RPE65 has an additional function as the lutein to *meso*-zeaxanthin isomerase in the vertebrate eye

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Discussion

RPE65 is an important enzyme in the visual cycle, responsible for the key all-*trans* to 11-*cis*-retinoid isomerization step of the visual cycle. Such diseases as Leber congenital amaurosis and retinitis pigmentosa arise from the loss of function of this gene. Here we show that RPE65 is capable of carrying out an additional function in which it converts lutein to *meso*-zeaxanthin, an eye-specific carotenoid with no common dietary sources. Since *meso*-zeaxanthin is accumulated in high concentrations at the fovea of the retina, a region crucial for visual acuity, its hypothesized function is to protect the region from blue light damage and oxidative stress and to potentially enhance visual function. In support of this hypothesis, a previous study has shown that *meso*-zeaxanthin has stronger antioxidant properties than lutein and zeaxanthin, and another study has shown that oral supplementation with all three macular carotenoids can improve contrast sensitivity in normal individuals. The process by which *meso*-zeaxanthin is produced in the eye has been a mystery. In the present study, we show in both in vitro and in vivo systems that RPE65 is the enzyme that catalyzes the conversion of lutein to *meso*-zeaxanthin.

After identifying RPE65 as a prime candidate for the *meso*-zeaxanthin isomerase in chicken RPE by RNA sequencing studies, we conducted overexpression experiments using both chicken and human *RPE65* plasmids in a nonocular cell culture system. Our studies in HEK293T cells show that RPE65 of both species is capable of producing *meso*-zeaxanthin from lutein, but not from zeaxanthin. HEK293T cells do not endogenously express LRAT, the acyl transferase enzyme essential to provide all-*trans* fatty acid ester retinoid substrates for RPE65 to catalyze their conversion into 11-*cis*-retinol. By overexpressing RPE65 in a system free of LRAT and treating these cells with HPLC-purified lutein, we were able to produce *meso*-zeaxanthin independent of LRAT's catalytic activity. The reaction is slow in cell culture, with no detectable product observed until several days after the addition of lutein. This is consistent with the relatively slow formation of *meso*-zeaxanthin during chicken eye development, which also takes several days.

Our structural modeling studies show that the epsilon ring of lutein can coordinate with the active site histidines and iron of RPE65 in a manner that could facilitate the double-bond shift reaction required to convert lutein to *meso*-zeaxanthin by a mechanism involving acid-base catalysis or some other mechanism. We also found that an RPE65 inhibitor, ACU-5200, was able to specifically inhibit formation of *meso*-zeaxanthin during chicken eye development without affecting lutein or zeaxanthin uptake into the RPE/choroid. Its close analog, emixustat, is currently in clinical trials as a visual cycle inhibitor for various eye diseases. Our finding of RPE65's additional role in macular carotenoid metabolism suggests that it may be of interest to examine whether this compound detectably alters macular pigment levels or distributions in the participants in these clinical trials.

Mutations in human *RPE65* are quite rare and typically cause severe visual function deficits, and we suspect that individuals with deleterious mutations in *RPE65* may also have abnormalities in their macular pigment levels and distributions. Interestingly, SNPs in human *RPE65*, along with other carotenoid-associated genes, such as *GSTP1*, *BCO1*, and *SCARB1*, were identified as determinants of macular optical density in women participating in the CAREDS study.

The notion that RPE65 is the *meso*-zeaxanthin isomerase is appealing, since its carotenoid oxygenase family members BCO1 and BCO2 are known carotenoid cleavage enzymes. In fact, RPE65's alternate name is BCO3. These three proteins share significant sequence homology, and each plays a crucial role in vertebrate retinoid and carotenoid physiology. BCO1 cleaves β-carotene at the central 15, 15′ site to produce two molecules of retinal. This newly formed all-*trans*-retinal undergoes reduction and conversion into retinyl esters that are substrates for RPE65-mediated production of 11-*cis*-retinol; alternatively, retinal can be oxidized to retinoic acid, which is used for cell signaling and gene regulation. BCO2 cleaves a variety of xanthophyll carotenoid substrates at the 9′, 10′ double bond and is involved in the homeostasis of non-provitamin A carotenoids.

In other species, a single enzyme can perform the functions of BCO1, BCO2, and RPE65. Arthropods encode a single carotenoid cleavage enzyme, NinaB, that performs the functions of all three BCO family members. Carotenoid cleavage enzymes in lower organisms have a range of substrate specificities. ACO from cyanobacteria is capable of cleaving carotenoids of various lengths, ranging from C_{20} to C_{27}. This enzyme binds to substrates with either aldehydes or alcohols at their terminal ends distal to the ionone ring, and it also can accept apocarotenoids with or without 3-hydroxyl groups on the ionone rings. Therefore, it is not unprecedented that RPE65, whose known interactions until now have been only with retinoids, may interact with structurally similar molecules, such as carotenoids.

Our findings show that *meso*-zeaxanthin production from lutein occurs in the RPE, and that it is catalyzed by RPE65. The specific accumulation of this carotenoid in the fovea may be mediated by specific transporters as well as binding proteins. IRBP and class B scavenger receptor proteins are capable of shuttling carotenoids to the retinal layers from the RPE via the interphotoreceptor space. GSTP1 is a known zeaxanthin-binding protein present in the primate RPE and foveal regions that binds *meso*-zeaxanthin with equally high affinity. It is plausible to hypothesize that newly formed *meso*-zeaxanthin from the RPE is shuttled into the subretinal space and then into the retinal layers by means of transport proteins, and that once in the retina, it may be held in place in the foveal region by specific binding proteins.

In the present study, we have described a novel function of RPE65 as the lutein to *meso*-zeaxanthin isomerase. We have shown that both chicken and human RPE65 are capable of converting lutein to *meso*-zeaxanthin. The reaction rate is slow, and *meso*-zeaxanthin isomerization is likely a secondary function of RPE65. The foveal presence of *meso*-zeaxanthin, especially given its lack of common dietary sources, has been a conundrum in the field of carotenoid biology. With the identification of RPE65 as the enzyme responsible for the production of *meso*-zeaxanthin, future studies can further delineate the physiological role of this macula-specific carotenoid.