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Expression and localization of sterol 27-hydroxylase (CYP27A1) in monkey retina

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Abstract

Sterol 27-hydroxylase (CYP27A1) is a mitochondrial P-450 enzyme with broad substrate specificity for C27 sterols including 7-ketocholesterol (7kCh). CYP27A1 is widely expressed in human tissues but has not been previously demonstrated in the retina. In this study, we examined the expression and localization of CYP27A1 in the monkey retina where it localized mainly to the photoreceptor inner segments. CYP27A1 was also observed in Müller cells with faint immuno staining detected in the RPE and choriocapillaris. We also determined that the 27-hydroxylation of 7-ketocholesterol (27OH7kCh) rendered it non-toxic to cultured RPE cells. Moreover, the 27OH7kCh when mixed with 7-ketocholesterol significantly reduced the toxicity of 7-ketocholesterol. These data, when taken in context of the known functions of CYP27A1 imply that expression in the retina serves to modify the biological activity of oxidized sterols that are either transported or generated locally by photo-oxidation. Published by Elsevier Ltd.

Keywords

cholesterol efflux; macula; 7-ketocholesterol; ARPE19; photoreceptors

The sterol 27-hydroxylase (CYP27A1:P450c27, Andersson et al., 1989) is a mitochondrial enzyme that performs multiple functions (Javitt, 2002, review): it is known to catalyze bile acid hydroxylations (Russell, 2003, review) and to activate vitamin D (Usui et al., 1990); furthermore, the formation of 27-hydroxycholesterol (27OHCh) by CYP27A1 has been demonstrated to stimulate cholesterol efflux in cultured cells (Escher et al., 2003). CYP27A1 is deficient in individual with the rare disease cerebrotendinous xanthomatosis (CTX) (Cali et al., 1991). CTX patients suffer from progressive neurological impairment as well as accelerated atherosclerosis and cataracts (Moghadasian, 2004, review).

CYP27A1 readily hydroxylates the oxysterol 7-ketocholesterol (7kCh) to 27-hydroxy-7-ketocholesterol (27OH7kCh) (Brown et al., 2000), which is highly toxic to a variety of cells (Lyons and Brown, 1999) including RPE cells (Rodriguez et al., 2004) and is the main cholesterol oxide in oxidized low density lipoprotein (Brown et al., 1996). The oxysterol 7kCh

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has been implicated in macrophage foam cell formation and atherosclerosis (Brown and Jessup, 1999).

The rationale for this study is threefold: (1) CYP27A1 is known to be involved in the cholesterol efflux pathway (Escher et al., 2003), (2) CYP27A1 is known to be involved in the metabolism of 7kCh (Brown et al., 2000; Lyons and Brown, 2001; Jessup and Brown, 2005) and (3) despite the extensive knowledge of cholesterol hydroxylation in the brain (Björkem and Meaney, 2004; Dietschy and Turley, 2004, reviews), there are no published reports concerning CYP27A1 (or other sterol hydroxylases) in the retina. The retina is known to perform de novo synthesis (Fliesler et al., 1993) as well as cholesterol uptake from circulating blood lipoproteins (Elner, 2002; Gordiyenko et al., 2004). However, little is known about the cholesterol efflux or how the retina maintains cholesterol homeostasis. CYP27A1 may be involved in cholesterol efflux by forming 27OHCh which can change the lipid composition of the plasma membrane and/or can alter gene expression by acting as a ligand for the X-activated receptor (Escher et al., 2003; Berkenstam and Gustafsson, 2005). Another aspect of retinal biochemistry which is not well understood is the formation of oxidized sterols and the biochemical pathways that metabolize these molecules. The highly oxidative retinal environment coupled with light exposure (photo-oxidation) provides excellent opportunities for lipid oxidation (Girotti, 1992; Girotti and Kriska, 2004, reviews). While the formation of oxidized sterols in the retina has not been studied in detail, oxidation and photo-oxidation products of cholesterol on other tissues and in vitro indicates that it readily forms 7 α - and 7 β -hydroxy derivatives (Bortolomeazzi et al., 1999), which are known precursors of 7-ketocholesterol (Dzeletovic et al., 1995).

The monkey retina was chosen for this study for several reasons. We can obtain fresh ocular tissue from young animals. This allows us to perform an immediate, but light fixation, preserving both excellent morphology and antigenicity for vibrotome sectioning. Young monkeys also have little lipofuscin accumulation which is highly desirable when detecting immunofluorescence in the retinal pigment epithelium (RPE). These conditions are very difficult to match with human postmortem tissues. Another important reason for using monkey tissue is the anatomical similarity to the human retina. Primates are the only mammals that have the macula-foveola structure, which affords them high visual acuity and color vision. Finally, the high cross-reactivity between human and monkey antigens increased chances of success with an anti-human CYP27A1 antibody.

Fresh monkey eye tissue was obtained from rhesus monkeys (*Macacca mulatta*, 2–3 years old) through the courtesy of the Center for Biologics Research and Testing, U.S. Food and Drug Administration (Bethesda, MD). The eyes were dissected and the retinal tissue was used for either biochemical or immunohistochemical studies. For immunoblotting retinas were dissected, and the RPE and choriocapillaris (RPE/CH) were carefully separated from the neural retina. The two areas of the retina were subfractionated by differential centrifugation, to obtain soluble (S1) and membrane associated fractions P2S (salt extracted P2 pellet) and P2D (detergent, triton X-100 extracted P2 pellet).

Immunohistochemical localization of CYP27A1 was performed using 100 μ m vibrotome sections of monkey retinas previously fixed in 4% paraformaldehyde for 4 h. The sections were incubated with anti-human CYP27A1 (1:250) and developed using goat anti-rabbit Cy5conjugated secondary antibody. The sections were imaged by confocal microscopy in a Leica Microsystems model SP2 (Leica Microsystems, Exton, PA). CYP27A1 immunoreactivity localized mainly to photoreceptor inner segments (Fig. 1A and B) although faint expression was observed throughout the retina including the RPE and choroid (Fig. 1B). Müller cells, ganglion cells and nerve fibers (Fig. 1A, see arrows) also demonstrated slight immunoreactivity. Immunoblotting was also performed on the monkey retina subfractions. The

proteins were separated by SDS-PAGE followed by blotting on to a PVDF membrane. The blot was probed with rabbit anti-human CYP27A1-specific antibody (1:2000 a kind gift from Dr. David Russell, Dallas TX. See also Cali and Russell, 1991). The blot was developed using a goat anti-rabbit HRPconjugated secondary antibody (1:55,000) with LumiGLO substrate (KPL, Gaithersburg, MD). The results of the immunoblot are shown in Fig. 1C. The ARPE19 cultured RPE cells showed a single band at 47 kDa similar to the previously reported size for CYP27A1 (Brown et al., 2004). In the neural retina, CYP27A1 immunoreactivity was observed mainly as a high molecular weight smear in the S1 (soluble) fraction. In the RPE/CH fractions CYP27A1 was seen mainly as a dimer in the S1 and P2S (P2 pellet salt extracted) fractions.

In order to determine if the hydroxylation of 7kCh reduced its cytotoxicity to ARPE19 cells, 27OH7kCh was synthesized (Carroll et al., 1998) and tested at 50 μ M (Fig. 2A). As mentioned above, 7kCh is cytotoxic to ARPE19 cells (Rodriguez et al., 2004). The sterol solutions (10 mM) were made in aqueous 45% (w/v) 2-hydroxypropyl β -cyclodextrin and filter sterilized. Cholesterol was used as a negative control (no cytotoxicity) and 7kCh was used as a positive control (almost complete cell death) at 50 μ M. The cells were incubated for 48 h, and cell viability was measured using the Cell Counting Kit-8 (Dojindo Molecular Technologies, Gaithersburg, MD). The 27OH7kCh demonstrated no cytotoxicity at the same concentration (50 μ M) and incubation time in which 7kCh essentially killed the entire culture. Moreover, when 27OH7kCh was mixed with 7kCh (at 50 μ M each) it reduced the cytotoxicity of 7kCh (Fig. 2A). No cytotoxicity was observed with 27OH7kCh even at 100 μ M for 96 h (Fig. 2B). Mixing of 7kCh with cholesterol failed to protect the cells (data not shown) suggesting that the protection by 27OH7kCh is not due to simple competition for hydrophobic sites in the plasma membrane. For the purpose of this article the term “cytotoxicity” refers to the measurable non-viable cell fraction as determined in our assay.

Using a well characterized antibody we demonstrated the presence and localization of CYP27A1 in the retina. This finding, however, raised a number of questions concerning the function of CYP27A1 in the retina. The broad range of sterols, which CYP27A1 can hydroxylate (Pikuleva and Javitt, 2003), and the complex pharmacological effects that these 27-hydroxylated sterols can induce in cells obscure the function of CYP27A1 in the retina. For instance, CYP27A1 by producing 27OHCh may promote cholesterol efflux from the retina. Cholesterol efflux would be expected at the RPE and Müller cells where the retina comes in contact with the circulating plasma. While CYP27A1 is present in the latter cells it appears to be more abundant in the photoreceptors (Fig. 1). The strong presence of CYP27A1 in the photoreceptor inner segment hints at an oxysterol neutralization function. Hydroxylation of toxic oxysterols like 7kCh may be necessary in the oxidative environment of the photoreceptors. There is significant *in vitro* evidence demonstrating photo-oxidation of cholesterol (Bortolomeazzi et al., 1999), although the presence of 7kCh and other oxysterols in the retina has not been demonstrated. Alternatively, CYP27A1 may be generating 27OHCh and/or other oxysterols for the purpose of activating X-receptors (Escher et al., 2003; Berkenstam and Gustafsson, 2005) and/or other molecules that could alter gene expression. The brain, in contrast with the retina synthesizes essentially all of its own cholesterol and uses cholesterol 24-hydroxylase (CYP46A1) to generate 24S-hydroxycholesterol which is excreted into circulation in order to maintain cholesterol homeostasis (Björkem and Meaney, 2004; Dietschy and Turley, 2004, reviews). The brain has also been shown to uptake circulating 27OHCh (which can readily cross blood-brain barrier, Heverin, et al., 2005) where it is suspected of regulating cholesterol-sensitive genes for the purpose of maintain cholesterol homeostasis (Heverin et al., 2005).

The attenuation of 7kCh cytotoxicity by 27OH7kCh has not been previously reported although a protective effect has been previously demonstrated by co-incubation of 7kCh with other oxysterols (Biasi et al., 2004); the authors suspect that the attenuation effect is due to

competition of oxysterols for NADPH oxidase (Biasi et al., 2004). Analysis of plasma and model membranes by X-ray diffraction (Phillips, et al., 2001), however, suggests that the mechanism for 7kCh toxicity may be due to crystal formation in the plasma membrane. Thus, the protection by 27OH7kCh (Fig. 2) must be viewed with caution. The delivery of 7kCh to cells in ethanol (Biasi et al., 2004) or in 2-hydroxypropyl β -cyclodextrin (Fig. 2) is unlikely to be physiologically relevant. In vivo the delivery of 7kCh is mainly via oxidized lipid particles like LDL, and the cytotoxicity of 7kCh is due to loss of mitochondrial transmembrane potential (Lizard et al., 1999). Conversely, the fact that CYP27A1 is a mitochondrial enzyme known to metabolize 7kCh to 27OH7kCh (Jessup and Brown, 2005) and that 7kCh can affect mitochondrial function, suggests that the protection by 27OH7kCh may be of physiological importance.

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Fig. 1.

Immunohistochemical localization of CYP27A1 in the monkey retina. Confocal image of a cone-rich area of the monkey retina. The anti-CYP27A1 immunoreactivity was detected using a Cy5 (RED) secondary antibody. Cy5 was excited with the 633 laser line and emissions collected between 650 and 750 nm. Nuclei were labeled with DAPI (BLUE). The green fluorescence is autofluorescence activated with the 488 laser line with emissions collected between 400 and 500 nm. (A) Low magnification of image of the entire retina cross section. (B) High magnification image of photoreceptor region. (C) Immunoblot demonstrating the expression of CYP27A1 in the subfractionated monkey retina and culture ARPE19 cells.

Fig. 2.

Treatment of ARPE19 cells with 27OH7kCh. (A) ARPE19 cells were treated with 50 μ M oxysterols 7kCh, 27OH7kCh and an equimolar mixture of both for 48 h. (B) ARPE19 cell was treated for 96 h with 27OH7kCh at different concentrations. The cell viability was determined using the Cell counting kit-8 (Dojindo Molecular Technologies, Gaithersburg, MD).