Cholestenic Acid Is an Important Elimination Product of Cholesterol in the Retina: Comparison of Retinal Cholesterol Metabolism with That in the Brain

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PURPOSE. Accumulating evidence indicates a link between cholesterol and age-related macular degeneration. Yet, little is known about cholesterol elimination from the retina and retinal pigment epithelium (RPE), the two layers that are damaged in this blinding disease. Several different pathways of enzymatic cholesterol removal exist in extraocular tissues. The authors tested whether metabolites from these pathways could also be quantified in the bovine and human retina and RPE. For comparison, they measured cholesterol oxidation products in two regions of the bovine and human brain and in the bovine liver and adrenal glands.

METHODS. Sterol quantification was carried out by isotope dilution gas chromatography-mass spectrometry. Bovine tissues were used first to optimize analytical procedures and to investigate postmortem changes in oxysterol concentrations. Then human specimens were analyzed for oxysterol concentrations.

RESULTS. Qualitatively, oxysterol profiles were similar in the bovine and human tissues. In the human retina and RPE, the authors could not detect 27-hydroxycholesterol but unexpectedly found that its oxidation product, 5-cholestenic acid, is the most abundant oxysterol, varying up to threefold in different persons. 24S-Hydroxycholesterol and pregnenolone were also present in the retina, but at much lower quantities and without significant interindividual variability. In the brain, the predominant oxysterol was 24S-hydroxycholesterol.

CONCLUSIONS. The oxysterol profile of the retina suggests that all known pathways of cholesterol elimination in extraocular organs are operative in the retina and that they likely vary depending on specific cell type. However, overall oxidation to

5-cholestenic acid appears to be the predominant mechanism for cholesterol elimination from this organ. (*Invest Ophthalmol Vis Sci.* 2011;52:594–603) DOI:10.1167/iovs.10-6021

Steady state levels of cholesterol in any organ represent a balance between the pathways of accumulation and elimination. These pathways have been studied extensively in the liver, the central organ for regulation of total body cholesterol homeostasis,¹ and less intensively in the brain and eye (see Refs. 2–7 for reviews), whose contribution to total body cholesterol balance are deemed not very significant. Cholesterol homeostasis in the brain and retina, however, is now drawing more attention because of the putative links between cholesterol and Alzheimer's disease and cholesterol and age-related macular degeneration (AMD).^{8,9} Because cholesterol cannot cross the blood-brain barrier, most of the brain's cholesterol is derived from local synthesis.¹⁰ The retina also has a barrier, the blood-retinal barrier,¹¹ and thus synthesizes cholesterol endogenously.^{12,13} Yet, in contrast to the brain, plasma low-density lipoprotein (LDL) containing high amounts of cholesterol can enter the retina through the underlying retinal pigment epithelium (RPE).¹⁴ The relative contributions of LDL-derived cholesterol and endogenously synthesized cholesterol to total retinal cholesterol are unknown.

It has also been established that cholesterol trafficking in the central nervous system is mediated by apolipoprotein E⁷ and that elimination is achieved by enzymatic conversion to 24S-hydroxycholesterol (24-OH) by cytochrome P450 46A1 (CYP46A1).^{15–17} 24-OH spontaneously diffuses across cellular membranes and the blood-brain barrier, becomes associated with plasma lipoproteins, and is delivered to the liver for further degradation to bile acids.² Systematic elucidation of cholesterol transport and metabolism in the retina began only recently. Many proteins involved in the reverse transport of cholesterol from extrahepatic tissues to the liver—apolipoproteins E and A1; cholesterol efflux transporter ABCA1; class B scavenger receptors SR-BI, SR-BII and CD36; lecithin-cholesterol acyltransferase; and cholesteryl ester transfer protein—were also found to be expressed in the retina.^{18–22} Based on the retinal localization of these proteins, it was proposed that lipids move within the retina in the form of high-density lipoprotein (HDL) and may be secreted back to the circulation to maintain homeostasis.¹⁹ The importance of HDL and cholesterol transport are also supported by two recent genetic studies that revealed association between AMD and several genes (hepatic triglyceride lipase, cholesteryl ester transfer protein, ABCA1, and lipoprotein lipase) involved in the metabolism of HDL.^{23,24} In parallel, significant efforts are being directed toward elucidating the source of age-dependent lipid deposition in Bruch's membrane (BrM), where drusen, the clinical hallmark of AMD, develop. BrM is located between the RPE and the choriocapillaris. Evidence from light microscopy, ultra-

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structural studies, lipid histochemistry, isolated lipoprotein assays, and gene expression analysis suggests that lipid deposition in BrM is, at least in part, due to the accumulation of apolipoprotein B-containing particles complexed with esterified cholesterol (see Refs. 5 and 6 for reviews). A model was developed according to which, in younger eyes, cholesterol-rich lipoprotein particles leave the RPE and cross the BrM for egress to the plasma. With advanced age, transit time across BrM increases, resulting in lipoprotein accumulation and modification. This eventually leads to the formation of drusen.^{5,6}

Incorporation into lipoprotein particles is not the only mechanism whereby extrahepatic cells dispose of excess cholesterol. Cholesterol is also eliminated enzymatically through metabolism to more polar 27-OH or 24-OH or by conversion to steroid hormones (Fig. 1). Cholesterol 27-hydroxylation, catalyzed by cytochrome P450 27A1 (CYP27A1), takes place in many extrahepatic cells, complementing HDL-mediated reverse cholesterol transport.^{25,26} CYP27A1, however, probably does not contribute significantly to cholesterol elimination from the brain under normal conditions, where hydroxylation at position 24 represents the major mechanism of cholesterol removal.^{2,27} Also expressed in the brain is cytochrome P450 11A1 (CYP11A1), which catalyzes the conversion of cholesterol to pregnenolone (Preg), the first step in steroid hormone biosynthesis.²⁸ Similarly to CYP27A1, CYP11A1 plays only a minor role in cerebral cholesterol removal; this enzyme is important for cholesterol metabolism in steroidogenic tissues. All three cholesterol-metabolizing P450s—CYPs 27A1, 46A1, and 11A1—were found to be expressed in the retina,^{29–33} but their significance for retinal cholesterol homeostasis has not yet been investigated, and it is still unknown whether the major mechanism for enzymatic elimination of cholesterol from the retina involves 24- or 27-hydroxylation or the conversion to Preg. To gain insight into the initial steps of retinal cholesterol biotransformations, we measured the levels of five oxysterols that represent the products of cholesterol-metabolizing P450s. Analytical procedures were first optimized on bovine tissues, followed by sterol quantifications in human specimens. Retinal and RPE sterols were also compared with sterol profiles in extraocular organs in which cholesterol-metabolizing P450s are the most abundant. Quite unexpectedly, 3 β -hydroxy-5-cholestenoic acid (27-COOH), an oxidation product of 27-OH, was found to be the predominant oxysterol in

the human retina. These and other data indicate that cholesterol metabolism in the retina is significantly different from that in the brain.

MATERIALS AND METHODS

Materials

Bovine organs were obtained from a local slaughterhouse 3 hours after animals were killed. Brain and liver samples were rinsed in cold 0.9% NaCl, blotted, and flash-frozen in liquid nitrogen. Adrenal glands were trimmed to remove fat and were cut in half longitudinally. The medulla and a minor portion of the adjacent zona reticularis were scraped and discarded. Then the cortex was scraped and flash-frozen in liquid nitrogen. To isolate ocular tissues, the neural retina was carefully separated from the RPE. RPE was next separated from BrM and choroid using a brush, followed by peeling of BrM and choroid from the underlying sclera. Samples from 10 eyes were combined and flash-frozen in liquid nitrogen. All tissues were stored at -80°C until analysis.

Human tissue use conformed to the Declaration of Helsinki and institutional reviews at the University of Texas Medical Branch and Case Western Reserve University. Brain and eye specimens were obtained from de-identified donors after informed consent of the respective families. Demographic information on the donors and pertinent medical history are summarized in Supplementary Table S1 (<http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6021/-DCSupplemental>). Brain samples were obtained during autopsy at the University of Texas Medical Branch 9 to 11 hours after death. These samples were immediately flash-frozen in liquid nitrogen and stored at -80°C . Eyes were acquired through the Cleveland Eye Bank and dissected within 10 to 14 hours after death. The anterior segment was removed, and fundus photographs were taken to confirm the absence of retinal pathology. These were evaluated by a retinal specialist. Thereafter, neural retina, RPE, and choroid were isolated, flash-frozen in liquid nitrogen, and stored at -80°C until analyzed.

Quantification of Sterols

Sterols were quantified by isotope dilution gas chromatography-mass spectrometry (GC-MS), as described.^{34–36} Sample processing is outlined in Figure 2. Thawed tissues were weighed and homogenized in 10 volumes (wt/vol) of 50 mM potassium phosphate buffer (KPi), pH 7.2, containing 300 mM sucrose, 0.5 mM dithiothreitol, 10 mM EDTA,

FIGURE 1. Schematic representation of cholesterol elimination pathways in different extrahepatic organs and cytochrome P450 enzymes involved in these pathways. **Bold lines and bold font** indicate the major contributors and products of cholesterol elimination.

FIGURE 2. Flow chart outlining sample processing for quantification of different oxysterols.

100 μ g/mL butyl hydroxytoluene and a cocktail of protease inhibitors. Tissue homogenization was followed by centrifugation at 1500g for 15 minutes to remove cell debris. Supernatants were then aliquotted, ~15 mg total protein per tube, and supplemented with a mixture of internal standards containing 500 nmol [25,26,26,26,27,27-²H₇]Chol (cholesterol-D₇), 2 nmol racemic [25,26,26,26,27,27,27-²H₇]24-OH (24-OH-D₇), 2 nmol [26,26,26,26,27,27-²H₅]27-OH (27-OH-D₅), 2.9 nmol [26,26,26,26,27,27,27-²H₇]22R-OH (22-OH-D₇), 5 nmol [17,21,21,21-²H₄]17 α -Preg (Preg-D₄) and 2 nmol 5-choleonic acid-3 β -ol (ChA). Lipids were extracted by the Folch method³⁷ using 19 volumes of chloroform/methanol (2:1, vol/vol) followed by centrifugation at 2000 rpm for 5 minutes. The lipid-containing chloroform layer was isolated and evaporated to dryness under nitrogen.

To quantify free sterols, two tubes with dried lipid extracts were used. Each tube was dissolved in 600 μ L methanol, and the methanol solution was applied to a Varian C18 column (1000 mg; Varian Inc., Lake Forest, CA) equilibrated with 5 mL CH₃OH/CH₃CN/H₂O (40:40:20, vol/vol/vol). The columns were washed with 20 mL CH₃OH/CH₃CN/H₂O (82:12:6, vol/vol/vol) resulting in the elution of the oxysterol fraction (24-OH, 27-OH, Preg, 22-OH, and 27-COOH). Cholesterol was eluted by washing the column with 20 mL methanol. All eluates were evaporated to dryness under nitrogen. One tube with the oxysterol fraction was used for quantification of 24-OH, 27-OH, Preg, and 22-OH after trimethylsilylation with 60 μ L bis-(trimethylsilyl) trifluoroacetamide/trimethylchlorosilane (TMS) at 60°C for 10 minutes. The other tube with the oxysterol fraction was used for quantification of 27-COOH. This fraction was first methylated with 3 mL freshly prepared ethereal diazomethane at room temperature for 30 minutes, then dried under nitrogen, and derivatized with TMS. The cholesterol-containing fraction was derivatized with TMS and analyzed by GC-MS.

Quantification of total sterols was similar to that of free sterols except that one tube containing dried lipid extract was used, and lipids were saponified before they were applied to the Varian C18 column. Saponification was carried out at room temperature for 2 hours in 2 mL of 1 N KOH/70% ethanol. After saponification, lipids were extracted three times with 4 mL hexane, and combined organic extracts were evaporated to dryness under nitrogen. The residue was dissolved in

600 μ L methanol and applied to the Varian C18 column (1000 mg) equilibrated with 5 mL CH₃OH/CH₃CN/H₂O (40:40:20, vol/vol/vol). Subsequent chromatography steps and sample processing were as described for quantification of free sterols.

GC-MS

The derivatized samples were analyzed using a mass spectrometer (5973N-MSD; Agilent) equipped with an gas chromatograph (6890; Agilent, Santa Clara, CA). A capillary column (60 m $\times$ 0.25 mm $\times$ 0.25 mm; ZB-5MS; Zebron, Charlotte, NC) was used for all analyses. The mass spectrometer was operated in the electron impact ionization mode. Gas chromatographic conditions were as follows: 2 μ L sample was injected in the splitless mode (inlet was kept at 270°C with the helium flow at 1.1 mL/min) at the initial 200°C (for quantification of cholesterol, 1 μ L with split 20 was used). The oven was first kept at 200°C for 1 minute, ramped at 20°C/min to 280°C, then ramped up to 310°C (3°C/min) and held for 14 minutes isothermally. The ion source filament was operated at 70 eV. Mass detector was set at 310°C. We used both SCAN (m/z range 100–800 Da) and SIM (selected ion monitoring) modes. The following ions (m/z) were monitored in the SIM mode: 368 (cholesterol), 375 (cholesterol-D₇), 145 (24-OH), 152 (24-OH-D₇), 417 (27-OH), 422 (27-OH-D₅), 388 (Preg), 392 (Preg-D₄), 173 (22-OH), 180 (22-OH-D₇), 331 (ChA), 373 and 412 (27-COOH). For quantification, calibration curves were generated using a fixed concentration of the internal standard and varying concentrations of the unlabeled sterols. Each extract was analyzed in triplicate with SD $\leq$ 10% for all the sterols, except those at low picomolar levels with SD $\leq$ 15% because these levels were close to the limits of detection (1 pmol/mg protein). In the case of the bovine samples, extracts from four different tissue preparations were used, with the inter-tissue SD $\leq$ 25%. In the case of the human brain, three extracts from the same tissue were analyzed, with the inter-extract SD $\leq$ 10%. Only a single extract was used to quantify oxysterols in the human retina and RPE.

RESULTS

Oxysterols in Bovine Retina and RPE

Initially, bovine samples were analyzed. Five cholesterol oxidation products were measured: 27-OH and 27-COOH formed by CYP27A1; 24-OH generated by CYP46A1; and 22-OH and Preg produced by CYP11A1 (Fig. 1). All oxysterols except 27-COOH were quantified in free (unconjugated) and total (conjugated) forms. 27-COOH was measured as a free sterol only because the ChA used as an internal standard for its quantification decomposes during saponification. Thus, the levels of 27-COOH could be underestimated. Results are summarized in Figure 3. Cholesterol and its metabolites were predominantly present in the free form, and their concentrations paralleled those of the corresponding total sterol pool. Cholesterol was by far the most abundant sterol in the retina (158 nmol/mg protein) and RPE

(136 nmol/mg protein), exceeding the amounts of the oxidation products by more than a thousand-fold (Figs. 3A, 3D). Enzymatically produced oxysterols were in the 0.007 to 0.036 nmol/mg protein range. In the retina the levels were 24-OH (0.036 nmol/mg protein), 27-COOH (0.025 nmol/mg protein), Preg (0.019 nmol/mg protein), and 22-OH (0.007 nmol/mg protein). The content in the RPE was approximately the same: Preg (0.029 nmol/mg protein), 24-OH (0.021 nmol/mg protein), 22-OH (0.010 nmol/mg protein), and 27-COOH (0.009 nmol/mg protein). Initially, we did not intend to measure 27-COOH because this oxysterol is formed only in the lung, macrophages, and vascular endothelium.^{38–40} However, we could not detect any 27-OH in the retina and decided to test whether 27-OH is further oxidized to 27-COOH. This indeed turned out to be the case, demonstrating that the retina and RPE are also capable of generating 27-COOH.

FIGURE 3. Effect of death-to-preservation interval on oxysterol concentrations in bovine retina, RPE, and choroid. Open bars: concentrations of the free sterols; dashed bars: concentrations of the total sterols. White: cholesterol; pink: 27-COOH; green: 22-OH; orange: Preg; blue: 24-OH.

Oxysterols in Bovine Choroid

During the isolation process, it is always possible to contaminate RPE with BrM, which underlies RPE, and with choroid, which lies beneath BrM. Therefore, we measured the oxysterol content in the choroid as well. The levels of 24-OH and 22-OH in the choroid were lower than those in the retina and RPE (0.018 and 0.001 nmol/mg protein, respectively), and 27-COOH and Preg were below the limits of detection (Fig. 3G). These data indicate that there is no gradient in oxysterol concentrations from the choroid to the RPE and retina. In fact, this gradient is reversed for all the oxysterols, suggesting that cholesterol oxidation products found in the retina and RPE are generated locally and do not represent contamination from the circulation.

Postmortem Changes in Oxysterol Concentrations

The bovine retina and RPE samples we analyzed initially were preserved 4 hours postmortem by snap-freezing in liquid nitrogen and storing at -80°C until use. Specimens from human cadavers, however, were preserved 9 to 14 hours postmortem. To investigate how the death-to-preservation interval affects sterol content, we used bovine eyes and left them at 4°C for an additional 8 and 20 hours before the retina and RPE were isolated and flash-frozen in liquid nitrogen. The death-to-preservation intervals for these eyes were 12 and 24 hours, respectively, and we designated them as 12- and 24-hour postmortem in Figure 3. By leaving bovine eyes at 4°C , we tried to model the retrieval of the human specimens: when patients die at the hospital (which was the case with our donors), their bodies are placed in a 4°C refrigerator within 4 hours after death. Comparison of the 8- and 12-hour bovine samples with the 4-hour

samples indicates that the changes in oxysterol levels were sterol-specific. The Preg content was decreased in both the retina and the RPE, and that of 22-OH was decreased in the retina and fluctuated in the RPE (Figs. 3B, 3C, 3E, 3F). The content of 27-COOH was increased in both the retina and the RPE, and these increases were similar in the two tissues: 2.8-fold in the 12-hour samples and 1.8- to 2.2-fold in the 24-hour samples. The level of 24-OH remained unchanged in the retina and was reduced up to 1.6-fold in the RPE. With respect to the choroid, we could not detect any oxysterols in the 12- and 24-hour samples (Figs. 3H, 3I). Thus, the data obtained indicate that the postmortem sterol content does change with time and that the death-to-preservation interval is an important factor that should be considered when one compares oxysterol concentrations in samples from different human donors.

Oxysterols in Bovine Brain, Liver, and Adrenal

The major sites of expression of cholesterol-metabolizing P450s are the brain (CYP46A1), liver (CYP27A1), and adrenal glands (CYP11A1). We determined oxysterol levels in these organs and compared them with those in the retina. In the brain, cholesterol metabolites were quantified in two regions: gray matter of the temporal lobe and gray matter of the cerebellum (Figs. 4A, 4B). 24-OH was the dominant oxysterol in the temporal lobe, exceeding the concentrations of the second most abundant cholesterol metabolite, Preg, by approximately 50-fold (0.8 nmol/mg protein vs 0.017 nmol/mg protein) and the levels of 27-OH and 22-OH by approximately 400-fold (0.8 nmol/mg protein vs 0.002 nmol/mg protein). In the cerebellum, the content of 24-OH (0.14 nmol/mg protein) was 5.7-fold lower than that in the temporal lobe but was at least eightfold higher than that of Preg (0.017 nmol/mg protein), 27-OH

FIGURE 4. Oxysterol concentrations in different bovine organs. White: cholesterol; pink: 27-COOH; green: 22-OH; orange: Preg; blue: 24-OH.

(0.006 nmol/mg protein), and 22-OH (0.002 nmol/mg protein). We also found 27-COOH in the cerebellum (0.024 nmol/mg protein) but could not detect this oxysterol in the temporal lobe. The high abundance of 24-OH in the temporal lobe and cerebellum is in good agreement with previous studies by others,^{2,16,41} demonstrating that CYP46A1 is the principal cholesterol hydroxylase in the brain. In the liver, the site of abundant CYP27A1 expression,⁴² 27-OH and 24-OH were below the limits of detection, and the concentrations of Preg, 22-OH, and 27-COOH were much lower than those in the ocular tissues (0.003, 0.001, and 0.001 nmol/mg protein, respectively; Fig. 4C). This result is consistent with the well-established fact that 7 α -, not C27-hydroxylation, is the first step in cholesterol degradation in the liver, and the major role of CYP27A1 in this organ is to hydroxylate bile acid intermediates rather than cholesterol.^{42,43} Finally, in the adrenals, where cholesterol is primarily converted to steroid hormones,⁴⁴ 22-OH and Preg were the major oxysterol metabolites (0.028 nmol/mg protein and 0.035 nmol/mg protein, respectively), exceeding the levels of 27-OH (0.001 nmol/mg protein) by more than 20-fold (Fig. 4D). Retina is a neural tissue, and it would not be surprising if the oxysterol profile in the retina was similar to that in the brain. However, the profile turned out to be different, indicating multiple mechanisms of cholesterol elimination from this organ.

Oxysterols in Human Retina and RPE

None of the enzymatic products of CYPs 27A1, 46A1, and 11A1 have yet been quantified in the human retina and RPE. In addition, it is unknown whether there is interindividual variability in retinal oxysterol concentrations in humans and how these concentrations are comparable to those in the bovine samples. We isolated retina and RPE from three human donors with no retinal pathology and measured the oxysterol content in these samples (Fig. 5). The most abundant oxysterol in the human retina was 27-COOH. Sterol concentrations were 0.037, 0.075, and 0.125 nmol/mg protein in donors 11, 8, and 9, respectively. Preg and 24-OH were detected as well but at only low picomolar concentrations (0.001–0.004 nmol/mg protein). 27-OH and 22-OH were below the limits of detection. In the RPE, the oxysterol content was lower than that in the retina, and we could measure only 27-COOH (0.002, 0.003, and 0.01 nmol/mg protein in donors 8, 11, and 9, respectively). Comparison of the human and bovine retinal oxysterol profiles (12 hours postmortem) indicated that qualitatively they were similar. In each species, 27-COOH was the most abundant cholesterol metabolite, whereas Preg and 24-OH were present

at lower concentrations. Thus, bovine eyes seem to be an acceptable model for studying cholesterol metabolism in the human retina and RPE.

Oxysterols in Human Brain

Analogous to the studies of the bovine samples, we tested two regions of the human brain: gray matter of the temporal lobe (three donors) and gray matter of the cerebellum (two donors; Fig. 6). In both regions, 24-OH was the most abundant oxysterol and was present at similar levels (1.4–1.9 nmol/mg protein). Preg content was also similar in the two regions but was much lower than that of 24-OH (0.009–0.016 nmol/mg protein). The range of 27-OH concentrations was slightly higher in the cerebellum than in the temporal lobe (0.036–0.045 nmol/mg protein vs 0.015–0.027 nmol/mg protein). Oxysterol profiles in the human brain resembled those in the bovine samples, except that the concentrations of 24-OH and 27-OH were higher in humans than in bulls.

DISCUSSION

Quantification of the oxysterols in the human retina revealed that 27-COOH is the most abundant enzymatic product of cholesterol oxidation in this organ, significantly exceeding the levels of Preg and 24-OH (0.037–0.125 nmol/mg protein vs 0.002–0.004 nmol/mg protein). The range of concentrations of 27-COOH varied among the three donors threefold, possibly reflecting differences in their past medical history, medications, or both. The lowest content was in donor 11 (0.037 nmol/mg protein), who died of complications due to end-stage chronic obstructive pulmonary disease (Supplementary Table S1, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6021/-/DCSupplemental>). Donor 8 had intermediate 27-COOH content (0.075 nmol/mg protein), chronic obstructive pulmonary disease, and pulmonary fibrosis. Only donor 9, who had the highest level of 27-COOH in the retina (0.125 nmol/mg protein), did not have a history of pulmonary problems. This donor had cardiovascular disease and died of cardiac arrest.

Knowledge of the clinical phenotype of those completely lacking sterol 27-hydroxylase activity may provide some insight into the ocular significance of cholesterol 27-oxygenation. CYP27A1 deficiency leads to a slowly progressive disease called cerebrotendinous xanthomatosis (CTX), which is characterized by a variety of symptoms (tendon xanthomas, neurologic dysfunction, and premature atherosclerosis).⁴⁵ Ocular manifestations are also diverse and include juvenile cataracts,

FIGURE 5. Oxysterol concentrations in the retina and RPE of donors 8, 9, and 11. White: cholesterol; pink: 27-COOH; green: 22-OH; orange: Preg; blue: 24-OH.

FIGURE 6. Oxysterol concentrations in two regions of the brains of donors 3 and 4. *White*: cholesterol; *pink*: 27-COOH; *green*: 22-OH; *orange*: Preg; *blue*: 24-OH; *violet*: 27-OH.

lipoid arcus, optic disc pallor, and premature retinal senescence with drusen and RPE changes.^{46–48} Cataracts are usually the earliest and most frequently observed clinical sign of CTX, preceding the systemic manifestations of the disease.^{47,48} Fundus examinations are difficult in patients with cataracts, which limit visualization of the retina. Therefore, for a number of years, cataracts were thought to be the only ocular symptom of CTX. However, it has become clear that ocular involvement in CTX is more widespread than previously described,⁴⁸ suggesting that CYP27A1 is as important for normal retinal function as it is for general health.

Many mammalian cells can metabolize cholesterol to 27-OH, but only the lung, macrophages, and vascular endothelium, where CYP27A1 is highly expressed, are known to further metabolize 27-OH to 27-COOH by two sequential oxidation reactions.^{38–40} 27-OH and 27-COOH are more polar than cholesterol and are transported more quickly from the cell by spontaneous diffusion across plasma membranes.^{39,49} In the circulation, 27-OH becomes associated with HDL, whereas 27-COOH is bound by albumin.^{39,50} Given that 27-OH is an intermediate in the conversion to 27-COOH, usually both 27-OH and 27-COOH are detected in extraocular cells producing 27-COOH; the ratio between the two metabolites depends on the level of CYP27A1, cholesterol availability, and presence of acceptor in the medium.⁵¹ In the retina we could not detect any 27-OH but found high amounts of 27-COOH. This finding raises a number of questions. Why is 27-COOH the preferential cholesterol oxidation product in the retina? What is the biochemical basis for retinal production of 27-COOH? Does 27-COOH play a specific role in the retina associated with unique retinal functions?

The retina is a multilayered organ composed of at least 12 different cell types, of which only Müller cells and RPE come in contact with the circulation. Müller cells surround the inner retinal vessels, and the RPE is fed by the choriocapillaris. Immunohistochemical localization of CYP27A1 performed on monkey retinas³¹ showed strong protein expression in photoreceptor inner segments and faint expression throughout the retina, including Müller cells, ganglion cells, and nerve fibers (Fig. 7). Faint CYP27A1 expression was also observed in the RPE and choriocapillaris.³¹ If CYP27A1 is highly abundant in photoreceptor inner segments and this site is the major source of retinal 27-COOH, then the 27-oxygenated cholesterol metabolite has to leave the photoreceptor inner segments and be transported within the retina to the site(s) where it can enter systemic circulation. Studies with cultured human macrophages showed that the nature of the acceptor outside the cell affects whether intracellular cholesterol is metabolized to 27-OH or 27-COOH; the presence of HDL favors the production of 27-OH, whereas with albumin 27-COOH is the major secreted product.³⁹ Apolipoprotein A1, a constituent of HDL, was recently shown by immunohistochemistry to be expressed in the retina in multiple cell types, including photoreceptor inner segments.¹⁹ Therefore, HDL is likely to be present in the retina, yet the predominant metabolite is 27-COOH. This suggests that factors other than acceptor presence in the interphotoreceptor matrix affect the metabolic fate of cholesterol in photoreceptor inner segments. One of these factors could be limited availability of cholesterol to CYP27A1, shown previously to facilitate the production of 27-COOH.³⁹ CYP27A1 resides in the inner mitochondrial membrane. Consequently, cholesterol has to be transported from the outer to the inner

FIGURE 7. Expression of cholesterol-metabolizing P450 in different cell types, as assessed by immunohistochemical studies.^{29–33} Illustration of the seven main classes of cell types and cell layers found in the vertebrate retina is reprinted by permission from Macmillan Publishers Ltd. Dyer MA, Cepko CL. Regulating proliferation during retinal development. *Nat Rev Neurosci.* 2001;2:333–342. Copyright 2001. RPE, retinal pigment epithelium; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; NFL, neural fiber layer.

membrane to be metabolized by CYP27A1. Intramitochondrial cholesterol transport has been studied extensively in steroidogenic tissues,^{52,53} but the nature of this process in photoreceptor inner segments is unknown. If it is similar to the process in steroidogenic tissues, the cholesterol amount should also be low in the inner mitochondrial membranes of photoreceptors. If it is, limited substrate availability coupled with high CYP27A1 expression creates a low substrate-to-enzyme ratio that also favors the production of 27-COOH.⁵¹ Another contributing factor could be the unique composition of the retinal membranes rich in n-3 polyunsaturated fatty acids.⁵⁴ CYP27A1 activity depends on the phospholipid content of the membranes in which this enzyme is embedded.⁵⁵ We established that the catalytic efficiency of CYP27A1 in vitro is much higher in the presence of retinal mitochondrial phospholipids than in the presence of liver mitochondrial phospholipids (I. Pikuleva, unpublished, 2010). These results suggest that C27-oxygenation of cholesterol is more efficient in the retina than in the liver and are consistent with the data of the present study. Thus, the production of 27-COOH in the retina could be explained from the biochemical standpoint, whereas the physiological significance of the conversion to 27-COOH requires further investigation. It should also be noted that enzymes other than CYP27A1 may contribute to further oxidation of 27-OH. Cholesterol derivative 5 β -cholestane-3 α ,7 α ,12 α , 27-tetrol was shown to be efficiently oxidized into the corresponding C-27 acid by horse liver alcohol dehydrogenase combined with aldehyde dehydrogenase.⁵⁶ In addition, short-chain dehydrogenases/reductases are also known to metabolize a wide range of substrates in vitro, including steroids.^{57–61} Some of these enzymes are expressed in the retina^{62,63} and, consequently, could be involved in the oxidation of 27-OH to 27-COOH.

Besides 27-COOH, Preg and 24-OH were also detected in the human retina, but at low picomolar concentrations. Retinal expression of CYP11A1 was investigated in rats and golden hamsters.^{29,30} In both species, only two layers were immunostained, the inner nuclear layer and the ganglion cell layer (Fig. 7), though with different intensities. Since the ganglion cell layer also expresses CYP27A1, the two mitochondrial P450s likely compete in the ganglion cells for the same substrate (cholesterol) and for the same redox partner (ferredoxin) to carry out the hydroxylation reaction. This competition may explain the low Preg content in the human retina. The low Preg content could also be the result of quick Preg use for the biosynthesis of steroid hormones. CYP46A1 is also present in the neurons of the ganglion cell layer, as indicated by immunohistochemical studies of rat and mouse retinas.^{32,33} In addition, P450 immunoreactivity was observed in the inner nuclear layer in rat retinas and in some but not all cells at the edge of this layer in mice.³³ In mice, CYP46A1 immunoreactivity was also detected in the RPE.³³ CYP46A1 is a microsomal enzyme that uses a different redox partner from CYPs 11A1 and 27A1, NADPH-cytochrome P450 oxidoreductase. CYP46A1 should not compete with mitochondrial P450s for cholesterol in cells in which these proteins coexpress; consequently, multiple oxysterols could be generated. Similar levels of Preg and 24-OH in the retina suggest approximately equal contribution of CYP46A1 and CYP11A1 to cholesterol metabolism in retinal neurons. This is different from brain neurons, where CYP46A1 is the main enzyme responsible for cholesterol elimination and the levels of 24-OH exceed the levels of other sterols by approximately 100-fold.

In summary, this is the first comprehensive investigation of oxysterol levels in the retina and RPE and comparison of the retinal oxysterol profile with that in the brain. Metabolites of all three cholesterol-metabolizing P450s—CYPs 27A1, 46A1, and 11A1—were found present in the retina and RPE, thus providing evidence that enzymatic cholesterol elimination indeed takes place in these organs and that lipoprotein-mediated cholesterol transport is not the only mechanism whereby retinal cells maintain cholesterol homeostasis. Cholesterol removal in the retina likely varies, depending on cell type; multiple oxysterols (Preg and 24-OH) are produced in the retinal neurons, and one predominant oxysterol, 27-COOH, is probably generated in the photoreceptor inner segments and RPE. Overall, oxidation to 27-COOH seems to be the predominant mechanism for cholesterol elimination from the retina and RPE, whereas in the brain cholesterol is eliminated primarily through 24-hydroxylation.

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References

- Dietschy JM, Turley SD, Spady DK. Role of liver in the maintenance of cholesterol and low density lipoprotein homeostasis in different animal species, including humans. *J Lipid Res.* 1993;34:1637–1659.
- Russell DW, Halford RW, Ramirez DM, Shah R, Kotti T. Cholesterol 24-hydroxylase: an enzyme of cholesterol turnover in the brain. *Annu Rev Biochem.* 2009;78:1017–1040.
- Bjorkhem I, Meaney S. Brain cholesterol: long secret life behind a barrier. *Arterioscler Thromb Vasc Biol.* 2004;24:806–815.
- Dietschy JM, Turley SD. Thematic review series: brain lipids: cholesterol metabolism in the central nervous system during early development and in the mature animal. *J Lipid Res.* 2004;45:1375–1397.
- Curcio CA, Johnson M, Huang JD, Rudolf M. Aging, age-related macular degeneration, and the response-to-retention of apolipo-

- protein B-containing lipoproteins. *Prog Retin Eye Res.* 2009;28:393–422.
6. Curcio CA, Johnson M, Huang JD, Rudolf M. Apolipoprotein B-containing lipoproteins in retinal aging and age-related macular degeneration. *J Lipid Res.* 2010;51:451–467.
 7. Vance JE, Hayashi H, Karten B. Cholesterol homeostasis in neurons and glial cells. *Semin Cell Dev Biol.* 2005;16:193–212.
 8. Bjorkhem I, Heverin M, Leoni V, Meaney S, Diczfalussy U. Oxy-sterols and Alzheimer's disease. *Acta Neurol Scand Suppl.* 2006;185:43–49.
 9. van Leeuwen R, Klaver CC, Vingerling JR, et al. Cholesterol and age-related macular degeneration: is there a link? *Am J Ophthalmol.* 2004;137:750–752.
 10. Dietschy JM, Turley SD. Cholesterol metabolism in the brain. *Curr Opin Lipidol.* 2001;12:105–112.
 11. Cunha-Vaz JG. The blood-ocular barriers: past, present, and future. *Doc Ophthalmol.* 1997;93:149–157.
 12. Fliesler SJ, Florman R, Rapp LM, Pittler SJ, Keller RK. In vivo biosynthesis of cholesterol in the rat retina. *FEBS Lett.* 1993;335:234–238.
 13. Fliesler SJ, Keller RK. Isoprenoid metabolism in the vertebrate retina. *Int J Biochem Cell Biol.* 1997;29:877–894.
 14. Tserentsoodol N, Szein J, Campos M, et al. Uptake of cholesterol by the retina occurs primarily via a low density lipoprotein receptor-mediated process. *Mol Vis.* 2006;12:1306–1318.
 15. Bjorkhem I, Lutjohann D, Diczfalussy U, Stahl L, Ahlborg G, Wahren J. Cholesterol homeostasis in human brain: turnover of 24S-hydroxycholesterol and evidence for a cerebral origin of most of this oxysterol in the circulation. *J Lipid Res.* 1998;39:1594–1600.
 16. Lutjohann D, Breuer O, Ahlborg G, et al. Cholesterol homeostasis in human brain: evidence for an age-dependent flux of 24S-hydroxycholesterol from the brain into the circulation. *Proc Natl Acad Sci U S A.* 1996;93:9799–9804.
 17. Lund EG, Guileyardo JM, Russell DW. cDNA cloning of cholesterol 24-hydroxylase, a mediator of cholesterol homeostasis in the brain. *Proc Natl Acad Sci U S A.* 1999;96:7238–7243.
 18. Hayes KC, Lindsey S, Stephan ZF, Brecker D. Retinal pigment epithelium possesses both LDL and scavenger receptor activity. *Invest Ophthalmol Vis Sci.* 1989;30:225–232.
 19. Tserentsoodol N, Gordiyenko NV, Pascual I, Lee JW, Fliesler SJ, Rodriguez IR. Intraretinal lipid transport is dependent on high density lipoprotein-like particles and class B scavenger receptors. *Mol Vis.* 2006;12:1319–1333.
 20. Duncan KG, Bailey KR, Kane JP, Schwartz DM. Human retinal pigment epithelial cells express scavenger receptors BI and BII. *Biochem Biophys Res Commun.* 2002;292:1017–1022.
 21. Amaratunga A, Abraham CR, Edwards RB, Sandell JH, Schreiber BM, Fine RE. Apolipoprotein E is synthesized in the retina by Muller glial cells, secreted into the vitreous, and rapidly transported into the optic nerve by retinal ganglion cells. *J Biol Chem.* 1996;271:5628–5632.
 22. Li CM, Chung BH, Presley JB, et al. Lipoprotein-like particles and cholesteryl esters in human Bruch's membrane: initial characterization. *Invest Ophthalmol Vis Sci.* 2005;46:2576–2586.
 23. Neale BM, Fagerness J, Reynolds R, et al. Genome-wide association study of advanced age-related macular degeneration identifies a role of the hepatic lipase gene (LIPC). *Proc Natl Acad Sci U S A.* 2010;107:7395–7400.
 24. Chen W, Stambolian D, Edwards AO, et al. Genetic variants near TIMP3 and high-density lipoprotein-associated loci influence susceptibility to age-related macular degeneration. *Proc Natl Acad Sci U S A.* 2010;107:7401–7406.
 25. Duane WC, Javitt NB. 27-Hydroxycholesterol: production rates in normal human subjects. *J Lipid Res.* 1999;40:1194–1199.
 26. Lund E, Andersson O, Zhang J, et al. Importance of a novel oxidative mechanism for elimination of intracellular cholesterol in humans. *Arterioscler Thromb Vasc Biol.* 1996;16:208–212.
 27. Kotti TJ, Ramirez DM, Pfeiffer BE, Huber KM, Russell DW. Brain cholesterol turnover required for geranylgeraniol production and learning in mice. *Proc Natl Acad Sci U S A.* 2006;103:3869–3874.
 28. Stoffel-Wagner B. Neurosteroid metabolism in the human brain. *Eur J Endocrinol.* 2001;145:669–679.
 29. Guarneri P, Guarneri R, Cascio C, Pavasant P, Piccoli F, Papadopoulos V. Neurosteroidogenesis in rat retinas. *J Neurochem.* 1994;63:86–96.
 30. Jalliffa CO, Howard S, Hoijman E, et al. Effect of neurosteroids on the retinal gabaergic system and electroretinographic activity in the golden hamster. *J Neurochem.* 2005;94:1666–1675.
 31. Lee JW, Fuda H, Javitt NB, Strott CA, Rodriguez IR. Expression and localization of sterol 27-hydroxylase (CYP27A1) in monkey retina. *Exp Eye Res.* 2006;83:465–469.
 32. Bretillon L, Diczfalussy U, Bjorkhem I, et al. Cholesterol-24S-hydroxylase (CYP46A1) is specifically expressed in neurons of the neural retina. *Curr Eye Res.* 2007;32:361–366.
 33. Ramirez DM, Andersson S, Russell DW. Neuronal expression and subcellular localization of cholesterol 24-hydroxylase in the mouse brain. *J Comp Neurol.* 2008;507:1676–1693.
 34. Bjorkhem I, Andersson O, Diczfalussy U, et al. Atherosclerosis and sterol 27-hydroxylase: evidence for a role of this enzyme in elimination of cholesterol from human macrophages. *Proc Natl Acad Sci U S A.* 1994;91:8592–8596.
 35. Dzeletovic S, Breuer O, Lund E, Diczfalussy U. Determination of cholesterol oxidation products in human plasma by isotope dilution-mass spectrometry. *Anal Biochem.* 1995;225:73–80.
 36. Axelsson M, Mork B, Sjoval J. Occurrence of 3 beta-hydroxy-5-cholestenoic acid, 3 beta,7 alpha-dihydroxy-5-cholestenoic acid, and 7 alpha-hydroxy-3-oxo-4-cholestenoic acid as normal constituents in human blood. *J Lipid Res.* 1988;29:629–641.
 37. Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem.* 1957;226:497–509.
 38. Reiss AB, Martin KO, Rojer DE, et al. Sterol 27-hydroxylase: expression in human arterial endothelium. *J Lipid Res.* 1997;38:1254–1260.
 39. Babiker A, Andersson O, Lund E, et al. Elimination of cholesterol in macrophages and endothelial cells by the sterol 27-hydroxylase mechanism: comparison with high density lipoprotein-mediated reverse cholesterol transport. *J Biol Chem.* 1997;272:26253–26261.
 40. Babiker A, Andersson O, Lindblom D, et al. Elimination of cholesterol as cholestenoic acid in human lung by sterol 27-hydroxylase: evidence that most of this steroid in the circulation is of pulmonary origin. *J Lipid Res.* 1999;40:1417–1425.
 41. Heverin M, Bogdanovic N, Lutjohann D, et al. Changes in the levels of cerebral and extracerebral sterols in the brain of patients with Alzheimer's disease. *J Lipid Res.* 2004;45:186–193.
 42. Wikvall K. Hydroxylations in biosynthesis of bile acids: isolation of a cytochrome P-450 from rabbit liver mitochondria catalyzing 26-hydroxylation of C27-steroids. *J Biol Chem.* 1984;259:3800–3804.
 43. Myant NB, Mitropoulos KA. Cholesterol 7 α -hydroxylase. *J Lipid Res.* 1977;18:135–153.
 44. Stone D, Hechter O. Studies on ACTH action in perfused bovine adrenals: site of action of ACTH in corticosteroids. *Arch Biochem Biophys.* 1954;51:457–469.
 45. Bjorkhem I, Boberg KM, Leitersdorf E. Inborn errors in bile acid biosynthesis and storage of sterols other than cholesterol. In: Scriver AL, Beaudet AL, Sly WS, Valle D, Childs B, eds. *The Metabolic and Molecular Bases of Inherited Disease*. New York: McGraw-Hill;1995:2073–2099.
 46. Cruysberg JR, Wevers RA, van Engelen BG, Pinckers A, van Spreken A, Tolboom JJ. Ocular and systemic manifestations of cerebrotendinous xanthomatosis. *Am J Ophthalmol.* 1995;120:597–604.
 47. Morgan SJ, McKenna P, Bosanquet RC. Case of cerebrotendinous xanthomatosis, I: unusual ophthalmic features. *Br J Ophthalmol.* 1989;73:1011–1014.
 48. Dotti MT, Rufa A, Federico A. Cerebrotendinous xanthomatosis: heterogeneity of clinical phenotype with evidence of previously undescribed ophthalmological findings. *J Inher Metab Dis.* 2001;24:696–706.
 49. Meaney S, Bodin K, Diczfalussy U, Bjorkhem I. On the rate of translocation in vitro and kinetics in vivo of the major oxysterols in human circulation: critical importance of the position of the oxygen function. *J Lipid Res.* 2002;43:2130–2135.

50. Babiker A, Diczfalussy U. Transport of side-chain oxidized oxysterols in the human circulation. *Biochim Biophys Acta*. 1998;1392:333-339.
51. Pikuleva IA, Babiker A, Waterman MR, Bjorkhem I. Activities of recombinant human cytochrome P450c27 (CYP27) which produce intermediates of alternative bile acid biosynthetic pathways. *J Biol Chem*. 1998;273:18153-18160.
52. Manna PR, Dyson MT, Stocco DM. Regulation of the steroidogenic acute regulatory protein gene expression: present and future perspectives. *Mol Hum Reprod*. 2009;15:321-333.
53. Rone MB, Fan J, Papadopoulos V. Cholesterol transport in steroid biosynthesis: role of protein-protein interactions and implications in disease states. *Biochim Biophys Acta*. 2009;1791:646-658.
54. Gulcan HG, Alvarez RA, Maude MB, Anderson RE. Lipids of human retina, retinal pigment epithelium, and Bruch's membrane/choroid: comparison of macular and peripheral regions. *Invest Ophthalmol Vis Sci*. 1993;34:3187-3193.
55. Murtazina DA, Andersson U, Hahn IS, Bjorkhem I, Ansari GA, Pikuleva IA. Phospholipids modify substrate binding and enzyme activity of human cytochrome P450 27A1. *J Lipid Res*. 2004;45:2345-2353.
56. Bjorkhem I, Jornvall H, Zeppezauer E. Oxidation of omega-hydroxylated fatty acids and steroids by alcohol dehydrogenase. *Biochem Biophys Res Commun*. 1973;52:413-420.
57. Duester G. Function of alcohol dehydrogenase and aldehyde dehydrogenase gene families in retinoid signaling. *Adv Exp Med Biol*. 1999;463:311-319.
58. Liden M, Eriksson U. Understanding retinol metabolism: structure and function of retinol dehydrogenases. *J Biol Chem*. 2006;281:13001-13004.
59. Chai X, Zhai Y, Napoli JL. cDNA cloning and characterization of a cis-retinol/3alpha-hydroxysterol short-chain dehydrogenase. *J Biol Chem*. 1997;272:33125-33131.
60. Wang J, Chai X, Eriksson U, Napoli JL. Activity of human 11-cis-retinol dehydrogenase (Rdh5) with steroids and retinoids and expression of its mRNA in extra-ocular human tissue. *Biochem J*. 1999;338(pt 1):23-27.
61. Keller B, Adamski J. RDH12, a retinol dehydrogenase causing Leber's congenital amaurosis, is also involved in steroid metabolism. *J Steroid Biochem Mol Biol*. 2007;104:190-194.
62. Martras S, Alvarez R, Martinez SE, et al. The specificity of alcohol dehydrogenase with cis-retinoids: activity with 11-cis-retinol and localization in retina. *Eur J Biochem*. 2004;271:1660-1670.
63. Haeseleer F, Jang GF, Imanishi Y, et al. Dual-substrate specificity short chain retinol dehydrogenases from the vertebrate retina. *J Biol Chem*. 2002;277:45537-45546.