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Alterations in glutamate cysteine ligase content in the retina of two retinitis pigmentosa animal models

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ABSTRACT

Retinitis Pigmentosa (RP) comprises a group of rare genetic retinal disorders in which one of several different mutations induces photoreceptor death. Oxidative stress and glutathione (GSH) alterations may be related to the pathogenesis of RP. GSH has been shown to be present in high concentrations in the retina. In addition, the retina has the capability to synthesize GSH. In this study, we tested whether the two subunits of glutamate cysteine ligase, the rate-limiting enzyme in GSH synthesis, and the concentrations of retinal GSH, oxidized glutathione (GSSG), cysteine (Cys) and glutamate are altered in the retina of two different RP mice models.

Retinas from C3H and rd1 mice at different postnatal days (P7, P11, P15, P19, P21 and P28) and from C57BL/6 and rd10 mice at P21 were obtained. Western blot analysis was performed to determine the protein content of catalytic and modulatory subunits from glutamate cysteine ligase (GCLC and GCLM, respectively). In another set of experiments, control and rd1 mice were administered buthinine sulfoximine, a glutathione synthase inhibitor, or paraquat. GSH, GSSG, glutamate and Cys concentrations were determined, by HPLC.

A decrease in retinal GCLC content was observed in C3H and rd1 mice with age, nevertheless, there was an increase in retinal GCLC in rd1 mice compared to control retinas at P19. No modifications in GCLM content with age and no difference between GCLM content in rd1 and control retinas were observed. The GSH concentration decreased in the rd1 retinas compared with control ones at P15, it increased at P19, and was again similar at P21 and P28. No changes in GSSG concentration in control retinas with age were observed; the GSSG levels in rd1 retinas were similar from P7 to P19 and then increased significantly at P21 and P28. Glutamate concentration was increased in the rd1 retinas compared to control mice from P7 to P15 and were comparable at P21 and P28. The Cys concentrations was measured in control and rd1 retinas, but no significant changes were observed between them.

BSO administration decreases GSH retinal concentration in control and rd1 mice, while paraquat administration induced an increase in GSH retinal concentration in control mice and a decrease in GSH in rd1 mice retina.

Retinal GCLC was significantly increased in rd10 mice at P21 as well as GSSG.

Our results suggest alterations in retinal GCLC content and GSH and/or its precursors in these two RP animal models. Regulation of the enzymes related to GSH metabolism and the retinal concentration of glutamate may be a possible target to delay especially cone death in RP.

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1. Introduction

Retinitis Pigmentosa (RP) is a group of genetic diseases of the retina in which one of several possible mutations causes the death of photoreceptor cells [1]. It usually follows a two-step process, where rods degenerate primarily followed by a secondary cone death [2]. However, the precise mechanisms causing death of photoreceptors are still unknown [2]. There is currently no treatment for this disease.

Oxidative stress has been implicated in the pathogenesis of RP. One hypothesis is that after rods die; the oxygen level in the outer retina becomes markedly elevated [3]. In this sense, Komeima et al. [4] showed that antioxidants decreased cone photoreceptor

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cell death in several RP models. Our group and others have also found significant protection when antioxidants are administered while rods are dying [5,6]. Our previous data suggest that thiol content and metabolism may have a role in RP, as we have observed a negative correlation between glutathione (GSH) retinal concentration and the number of TUNEL positive cells in RP mice that were treated with several antioxidants (zeaxanthin, lutein, alpha-lipoic acid, glutathione, and Lycium barbarum extract) [7].

GSH is the most abundant non-protein thiol and a major component of the defence against oxidants [8–10]. It also transports amino acids across the cellular membrane, removes foreign agents [11] and plays a role in cysteine (Cys) transport and storage [12]. GSH has an active role in the regulation of apoptosis rather than simply acting as an antioxidant against oxidative stress [13]. An increase in oxidized glutathione (GSSG) has been shown to directly induce apoptosis, because caspases can suffer S-glutathiolation [14]. Therefore, an imbalance in GSH and other related products and/or enzymes have been implicated in several diseases such as cancer, neurodegenerative diseases, cystic fibrosis, diabetes, aging, etc. [15]. In mammalian cells, three mechanisms serve to maintain the GSH homeostasis, *de novo* synthesis, extracellular uptake and GSSG-reductase catalysed reduction of GSSG [10]. GSH is synthesized from the amino acids Cys, glutamate, and glycine. *De novo* synthesis of GSH occurs in the cytosolic compartment [14]. It is initiated by the synthesis of γ-glutamylcysteine from glutamate and Cys by the glutamate cysteine ligase and then glycine is subsequently added by the activity of GSH synthetase [11]. Glutamate cysteine ligase is the rate-limiting enzyme of GSH biosynthesis [14]. Glutamate cysteine ligase is composed of two subunits, each one encoded by a different gene. One subunit (GCLC) is the relatively heavy (73 kDa) subunit, which has catalytic activity and can be inhibited by GSH [16]. The lower molecular weight (28 kDa) modulatory subunit (GCLM) is enzymatically inactive but it increases the affinity for glutamate and ATP as well as the GSH concentration required for glutamate cysteine ligase inhibition [17,18].

The aim of this study was to examine whether the retinal content of glutamate cysteine ligase and the concentrations of GSH, GSSG, glutamate and Cys are altered in the retina of two RP animal models.

Studies on retinal degeneration rely on the use of appropriate animal models. The retinal degeneration 1 (rd1) mouse is characterized by a mutation in exon 7 of the gene encoding for the beta subunit of the rod phosphodiesterase (PDE6), which leads to the nonprotein expression, PDE6 dysfunction [19,20] and an increase of cyclic guanosine monophosphate (cGMP) [21]. It can be supposed that the cGMP accumulation induces a continuous depolarization of rods because cGMP-gated cationic channels are maintained open [22]. The subsequent activation of the voltage-sensitive calcium channels induces glutamate release [22]. In this animal model, rods develop but then they suffer a rapid degeneration between postnatal days P12 and P21, with a peak of cell death around P11-12 [4].

Mice homozygous for the rd10 mutation display autosomal recessive hereditary retinal degeneration due to a missense mutation in exon 13 of the beta subunit of PDE6 gene [23]. The rd10 mutation appears milder when compared to the rd1 one [24], probably because rd10 retinal degeneration is not caused by the complete absence of PDE6 protein but may be induced by a deficient expression or a decrease enzymatic activity of this protein [23]. This could lead to a slower intracellular cGMP accumulation than the one observed in rd1 retina. In rd10 mice, rod photoreceptor cells start degenerating at 16 days of age in the central retina, at 20 days of age in the peripheral retina, and by 60 days no photoreceptors are left, in a center-to-periphery gradient [23]. Secondarily cones degenerate at a slower rate [24].

2. Material and methods

2.1. Animals

Mice were housed under standard white cyclic lighting (12 h); they had free access to food and water, and were used independently of gender. Rd1 and rd10, together with their respective controls, C3H and C57BL/6 were the mice used for this study. Animal care and protocols were in accordance with, and approved by, the Animal Ethics Committee of the university and they were performed in accordance with the ARVO statement for the use of animals in ophthalmic and visual research.

Day of birth was considered P0; retinas were obtained from the rd1 and C3H mice at P7, P11, P15, P19, P21, and P28, and from rd10 and C57BL/6 mice at P21. At least three animals were used on each post-natal day.

Eyes were quickly enucleated, and half of the retinas were used for western blot analysis. Pools of two retinas were homogenized in 50 μL RIPA (Radio-Immunoprecipitation Assay) buffer which was composed of 150 mM NaCl (Sigma-Aldrich, Madrid, Spain), 1% Nonidet P40 *substitute* (Sigma-Aldrich, Madrid, Spain), 0.1% sodium dodecyl sulfate (SDS) (Sigma-Aldrich, Madrid, Spain), 50 mM de Tris(hydroxymethyl)aminomethane (Sigma-Aldrich, Madrid, Spain), 0.5% sodium deoxycholate (Sigma-Aldrich, Madrid, Spain), 1 mM Dithiothreitol (DTT) (Sigma-Aldrich, Madrid, Spain), protease inhibitor cocktail (Complete Mini, Roche, Mannheim, Germany) and a phosphatase inhibitor mixture (10 mM NaF (Sigma-Aldrich, Madrid, Spain), 1 mM Na_3VO_4 (Sigma-Aldrich, Madrid, Spain)). Homogenates were centrifuged at 13,000 rpm for 10 min at 4 °C, and the supernatants were collected. The protein concentrations in supernatants were determined using the Bradford Protein Assay [25].

The other half of the retinas were used for biochemical analysis (GSH, GSSG, glutamate and Cys determination). Retinas were dissected and homogenized at 13,000 rpm for one minute in an ULTRA TURRAX T25 dispersing machine (Labortech, Staufen im Breisgau, Germany) in pre-chilled 0.2 M potassium phosphate buffer, pH 7.0. The homogenate was then centrifuged at 6000 rpm for two minutes at 4 °C, and 180 μL of the homogenate supernatant was acidified with 20 μl of 20% perchloric acid (PCA) (Panreac, Barcelona, Spain). The supernatant was collected and stored at −20 °C until GSH, GSSG, glutamate and Cys determination was performed. The rest of the homogenate was used for protein determination.

2.2. Western blot analysis

The protein content of the GCLC and GCLM subunits of glutamate cysteine ligase was determined by western blotting. Homogenate supernatants containing equal amounts of protein (30 μg) were loaded onto 10% sodium dodecyl sulfate (SDS) (Sigma-Aldrich, Madrid, Spain) polyacrylamide gels and transferred to nitrocellulose membranes (Amersham Biosciences, Buckinghamshire, UK), which were blocked in 5% skimmed milk in Tris Buffered Saline (TBS) (Sigma-Aldrich, Madrid, Spain) and 0.1% of Tween 20 (Sigma-Aldrich, Madrid, Spain), for one hour. Thereafter, samples were incubated with the primary antibody against GCLC or GCLM (Abcam, Cambridge, UK) overnight at 4 °C. Bound antibody was visualized using horseradish peroxidase-coupled secondary anti-rabbit ($F(ab')_2$ – HRP, goat anti-rabbit) (Santa Cruz Biotechnology, Santa Cruz, USA), incubating the membrane one hour at room temperature. Finally, the signal was detected with the enhanced chemiluminescence (ECL) developing kit (Amersham Biosciences, Buckinghamshire, UK). Blots were quantified by densitometry using ImageQuant™TL (GE Healthcare Life Sciences, Barcelona, Spain).

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