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Features of the retinal environment which affect the activities and product profile of cholesterol-metabolizing cytochromes P450 CYP27A1 and CYP11A1

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Abstract

The retina is the sensory organ in the back of the eye which absorbs and converts light to electrochemical impulses transferred to the brain. Herein, we studied how retinal environment affects enzyme-mediated cholesterol removal. We focused on two mitochondrial cytochrome P450 enzymes, CYPs 27A1 and 11A1, which catalyze the first steps in metabolism of cholesterol in the retina and other tissues. Phospholipids (PL) from mitochondria of bovine neural retina, retinal pigment epithelium, liver and adrenal cortex were isolated and compared for the effect on kinetic properties of purified recombinant CYPs in the reconstituted system *in vitro*. The four studied tissues were also evaluated for the mitochondrial PL and cholesterol content and levels of CYPs 27A1, 11A1 and their redox partners. The data obtained were used for modeling the retinal environment in the *in vitro* enzyme assays in which we detected the P450 metabolites, 22R-hydroxycholesterol and 5-cholestenoic acid, unexpectedly found by us in the retina in our previous studies. The effect of the by-product of the visual cycle pyridinium bis-retinoid A2E on kinetics of CYP27A1-mediated cholesterol metabolism was also investigated. The results provide insight into the retina's regulation of the enzyme-mediated cholesterol removal.

Keywords

CYP11A1; CYP27A1; CYP46A1; retina; retinal pigment epithelium; cholesterol; 5-cholestenoic acid; 22R-hydroxycholesterol; phospholipids; ferredoxin; ferredoxin reductase; A2E

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Introduction

In all extrahepatic organs, the first step in cholesterol biotransformations, the hydroxylation reaction, is always carried out by cytochrome P450 enzymes (CYP or P450) 27A1, 46A1, and 11A1 that generate more polar forms of cholesterol, oxysterols, for elimination or regulation (reviewed in [1]). CYP27A1 is expressed in the inner mitochondrial membranes of many tissues where it converts cholesterol to 27-hydroxycholesterol, the first step in a pathway of cholesterol removal that complements lipoprotein-mediated reverse cholesterol transport [2–4]. In the lung, macrophages and vascular endothelium, however, CYP27A1 performs additional oxidations of 27-hydroxycholesterol yielding 5-cholestenoic acid [5–7], whose production is favored by the high mitochondrial CYP27A1 to cholesterol ratio and the presence of the extracellular acceptor, albumin, outside the cell [6]. Lack of CYP27A1 underlies a slowly progressive, if untreated, disease cerebrotendinous xanthomatosis characterized by multiple symptoms including tendon xanthomas, neurological and retinal abnormalities, juvenile bilateral cataracts, and premature atherosclerosis [8–11]. Microsomal CYP46A1 is a brain-specific enzyme that catalyzes cholesterol 24-hydroxylation, the major mechanism whereby cholesterol is disposed from the brain [12–14]. Medical significance of CYP46A1 is not yet established but could involve effects on memory and cognition as indicated by animal studies [15, 16]. CYP11A1 is abundant in the inner mitochondrial membranes of steroidogenic tissues where it initiates the production of all steroid hormones by sequential metabolism of cholesterol to 22R-hydroxycholesterol, 20, 22R-dihydroxycholesterol and pregnenolone [17, 18]. Partial or complete deficiency of CYP11A1 leads to congenital lipid adrenal hyperplasia [19–21], a severe endocrine disorder, lethal without a lifetime hormone replacement therapy. In the adrenal cortex, mitochondria contain cholesterol at subsaturating for CYP11A1 levels [22]; hence cholesterol availability to CYP11A1 (i.e. transfer from the outer to the inner mitochondrial membrane) is the rate-limiting step in the overall steroidogenesis in this tissue [23]. In contrast, in placental mitochondria, cholesterol is at near-saturating concentrations for CYP11A1 [24], yet another factor, electron supply by the P450 redox partners, limits the rate at which cholesterol is metabolized to pregnenolone in the placenta [25, 26]. Thus, activity of CYP11A1 as well as CYP27A1 is affected by tissue-specific environment which, along with other mechanisms, controls the rate of enzyme-mediated cholesterol metabolism.

The retina is a light-sensitive tissue lining the inner surface of the eye. The retina has a complex multilayered structure and is composed of the neural retina (NR), formed by 9 layers of different cell types, and a single layer of the underlying epithelial cells called retinal pigment epithelium (RPE). RPE is considered a part of the retina because of its close association with the NR. The retina was found to express all extrahepatic cholesterol-metabolizing P450s as demonstrated initially by immunohistochemical stainings by others [27–31] and then by us by mass spectrometry quantifications [32, 33]. We measured CYPs 27A1 and 46A1 in human NR and CYP11A1 in bovine NR and found that CYP27A1 is the most abundant cholesterol-metabolizing P450 in the NR expressed at ~8-fold higher levels than CYP46A1 and ~70-fold higher levels than CYP11A1 [32, 33]. Parallel evaluation of the oxysterol profiles demonstrated that in human and bovine NR and RPE the product of CYP27A1 activity is a rare metabolite 5-cholestenoic acid rather than a more common metabolite 27-hydroxycholesterol [34]. Enzymatic products of CYP46A1 and CYP11A1 (24-hydroxycholesterol and pregnenolone, respectively) were detected in the NR and RPE as well [34], at comparable levels despite CYP11A1 is a low abundance retinal P450. Interestingly, NR and RPE contained the CYP11A1 reaction intermediate and biologically active oxysterol 22R-hydroxycholesterol, usually not released from the enzyme active site during the formation of pregnenolone [35, 36]. The unexpected conversion of retinal cholesterol to 5-cholestenoic acid and detection of 22R-hydroxycholesterol are important findings because they may pertain to the mechanisms whereby the retina regulates

cholesterol elimination. Therefore, in the present study, we investigated how organ-specific lipid and cholesterol content and the P450-redox partner ratio affect the activities and product profile of cholesterol-metabolizing mitochondrial CYPs 27A1 and 11A1. Our results establish the biochemical basis for the production of 5-cholestenoic acid and 22R-hydroxycholesterol in the retina and identify key retinal features that affect enzyme-mediated cholesterol removal from the NR and RPE.

Materials and Methods

Materials

Cholesterol and [^3H]cholesterol were from Steraloids, Inc (Newport, RI) and American Radiolabeled Chemicals, Inc (St. Louis, MO), respectively. All other chemicals were from Sigma-Aldrich unless otherwise specified. Bovine organs were obtained from a local slaughterhouse 3 hrs after animals were sacrificed. The liver was rinsed in cold 0.9% NaCl and blotted. Adrenal glands were trimmed to remove fat and cut in half longitudinally. The medulla and a minor portion of the adjacent zona reticularis were scraped and discarded followed by scraping of the adrenal cortex. The dissection of the eyes and isolation of NR and RPE were as described [37]. Preparation of tissue homogenates, isolation of mitochondria and mitochondrial PLs and preparation of liposomes for enzyme assays were also as in our previous studies [37]. Briefly, mitochondria were isolated by differential centrifugation; mitochondrial PLs were obtained from mitochondrial lipid extracts after chromatography on Sep-Pak Silica cartridges (Waters Corporation, Milford, MA); liposomes from mitochondrial PLs were prepared by sonication. PLs were free of cholesterol and non-phosphorous lipids as assessed by thin layer chromatography (Fig. S1). The PL and cholesterol concentrations were measured using ammonium ferrothiocyanate [38] and commercial kit (Stanbio Laboratory, Boerne, TX), respectively. Natural isotopic abundance and ^{15}N -labeled recombinant human CYP27A1, bovine CYP11A1, human CYP46A1 and bovine ferredoxin (Fdx) and bovine ferredoxin reductase (Fdr)2 were expressed and purified as described [32, 33, 39–42]. Antibodies for Western blot analyses were produced in the laboratory. N-retinylidene-N-retinylethanolamine (A2E) was synthesized from all-*trans*-retinal and ethanolamine and purified by silica gel column chromatography and HPLC [43]. The absolute spectrum of A2E and the ratio of the peaks at 336 nm and 439 nm were as those published in the literature [43]. Stock solution of A2E in dimethylsulfoxide (1 mg/ml) was stored under argon at -20°C in a light-blocking container.

Enzyme Assays

Incubations were carried out as described [44, 45], always in 1 ml of 40 mM potassium phosphate buffer (KP_i), pH 7.2, containing 1 mM EDTA. Liposomes were added first followed by addition of cold and [^3H]cholesterol and proteins and reconstitution procedure after which NADPH was added to initiate the enzymatic reaction. The enzymatic reaction was terminated by addition of CH_2Cl_2 (two times by 2 ml) which also served to extract reaction products and unused cholesterol. Organic extract was evaporated, dissolved in CH_3CN , and analyzed by HPLC connected to the flow-through detector of γ -radioactivity. The concentrations of PLs, cholesterol, and proteins as well as the reconstitution procedure and reaction time varied depending on the assay and P450 and are indicated in the sections below.

Kinetic studies with different PLs—Reconstitution into liposomes was optimized for each CYP11A1 and CYP27A1. CYP11A1 was incubated with PL vesicles for 20 min at

²Ferredoxin and ferredoxin reductase are frequently called Adx (adrenodoxin) and AdR (adrenodoxin reductase) because they were first discovered in adrenal glands.

room temperature and then for 2 min at 30°C followed by initiation of the enzymatic reaction which was carried out at 30°C. Reconstitution of CYP27A1 required 10 min at 37°C with subsequent enzymatic reactions performed at 37°C. For each P450-PL pair, preliminary experiments were conducted to find the optimal PL concentration at which the maximal enzyme activity was observed. In the case of CYP11A1 and PLs from NR and RPE, this concentration was 20 g/ml; adrenal PLs were used at 50 g/ml. In the incubations with CYP27A1, the concentrations of PLs from NR, RPE and liver were the same and equal to 10 g/ml. The CYP11A1 assays contained 0.1 M CYP11A1, 1.6 M Fdx, and 0.4 M FdR, whereas the protein concentrations in the CYP27A1 assays were 0.02 M CYP27A1, 0.6 M Fdx and 0.08 M FdR. For both P450s, a range of concentrations of cold cholesterol was the same (0 – 50 M) and [³H]cholesterol was at trace amounts. Reaction conditions in the incubations with CYP11A1 and CYP27A were optimized for the formation of only one product, pregnenolone and 27-hydroxycholesterol, respectively with the product formation being always linear with time and protein and not exceeding 16%. Data were analyzed with Graph-Pad Prism software.

Effect of Redox Partner Ratios on Activity of Mitochondrial P450s—Incubations were carried out using a fixed concentration of P450 (0.05 M), cholesterol (1.5 M), and PLs (~15 M or 10 g/ml), and varying concentrations of Fdx and FdR at ratios to P450s indicated in Table 4. The reaction time and temperature were 10 min and 37°C, respectively.

Conditions that Model Those in the Mitochondria of NR—Regardless of whether CYP11A1 and CYP27A1 were incubated separately or together, their concentrations were always 0.007 M (CYP11A1) and 0.25 M (CYP27A1) and those of the redox partners were 0.84 M (Fdx) and 0.73 M (FdR). Concentrations of PLs (from NR) and cholesterol were 75 M and 7.5 M, respectively, and the reaction temperature was 37°C.

Kinetics of CYP27A1-Mediated Cholesterol Hydroxylation in the Presence of A2E—The assay conditions were the same as in kinetic studies evaluating the effects of different PLs except A2E was present. The reaction components were mixed in the following order: KPi, liposomes (from the RPE PLs), [³H]cholesterol, cholesterol, A2E, Fdx, FdR, P450 and NADPH.

Quantifications by Western blotting

Proteins from bovine tissue homogenates and suspensions of mitochondria were separated by 10% SDS-PAGE and transferred to PVDF membrane for 1.5 hr at room temperature at 15V using semi-dry transfer system (Bio-Rad, Hercules, CA). Membranes were incubated for 1 hr at room temperature with the Odyssey blocking buffer (Li-cor Biosciences, Lincoln, NE), and then with the primary antibodies overnight at 4°C in the blocking buffer containing 0.1% Tween-20. Sera of rabbits immunized with CYP11A1, CYP27A1 and FdR were used at 1 to 5,000 dilution and that against CYP46A1 at 1 to 2,000 dilution. Purified IgG (10 µg/ml) were used to detect Fdx. After incubation with the primary antibodies, membranes were washed 4 times for 15 min at room temperature with the phosphate buffer saline containing 0.1% Tween-20 and incubated with the secondary IRDye goat anti-rabbit IgG (Li-cor Biosciences, Lincoln, NE, 1 to 20,000 dilution) for 1 hr at room temperature in the blocking buffer containing 0.1% Tween. After 4 washes with the phosphate buffer saline containing 0.1% Tween-20, membranes were analyzed by the Odyssey infrared imaging system and Li-cor Odyssey software (Li-cor Biosciences). Protein expression was estimated based on the fluorescence of the known amounts of purified recombinant proteins used as standards for quantifications in bovine tissues.

Quantifications by MS spectrometry

This was carried out by multiple reaction monitoring as described [32, 33] using ^{15}N -labeled proteins as internal standards: bovine CYP11A1 and FdR and human CYP46A1. Transitions (3 per peptide) were from the two peptides from CYP11A1 (NFIPLLNPVSQDFVSLHK and LHPISVTLQR), two peptides from FdR (FGVAPDHPEVK and SPQQVLPSPDGR) and three peptides from CYP46A1 (VLQDVFLDWAK, TSVIVTSPESVK, and LLEEETLIDGVR). All these peptides overlap between human and bovine enzymes.

Results

Effect PLs on Kinetic Properties of Mitochondrial CYPs 11A1 and 27A1

The uniquely high content of n-3 polyunsaturated fatty acids in the retina [46] served as a rationale for comparison of cholesterol metabolism in the presence of PLs from the NR and RPE to those from the liver and adrenal cortex (Table 1), tissues with the highest expression of CYPs 27A1 and 11A1, respectively. Purified recombinant P450s were reconstituted into liposomes prepared from PLs isolated from the mitochondria of different organs followed by the *in vitro* enzyme assays. Relative to kinetic constants in the presence of PLs from adrenal cortex, PLs from NR and RPE decreased both K_m and k_{cat} of CYP11A1 and the overall efficiency (the k_{cat}/K_m ratio) of CYP11A1-mediated cholesterol metabolism. The effect on CYP27A1 was different when compared to the kinetics in the presence of liver PLs. The enzyme's K_m was unaffected, whereas the k_{cat} either increased (NR PLs) or remained unchanged (RPE PLs). Accordingly, the overall catalytic efficiency of CYP27A1 was the same in the presence of RPE PLs and increased in the presence of NR PLs.

Cholesterol and PL Content in Mitochondria of Different Bovine Tissues

CYPs 11A1 and 27A1 likely hydroxylate cholesterol within the lipid bilayer [47], hence, membrane concentrations of cholesterol, absolute and relative to the concentration of PLs, affect the product formation by these P450s [22, 47]. Of the four bovine tissues analyzed for the mitochondrial lipid content (Table 2), PL and cholesterol levels were the highest in the RPE and lowest in the liver with the NR and adrenal cortex having intermediate lipid amounts (Table 3). Despite differences in absolute concentrations, the PL to cholesterol ratios were similar in the NR and RPE and lower than those in the adrenal cortex and liver. These results suggest that more cholesterol is available to mitochondrial P450s 11A1 and 27A1 in the NR and RPE than in the liver and adrenal cortex with the latter known to contain subsaturating for CYP11A1 cholesterol concentrations [22].

Quantifications of P450s and Mitochondrial Redox Partners in Bovine Tissues

Mitochondrial P450s receive electrons from a small soluble protein Fdx which in turn is reduced by the membrane-bound protein FdR obtaining electrons from NADPH. The concentrations of FdR and Fdx determine electron supply to mitochondrial P450s and could limit cholesterol metabolism by these enzymes *in vivo* [25, 26]. In our prior studies, we quantified some (CYP11A1 and FdR) but not all of the components of the mitochondrial electron transfer chain in bovine NR and adrenal cortex [32]. Therefore, in the present work we extended our measurements to other proteins, CYP27A1 and Fdx, and other tissues, the RPE and liver. CYP46A1 was determined as well for completeness of the P450 data sets, yet this microsomal enzyme receives electrons from a different redox partner P450 oxidoreductase. Two methods were used: Western blotting to estimate the amounts of the high abundance components of the P450 system, and a mass spectrometry technique multiple reaction monitoring to quantify the low abundance system components (Table 3). In the case of CYP11A1 and FdR in adrenal cortex, protein concentration were assessed by two methods which showed comparable results, thus lending the support to the validity of

our quantifications. In bovine NR and RPE, CYP27A1 was the most abundant cholesterol-metabolizing P450 present at 2–3 fold higher levels than CYP46A1 and 30-fold higher levels than CYP11A1. Despite this relatively high abundance, absolute concentrations of CYP27A1 in the NR and RPE were more than 10-fold lower than the enzyme's concentrations in the liver. Accordingly, retinal concentrations of a low abundance CYP11A1 were more than 90-fold lower than those in the adrenal cortex. Our quantifications also revealed that the ratio between mitochondrial P450s and their redox partners vary in different tissues. In the NR, Fdx and FdR were in excess over CYP27A1 and CYP11A1; in the RPE, they were at about equimolar concentrations relative to a sum of CYP27A1 and CYP11A1; and in the liver, FdR was at 10-fold lower levels than CYP27A1 and Fdx.

Effect of Redox Partner Ratios on Activity of Mitochondrial P450s

We reproduced the PL composition and tissue-specific P450/redox partner ratios in the *in vitro* enzyme assays to evaluate how these factors affect the activity of CYPs 27A1 and 11A1 under the conditions when same amount of P450 (0.05 M), cholesterol (1.5 M), and PL (15 M) are used and the enzyme reactions are carried out for the same period of time (10 min). Pregnenolone formation by CYP11A1 was almost 6-fold higher under the conditions modeling the PL and redox partner ratio in the NR than under the conditions modeling these parameters in the RPE (Table 4). Since the kinetic parameters of pregnenolone formation are similar in the presence of PLs from the RPE and NR (Table 1), the data obtained indicate that the 1:1 molar ratio of Fdx to mitochondrial P450s in the RPE may limit the activity of CYP11A1 as compared to the 3:1 redox partner ratio in the NR. This conclusion is supported by the experiments in the presence of adrenal PLs and the 3:1 Fdx/P450 ratio when the amount of pregnenolone formed was close to that in the NR. The effect of Fdx/P450 ratio on activity of CYP27A1 was similar (Table 4) with the 1:1 molar ratio modeling that in the liver and RPE yielding much less (~4-fold) product than the 3:1 molar ratio in the NR. Since the kinetics of 27-hydroxycholesterol formation is very similar in the presence of hepatic and RPE PLs (Table 1), similar percent of product formation under the same Fdx/P450 ratio (1:1) but different Fdx/FdR ratios (1:0.1 and 1:2) suggests that low FdR concentrations may not limit the activity of CYP27A1 in the liver.

In vitro Activities of CYPs 11A1 and 27A1 Under the Conditions that Model Those in the Mitochondria of NR

Reaction mixtures contained purified P450s, FdR and Fdx at ratios similar to those in mitochondria of NR (Table. 3) incorporated into liposomes that model the PL composition and PL to cholesterol ratio in mitochondrial membranes of NR (10:1, M/M, Table 2). Both reaction kinetics and product profile were monitored. When CYPs 11A1 and 27A1 were assayed separately, pregnenolone was the only detectable product in the incubations with CYP11A1 (Fig. 1A), and 27-hydroxycholesterol and 5-cholestenoic acid were the two products in the incubations with CYP27A1 (Fig. 1B). The kinetics of pregnenolone and 5-cholestenoic acid formation was similar and hyperbolic, whereas that of 27-hydroxycholesterol was different and consistent with further oxidation of 27-hydroxycholesterol to 5-cholestenoic acid. When CYPs 11A1 and 27A1 were co-incubated (Fig. 1C), additional CYP11A1 metabolite 22R-hydroxycholesterol was detected, whereas the product profile of CYP27A1 remained unchanged and contained 27-hydroxycholesterol and 5-cholestenoic acid. In the co-incubations, percent of formation of each pregnenolone, 27-hydroxycholesterol and 5-cholestenoic acid depended on the reaction time: it was either the same as in individual incubations (when the reaction time was short, up to 10 min) or was decreased (when the reaction time was longer). Indeed, at 60 min, only 16% of cholesterol was converted to pregnenolone in the co-incubation (Fig. 1C) and 33% in the individual incubation with CYP11A1 (Fig. 1A). Likewise, 13% vs 16% and 44% vs 68% of

cholesterol was oxidized to 27-hydroxycholesterol and 5-cholestenoic acid, respectively, in the co-incubation (Fig. 1C) as compared to individual incubation with CYP27A1 (Fig. 1B). Yet, the total percent of metabolized cholesterol was the same at each time point in the co-incubation and individual incubation with CYP27A1 because in the former pregnenolone and 22R-hydroxycholesterol were generated by CYP11A1. Furthermore, in the co-incubations cholesterol was likely at subsaturating concentrations for both enzymes thus limiting the formation of their products. The data obtained demonstrate that CYP11A competes successfully with CYP27A1 for cholesterol even when its concentration is >35-fold lower than that of CYP27A1, but under these conditions begins to release 22R-hydroxycholesterol leading to the production of less pregnenolone.

Effect of A2E on CYP27A1

A2E is a lipophilic endogenous compound, N-retinylidene-N-retinylethanolamine, formed during the vision cycle and present in substantial quantities in the RPE [48, 49]. Cell culture experiments indicate that A2E could target mitochondria inducing apoptosis, inhibiting cytochrome c oxidase and detaching cytochrome c [50]. We investigated whether A2E affects CYP27A1 *in vitro* when the enzyme is reconstituted into liposomes prepared from the RPE mitochondrial PLs. Kinetic analysis of the CYP27A1-mediated cholesterol hydroxylation at several A2E concentrations revealed inhibition of the enzyme activity in a concentration-dependent manner (Fig. 2) with a pattern of line intersection in the Lineweaver-Burk plots corresponding to non-competitive inhibition [51]. Secondary plots (data not shown) constructed to determine the K_i [51] yielded a value of 2.9 M suggesting that A2E is a relatively strong non-competitive CYP27A1 inhibitor under the experimental conditions used.

Discussion

The present work represents a part of a larger study by this laboratory in which we delineate the putative link between cholesterol and age-related macular degeneration (AMD), a major cause of irreversible vision loss in the elderly. With age, cholesterol and other lipids form extracellular deposits beneath the RPE called drusen, and in people with AMD drusen formation is more prominent and a hallmark of the disease. The reasons for the age- and disease-dependent drusen deposition are currently unknown but under investigation [52, 53] and likely include, at least in part, cholesterol elimination from the NR and RPE. Yet, in contrast to many other tissues, where cholesterol maintenance is well characterized, the mechanisms whereby cholesterol is removed from the NR and RPE are still not fully understood, although known to involve the lipoprotein-mediated transport as demonstrated by others [52–54] and metabolism to oxysterols by P450s as shown by us [34]. In our previous studies we identified the major cholesterol-metabolizing P450 (CYP27A1) and major oxysterol (5-cholestenoic acid) in the NR and RPE and by comparing the oxysterol profiles in different organs obtained an indication that the retina has peculiar features that affect the product profile of the two mitochondrial cholesterol-metabolizing P450s, CYP11A1 and CYP27A1. CYP11A1 was found to release 22R-hydroxycholesterol during the cholesterol conversion to pregnenolone and CYP27A1 oxidize cholesterol at position C27 three times yielding 5-cholestenoic acid [34]. The release of 22R-hydroxycholesterol could represent the retina-specific regulation of cholesterol elimination because 22R-hydroxycholesterol is a biologically active oxysterol and potent ligand for the liver X receptor [55] controlling the transcription of many cholesterol-related genes [56, 57]. Likewise, the formation of 5-cholestenoic acid, also demonstrated to be a ligand for the liver X receptor in cell cultures [58], could be important as well and represent retina's adaptations to cholesterol removal via 27-hydroxylation and putative neurodegenerative properties of 27-hydroxycholesterol. 27-Hydroxycholesterol was shown to counteract the inhibiting effect

of 24-hydroxycholesterol in generation of amyloid in cultured neuroblastoma cells [59]; increase - amyloid formation and oxidative stress in RPE cells [60]; increase -amyloid in hippocampal slices [61]; and reduce the production of the “memory protein” arc in mouse brain [62]. Thus, it is possible that 27-hydroxycholesterol may accelerate neurodegeneration *in vivo*, and if so, this could be damaging to the retina which similar to the brain is a neural tissue composed of different types of neurons. Accordingly, maintaining low levels of 27-hydroxycholesterol and production of a metabolite, 5-cholestenoic acid, presumably less toxic than 27-hydroxycholesterol and leaving the cell much faster than 27-hydroxycholesterol [6], could be one of the reasons for the retinal conversion of 27-hydroxycholesterol to 5-cholestenoic acid.

To elucidate the features of the retinal environment that may affect the activities and product profile of CYP27A1 and CYP11A1, we used bovine tissues since retinal bovine oxysterol profile is qualitatively similar to that in humans [34]. Bovine organs are always available in large quantities from local slaughterhouses and could be obtained much easier and faster than post-mortem human specimens. In our investigation, we focused first on PL environment and cholesterol content that have been previously shown to modulate the activities of CYPs 11A1 and 27A1 *in vitro* and in tissues outside the eye [6, 22–25, 47, 63, 64]. We also kept in mind studies of CYPs 11A1 and 27A1 membrane topology indicating that the entrance of the substrate access channel in these enzymes is embedded in the inner mitochondrial membrane and that cholesterol enters the enzyme active site directly from the lipid bilayer [45, 65, 66].

Previously, the effect of different head groups and fatty acyl chains was assessed mainly by using commercially available individual PLs or their mixtures and only one mitochondrial P450 [47, 63, 64, 67–69]. It has never been compared how kinetic constants of CYP11A1 and CYP27A1 are affected by PLs isolated from different organs (NR, RPE, liver and adrenal cortex) and, moreover, how the same source of PLs affects these two mitochondrial enzymes (Table 1). This was done in the present work and demonstrated that the effect is mainly on activity (k_{cat}) rather than affinity (as reflected by K_m) and activity as observed with several extraocular PLs [69, 70]. Furthermore, PLs had differential effects on CYPs 11A1 and 27A1, an unexpected finding because one would envision that the higher content of polyunsaturated fatty acids in the NR and RPE PLs relative to adrenal and liver PLs would have the same effect on the two mitochondrial P450s. However, PLs from the NR inhibited the activity of CYP11A1 (as compared to adrenal PLs) and stimulated the activity of CYP27A1 (as compared to hepatic PLs); the effect on K_m was only minor for both P450s. This data provide the first insight into the presence of 22R-hydroxycholesterol and 5-cholestenoic acid in the retina; when the rate of catalysis is slower, this could lead to a release of the reaction intermediate 22R-hydroxycholesterol from the CYP11A1 active site, and when catalysis is faster, CYP27A1 can further oxidize 27-hydroxycholesterol to 5-cholestenoic. Previous studies of CYP11A1 may provide some explanation as to how PLs modulate the activities of P450s. PLs were shown to affect the CYP11A1 spin state [67, 69] which reflects the coordination of the heme iron with water molecule. These results suggest that either directly (through opening or closure of the membrane-embedded substrate access channel) or indirectly (by changing the conformation of the active site) PLs change the environment within the active site and thereby affect, at least in part, the rate of P450 catalysis. Apparently, further studies are required to elucidate the effect of PLs on activity of P450s and why NR PLs have distinct effects on CYPs 27A1 and 11A1.

Comparative measurements of the PL and cholesterol content in different organs (Table 2) provided another important insight into cholesterol maintenance in the retina. The quantities of both lipids per milligram of total protein as well as the cholesterol concentrations in the PLs were found to be higher in the NR and RPE than in the adrenal cortex and liver.

Cholesterol is at subsaturating concentrations for CYP11A1 in the adrenal cortex [23] and possibly for CYP27A1 in the liver because the majority of hepatic cholesterol is metabolized by a different P450, microsomal CYP7A1. Therefore, a higher cholesterol content in the NR and RPE relative to the adrenal cortex and liver suggests that the activity of CYPs 11A1 and 27A1 in the NR and RPE may not be limited by the availability of cholesterol. However, this pertains only to those cell types where the two P450s are not co-expressed and compete for cholesterol. There may be a number of such retinal cells because expression of CYP11A1 is limited to only two layers (the inner nuclear and ganglion cell layers) in contrast to that of CYP27A1 which seems to be ubiquitous in the retina as indicated by immunohistochemistry stainings [27–29].

Besides the lipids, we studied the P450 redox partners FdR and Fdx as the activity of CYPs 11A1 and 27A1 was shown to depend in some organs on their availability [25, 26]. Along with NADPH, FdR and Fdx form the chain, NADPH FdR Fdx P450, that provides electrons to all mitochondrial P450s through the Fdx binding site located on the cytosolic side of the P450 molecule [71]. The data obtained (Table 3) enabled direct comparisons of the P450 and redox partner expression in the same organ as well as between different organs. Subsequent *in vitro* enzyme assays modeling the P450 redox partner ratios in different tissues (Table 4) indicate that similar to cholesterol, FdR and Fdx may not be at limiting amounts in the NR but could reduce the enzyme activity in the RPE.

Perhaps most exciting was the result of our *in vitro* co-incubations of CYPs 11A1 and 27A1 (Fig. 1C) when we used NR PLs and modeled PL to cholesterol and P450 to redox partner ratios as well as the ratio of CYPs 11A1 and 27A1. Under these conditions, when CYP11A1 was at 35-fold lower concentrations than CYP27A1, CYP11A1 was still able to metabolize cholesterol to pregnenolone and, in addition, began to release 22R-hydroxycholesterol. The major product of CYP27A1 was 5-cholestenoic acid. This *in vitro* product profile is consistent with that observed in the retina indicating that it is the specific retinal environment (PL composition and content, amounts of cholesterol and P450 redox partners as well as the ratio between CYPs 11A1 and 27A1 in the cells where they are co-expressed) that underlie the oxysterol profile of the NR. Simultaneous presence of pregnenolone and 22R-hydroxycholesterol suggests that the role of CYP11A1 in the retina extends beyond the production of steroid hormones and also involves maintenance of cholesterol levels and possibly regulation of cholesterol homeostasis. With respect to CYP27A1, previous studies with cultured macrophages showed that the production of 5-cholestenoic acid by CYP27A1 is enhanced when cholesterol availability is limited and CYP27A1 expression is high, and when there is an extracellular acceptor, albumin, outside the cells that binds 5-cholestenoic acid [6]. Our investigation of the retina complements the previous work and identifies additional parameters that favor the production of 5-cholestenoic acid. These parameters include PL environment and the levels of the redox partners that may stimulate the CYP27A1 catalysis.

Finally, we also show that A2E present in the RPE inhibits non-competitively CYP27A1. The primary site of A2E deposition in the RPE is the lysosomal compartment. Therefore, there is considerable skepticism in the field about mitochondrial localization of A2E and whether it happens under normal conditions. Yet, with the high loading levels used in cell culture studies [50], it is not surprising that a small amount of A2E localizes to the mitochondria. While it remains to be determined whether A2E does affect mitochondria and mitochondrial function under certain conditions *in vivo*, if this is the case, we demonstrate that A2E may also inhibit CYP27A1 and affect enzyme-mediated cholesterol elimination from the RPE.

In summary, we demonstrate that the retina-specific composition of mitochondrial PLs and levels of the mitochondrial P450 redox partners promote the three-step cholesterol oxidation by CYP27A1 to 5-cholestenoic acid. In contrast, the catalysis of CYP11A1 is slowed down by retinal PLs and in addition by the competition with CYP27A1 for cholesterol and redox partners leading to the release of 22R-hydroxycholesterol. Collectively, the data obtained provide insight into the regulation of enzyme-mediated cholesterol elimination from the back of the eye.

Highlights

Effect of phospholipids on catalytic efficiency of CYPs 11A1 and 27A1 is organ- and P450-specific

Cholesterol content is higher in the retinal mitochondria than in the adrenal and liver mitochondria

The redox partners for mitochondrial P450s are not at limiting amounts in the retina

In the retina, competition with CYP27A1 alters the product profile of CYP11A1

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

A2E	N-retinylidene-N-retinylethanolamine
CYP or P450	cytochrome P450
FdR	ferredoxin reductase
Fdx	ferredoxin
KP_i	potassium phosphate buffer
NR	neural retina
PL	phospholipids
RPE	retinal pigment epithelium

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Fig. 1.

Product formation in the incubations of cholesterol with CYPs 11A1 and 27A1 when the two enzymes were assayed separately (A and B) and together (C). Assay conditions are described in Materials and Methods. The results are the means of three independent measurements. The error bars are not seen because they are smaller than the symbol size. The insets show the HPLC product profiles in the 60-min incubations. The insets show the HPLC product profiles in the 60-min incubations. Different colors reflect the formation of different products: pregnenolone (Preg) is in green, 27-hydroxycholesterol (27-OH) is in purple, 5-cholestenoic acid (27-COOH) is in orange and 22R-hydroxycholesterol (22R-OH) is in light blue.

Fig. 2.

Kinetics of CYP27A1-mediated cholesterol hydroxylation at several A2E concentrations presented in the form of double-reciprocal (Lineweaver-Burk) plots of reaction velocity (nmol product/nmol P450/min) vs. cholesterol concentration. The results are the means of three independent measurements. The error bars are not seen because they are smaller than the symbol size. The insets show the HPLC product profiles in the 60-min incubations. The $1/v$ values on the y axis increase with increased concentrations of A2E indicating inhibition of CYP27A1, and the lines intersect on the x axis revealing that this inhibition is of a non-competitive type. Assays were performed as described in Materials and Methods.

Table 1

Effects of PLs on kinetic constants for cholesterol hydroxylation by purified recombinant CYPs 11A1 and 27A1^a

P450	Source of mitochondrial PLs	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($\mu\text{M}^{-1}\text{min}^{-1}$)
CYP11A1	NR	0.66 ± 0.01	4.88 ± 0.16	7.4
	RPE	0.79 ± 0.08	5.18 ± 0.52	6.6
	Adrenal cortex	1.05 ± 0.07	9.68 ± 0.83	9.2
CYP11A1	NR	2.67 ± 0.32	13.05 ± 1.09	4.9
	RPE	2.03 ± 0.23	6.78 ± 0.13	3.4
	liver	2.66 ± 0.43	7.83 ± 1.20	3.0

^a Assay conditions are described in Materials and Methods. The results are the means of three independent measurements SD.

Table 2Cholesterol and PL content in mitochondria of different bovine tissues^a

Lipid	Concentration (mol/mg of mitochondrial protein)			
	NR	RPE	Adrenal cortex	Liver
PL ^b	1.2	2.0	0.9	0.8
Cholesterol	0.15	0.20	0.06	0.04
PL/Cholesterol, M/M	8.0	10	15	20

^a Entire tissues (NR, RPE and adrenal cortex) or tissue samples (the liver) from several animals were pooled and the lipid content in these pooled samples was determined. The results are the means of two independent measurements which are different by no more than 30%

^b Molar concentrations of PLs were calculated assuming average molecular weight of 650.

Table 3

Quantification of cholesterol-metabolizing P450s and related proteins in different bovine tissues (numbers in parenthesis in bold indicate approximate protein ratios in the same tissue)

Protein	Concentration (pmol/mg of total protein)			
	NR	RPE	Adrenal cortex	Liver
CYP11A1	nd ^{a,b}	nd ^{a,b}	160 ^b	nm ^c
	0.007 ^{d,e} (0.03)	0.012 ^{e,f} (0.03)	96 ^{d,e} (1)	nm ^c
CYP27A1	0.24 ^b (1)	0.46 ^b (1)	nm	5.3 ^b (1)
CYP46A1	nd ^{a,b}	nd ^{a,b}		
	0.11 ^e (0.5)	0.13 ^e (0.3)	nm	nm
FdR	nd	nd	90 ^b	nd
	0.73 ^{d,e} (3)	0.75 ^e (1.6)	90 ^{d,e} (1)	0.6 ^e (0.1)
Fdx	0.84 ^b (3.5)	0.43 ^b (1)	285 ^b (3)	4.6 ^b (1)

^a Nd, not detectable; limits of detection varied for different proteins.

^b Assessed by Western blotting; if the protein under study was above the limits of detection, the results are the means of three independent measurements; SD is no more than 30%; the representative Western blots are shown in Fig. S2.

^c Nm, not measured.

^d Taken from ref. 32.

^e Assessed by mass-spectrometry in the present work (unless otherwise indicated); the results (unless otherwise indicated) are the means of three independent measurements in which three transitions per 2–3 individual target peptides were monitored; SD is no more than 25%.

^f Assessed by mass-spectrometry in the present work by monitoring one transition from one peptide.

Table 4Effect of Redox Partner Ratios on Activity of Mitochondrial P450s^a

Source of mitochondrial PLs	P450:Fdx:FdR, M/M	% of product formation
	CYP11A1	Pregnenolone
Adrenal cortex	1 : 3 : 1	45.2 ± 3.2
NR	1 : 3 : 3 ^b	58.7 ± 1.0
RPE	1 : 1 : 2 ^b	11.9 ± 0.6
	CYP27A1	27-Hydroxycholesterol
Liver	1 : 1 : 0.1	6.4 ± 0.1
NR	1 : 3 : 3 ^b	29.7 ± 2.4
RPE	1 : 1 : 2 ^b	7.3 ± 0.8

^a Assay conditions are described in Materials and Methods. The results are the means of three independent measurements SD.^b Molar ratio of a sum of CYPs 27A1 and 11A1.