serum of ApoA1 $^{-/-}$ mice, there are other lipoproteins except for HDL that can interact with carotenoids. Therefore, the possible interference from other receptors and lipoproteins cannot be excluded completely from the results of this work. Finally, we want to point out that SR-BI expression did not significantly impact the uptake of lutein, although a tendency of increase can be clearly seen. To clarify the role of SR-BI in lutein absorption, more carotenoid assays and animal experiments are warranted.

The uptake of macular carotenoids is a complex process in which multiple factors control the selective uptake of lutein and zeaxanthin. Besides SR-BI, HDL, and LIPC, the two carotenoid cleavage enzymes and the two specific ocular carotenoid-binding proteins are also thought to play roles in the selective uptake of carotenoids. Human β-carotene oxygenase 1 (BCO1) can efficiently cleave carotenes but not the xanthophylls (dela Sena et al., 2013; Raghuvanshi et al., 2015). In contrast, we and others have reported that human β-carotene oxygenase 2 (BCO2) cannot efficiently break down either xanthophylls or carotenes (dela Sena et al., 2016; Li et al., 2014). Thus, the specific enzymatic activities of BCO1 and BCO2 are already able to create selectivity of xanthophylls. In addition, lutein can be converted into *meso*-zeaxanthin by RPE65 (Shyam et al., 2017a), thereby reducing lutein and increasing zeaxanthins in the eye. When reaching the retina, lutein and zeaxanthin will be captured by their specific binding proteins, StARD3 and GSTP1 (Bhosale et al., 2004; Li et al., 2011). Moreover, we found that overexpression of human GSTP1 in *Bco2* knockout mice can specifically increase retinal zeaxanthin (Li et al., 2017), suggesting that binding proteins could also contribute to the selective uptake of lutein and zeaxanthin. In addition, the newly identified intracellular carotenoid transporter Aster protein might be a contributor to this selective process, too (Bandara et al., 2022). It is also known that the digestive system, the lymph system, the circulation, and the visual system are involved in the transport and uptake of macular carotenoids (Erdman et al., 1993; Harrison, 2019). Therefore, there must be additional factors that control the selective uptake of carotenoids.

It is crucial to uncover the entire macular carotenoid transport and uptake pathway in order to better utilize carotenoids to prevent eye diseases such as age-related macular degeneration (AMD) and improve visual performance. For example, if the intestinal carotenoid transporter loses its function, we may consider supplying lutein and zeaxanthin parenterally. Additionally, a gene-augmentation strategy focused on carotenoid-binding

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proteins may be a potential approach to increase macular carotenoid content. On the other hand, we noticed that many HDL-related proteins in reverse cholesterol transport also play roles in macular carotenoid transport and uptake. Dysfunction of these proteins may reduce carotenoids in the eye; meanwhile, it could cause the accumulation of bad cholesterol in the human body, and leading to atherosclerosis. Therefore, measuring carotenoid levels in the retina noninvasively may help to detect diseases unrelated to the eye, such as coronary artery disease and other types of atherosclerosis at very early stages.

In conclusion, we investigated the mechanism of SR-BI-mediated selective uptake of macular carotenoids from the bloodstream to the RPE. Our work demonstrates that the hydrophobic cholesterol tunnel in SR-BI may control the preferential uptake of xanthophylls, and SR-BI's partners HDL and LIPC also contribute to the selective uptake of macular carotenoids. These results may provide further insights into macular carotenoid transport and uptake, offering the potential for improved treatments against human retinal disease.

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Abbreviations:

AMD	age-related macular degeneration
BCO1	β-carotene oxygenase 1
BCO2	β-carotene oxygenase 2
BHT	butylated hydroxytoluene
DIPEA	N, N-diisopropylethylamine
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
FBS	fetal bovine serum
GSTP1	glutathione-S transferase P1
GWAS	genome-wide association studies
HDL	high-density lipoprotein
ICC	Immunocytochemistry
ISX	intestine-specific homeobox transcription factor
IRBP	interphotoreceptor retinoid-binding protein

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LIPC	hepatic lipase
RPE	retinal pigment epithelium
siRNA	small interfering RNA
SPR	surface plasmon resonance spectroscopy
SR-BI	scavenger receptor BI
THF	tetrahydrofuran
WT	wild-type

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Highlights

- SR-BI's cholesterol domain controls the selective uptake of macular carotenoids.
- HDL lipoproteins bind zeaxanthin and β-carotene stronger than lutein.
- LIPC facilitates SR-BI-mediated carotenoid uptake.

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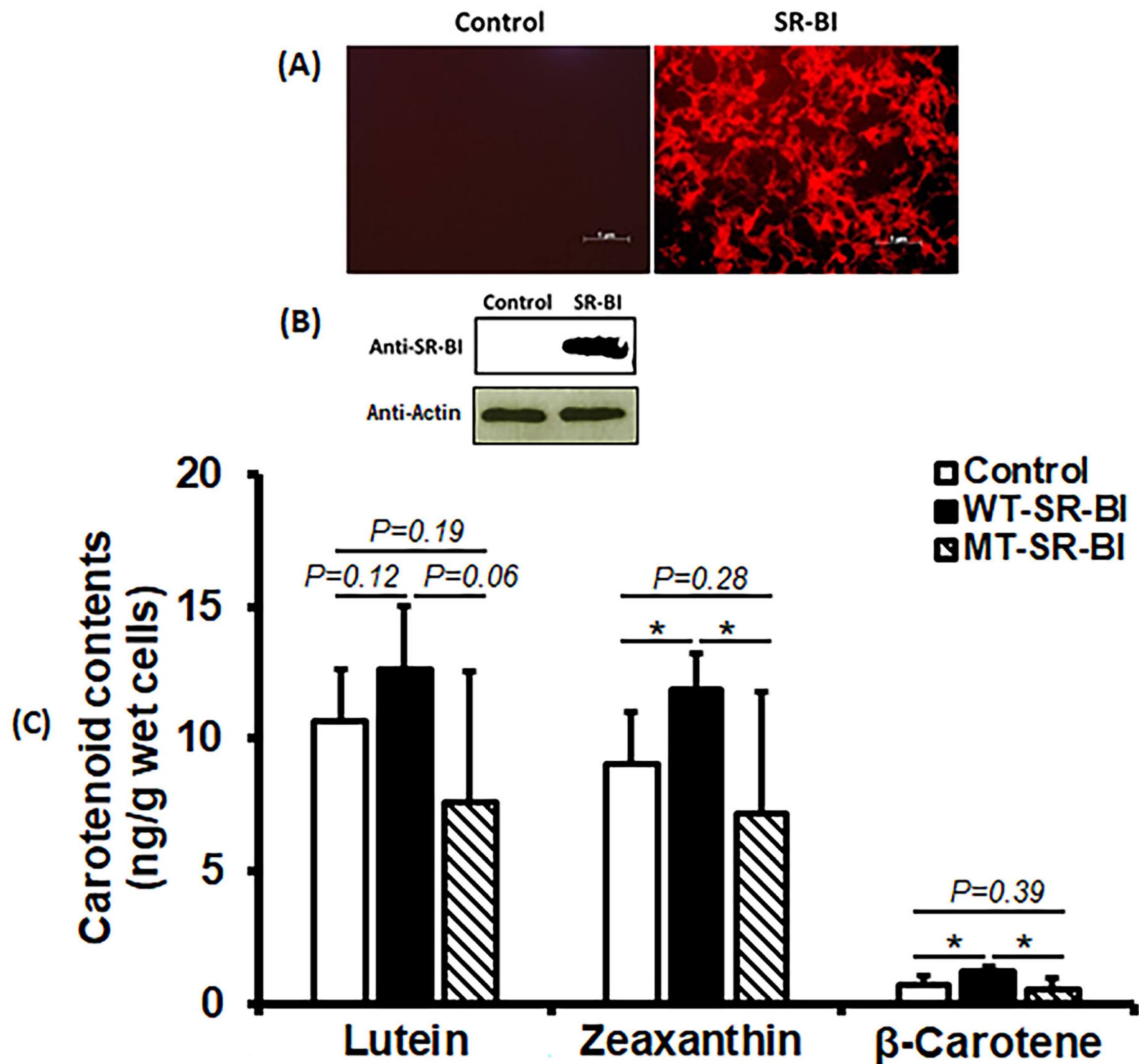

Figure 1. Effects of SR-BI mutant on the uptake of carotenoids.

Overexpression of WT-SR-BI increased lutein, zeaxanthin, and β-carotene in HEK293 cells, which was canceled out by replacing the cysteine amino acid (C) positioned at 384 with a tyrosine (Y) residue in the cholesterol transport tunnel of SR-BI. **(A)** Images of HEK293 cells transfected with and without (control) mCherry-tagged WT-SR-BI by Fluorescence microscopy; red, mCherry-tagged SR-BI protein. **(B)** Western blot results of cells from **(A). (C)** Carotenoid contents in HEK293 cells expressing wild-type (WT) SR-BI, C384Y-mutant SR-BI (MT), and empty vector (control). Twenty-four hours after being transfected with expression vectors WT-SR-BI, MT-SR-BI, and control vector, HEK293 cells were incubated with mixed carotenoids of lutein, zeaxanthin, and β-carotene (1:1:1) for another 24 hours, then collected for carotenoid analysis by HPLC. All assays were repeated 3 to 5 times. *, $P<0.05$.

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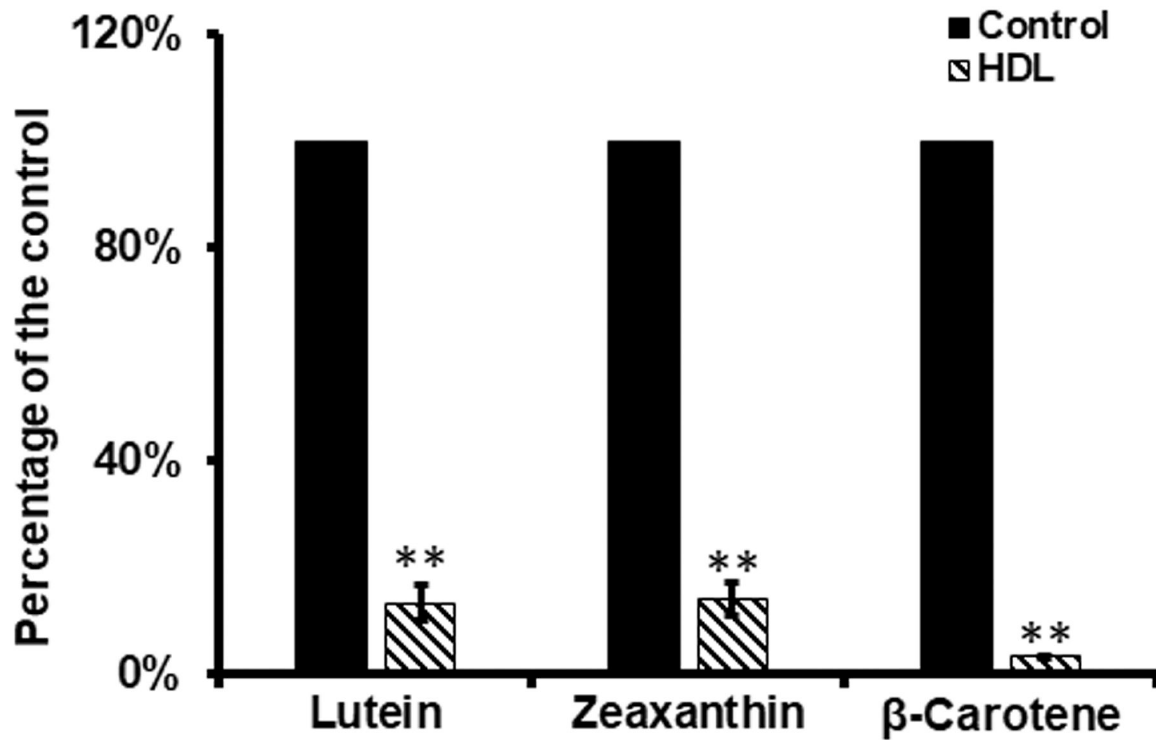

Figure 2. Effects of HDL on SR-BI-mediated uptake of carotenoids.

HDL addition significantly reduced lutein, zeaxanthin, and β-carotene in HEK293 cells expressing WT-SR-BI, while lutein and zeaxanthin contents were higher than than β-carotene. HEK293 cells were cultured in a complete cell culture medium, whose FBS was replaced with lipoprotein-depleted FBS, and incubated with the mixed carotenoids of lutein, zeaxanthin, and β-carotene (1:1:1) with and without (control) the addition of HDL for 24h, then collected for carotenoid analysis by HPLC. All assays were repeated 3 to 5 times. *, $P<0.05$; **, $P<0.001$.

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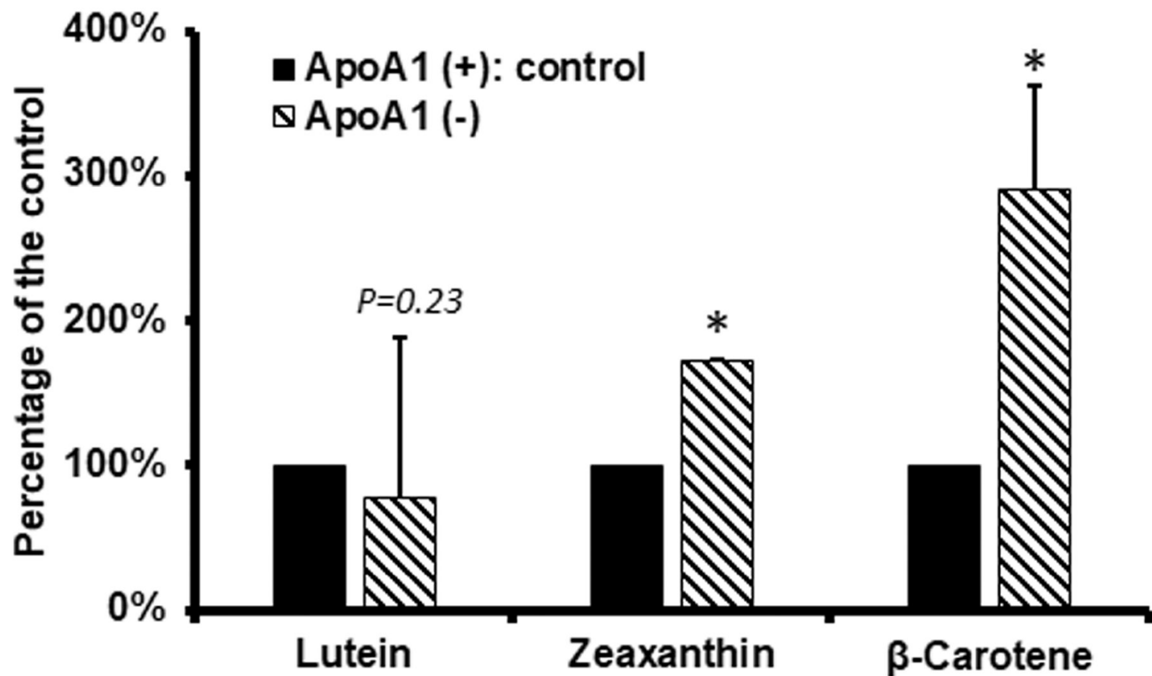

Figure 3. Effects of serum of *ApoA1* knockout (*ApoA1*$^{-/-}$) mice on SR-BI-mediated uptake of carotenoids.

The *ApoA1*$^{-/-}$ mice serum significantly increased zeaxanthin and β-carotene but not lutein in HEK293 cells expressing WT-SR-BI. HEK293 cells were cultured in a complete cell culture medium without FBS and incubated with the mixed carotenoids of lutein, zeaxanthin, and β-carotene (1:1:1) with the addition serum from the *ApoA1*$^{-/-}$ *mice* or *the ApoA1*$^{+/+}$ mice (C57BL/6, control) for 24h, then collected for carotenoid analysis by HPLC. All assays were repeated 3 to 5 times. *, $P<0.05$.

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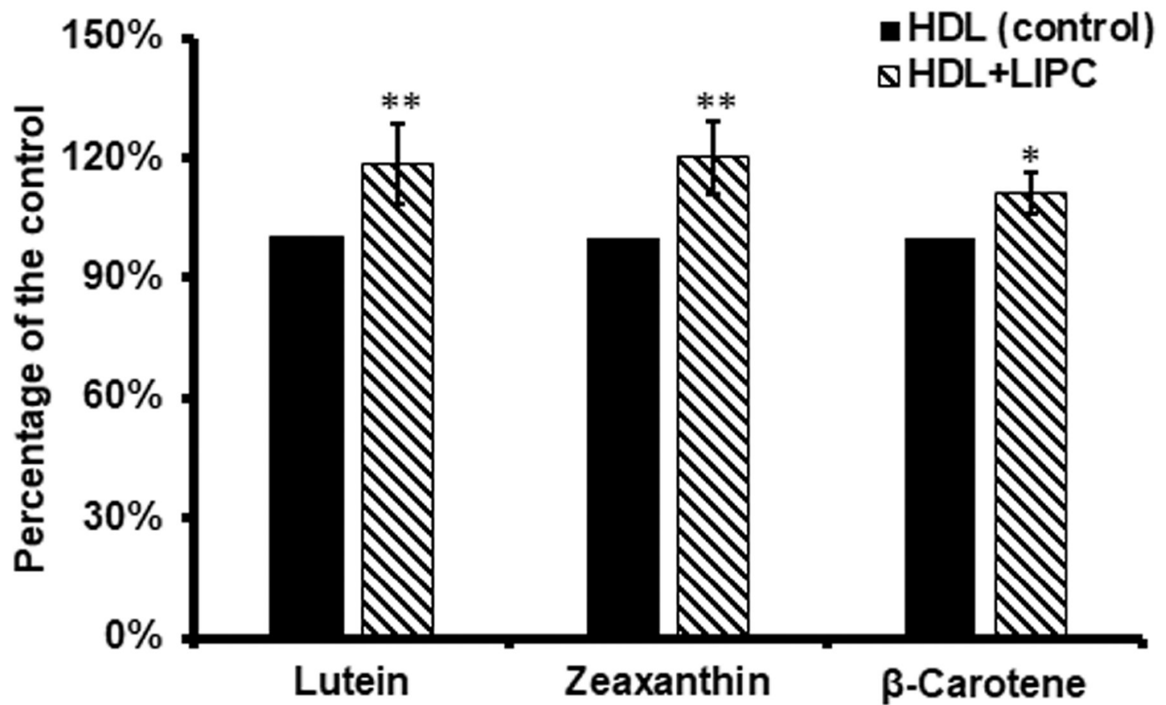

Figure 4. Effects of hepatic lipase (LIPC) on SR-BI-mediated uptake of carotenoids.

The addition of LIPC facilitated the uptake of lutein, zeaxanthin, and β-carotene in HEK293 cells expressing WT-SR-BI. HEK293 cells were cultured in the lipoprotein-depleted-FBS culture medium containing HDL, and incubated with lutein, zeaxanthin, and β-carotene (1:1:1) with and without (control) the addition of LIPC for 24h, then collected for carotenoid analysis by HPLC. All assays were repeated 3 to 5 times. *, $P<0.05$; **, $P<0.001$.

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Table 1.

Binding affinities between proteins and carotenoids determined by surface plasmon resonance (SPR) spectroscopy

Carotenoids	Human IRBP (μM)	GSTP1 (μM)	Human SR-BI (μM)
Lutein	1.06 ± 0.20	N.D.	1.03 ± 0.002
Zeaxanthin	1.64 ± 0.50	0.053 ± 0.01	1.41 ± 0.001
meso-Zeaxanthin	1.85 ± 0.30	0.055 ± 0.03	1.09 ± 0.008
β-Carotene	0.92 ± 0.10	N.D.	0.94 ± 0.070
Astaxanthin	1.64 ± 0.06	0.15 ± 0.02	1.08 ± 0.0003

N.D., no detectable interaction.

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Table 2.

SPR equilibrium binding constants (K_D) between HDL apolipoproteins and carotenoids

Lipoproteins \ Carotenoids	Lutein (μM)	Zeaxanthin (μM)	*meso*-Zeaxanthin (μM)	β-Carotene (μM)
Apo-AI	12.14 ± 0.00	5.62 ± 0.00	7.90 ± 0.20	0.33 ± 0.03
Apo-E	21.00 ± 1.00	9.25 ± 0.00	6.22 ± 0.00	0.40 ± 0.06
Apo-AII	6.10 ± 0.30	1.84 ± 0.09	1.60 ± 0.70	N.D.
Apo-D	84.00 ± 1.00	68.15 ± 0.00	45.58 ± 0.00	0.01 ± 0.06
Apo-M	35.00 ± 1.00	81.00 ± 9.00	35.40 ± 0.80	0.05 ± 0.00

N.D., no detectable interaction.